I, Soumyarwit Manna, hereby submit this original work as part of the requirements for the degree of Doctor of Philosophy in Materials Science.

It is entitled:
Sustained Release Micro-implants for Delivery of Hydrophilic Drugs to Treat Vitreoretinal Diseases

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SUSTAINED RELEASE MICRO-IMPLANTS FOR DELIVERY OF HYDROPHILIC DRUGS TO TREAT VITREORETINAL DISEASES

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ABSTRACT

A drug delivery device (micro-implant) providing sustained release of hydrophilic drugs has the potential to improve the therapeutic outcome for treatment of vitreoretinal (VR) diseases such as primary intraocular lymphoma, uveitis and proliferative retinopathy. At present, the preferred treatment of VR diseases is intravitreal methotrexate (MTX) injection. Each intravitreal injection of MTX is associated with potentially toxic and sub-therapeutic MTX concentrations. Repetitive intravitreal injections are required to maintain therapeutic MTX concentration. A drug delivery system is desired for sustained therapeutic release (0.2-2.0 µg/day) of MTX for >1 month to achieve effective treatment of VR diseases.

In an in vitro study, chitosan (CS) and polylactic acid (PLA)-based micro-implants were fabricated for different MTX loadings (10%, 25% and 40% w/w). The micro-implant structure was characterized using optical and scanning electron microscopy, time of flight-secondary ions mass spectroscopy and differential scanning calorimetry techniques. The MTX release rate studies were evaluated using a UV-Visible Spectrophotometer. It was observed that uncoated CS-MTX micro-implant released MTX rapidly (~1 day) because of the hydrophilic nature of both CS and MTX. However, the CS-MTX micro-implant with a lipophilic coating of PLA showed therapeutic MTX release (0.2 – 2 µg/day) for >50 days. The MTX release kinetics from the coated micro-implants is explained by a) the Korsmeyer Peppas and zero order model fit (R² ~ 0.9) of the first 60% of MTX release which indicates the swelling of polymer and initial burst release of MTX; and b) the first order and Higuchi model fit (R² ~ 0.9) from the 10th day to the end of drug release, implying the therapeutic MTX release depends on its concentration and follows diffusion kinetics.
The pharmacokinetics and toxicity of the PLA-coated CS-MTX micro-implant (40% w/w MTX) was evaluated in rabbit eyes. High performance liquid chromatography showed a therapeutic release of MTX (0.1 – 1.0 µM) in the vitreous of the rabbit eyes for 33 days following first order kinetics ($R^2 \sim 0.88$). Histopathology analysis of the enucleated eyes failed to show any signs of toxicity and clinically significant inflammation. Electroretinography (ERG), a non invasive technique, confirmed no functional toxicity in the retina for the entire duration of study. Ultrasonography (US) showed the micro-implant did not disintegrate or dislocate during the course of the study. The PLA-coated CS-MTX micro-implant is therefore non-toxic and the lipophilic PLA coating enables sustained release of MTX (>1 month) in vivo.

Further lipophilic surface modification of the CS-MTX micro-implant surface is shown to improve and optimize the release duration of MTX. It was investigated that with an increase in the PLA content in poly-(lactic-co-glycolic acid) PLGA and with an elevated molecular weight of PLA, a) the initial burst of MTX and the mean release rate of MTX from the micro-implants can be reduced; b) the release duration can be improved from 2 to >5 months; and c) the swelling and biodegradation of the micro-implants can be delayed. The findings of this study can be used to develop a generic platform for sustained release of hydrophilic drugs with widespread clinical application.
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NOMENCLATURE

C: Concentration of drug (µM)
CS: Chitosan
I: Intensity of the light stimulus (mcd.s/m²)
K: Retinal sensitivity
M: Mass of drug (µg)
MTX: Methotrexate
PIOL: Primary intraocular lymphoma
PLA: Poly-lactic acid
PLGA: Poly-(lactic-co-glycolic) acid
t: Time (days)
V: Amplitude of the B-wave (µV)
VR: Vitreoretinal
Chapter 1. Introduction

1.1 Vitreoretinal diseases

Vitreoretinal (VR) diseases refer to the ocular disorders diagnosed in the back of the eye (posterior ocular segment), the part of the eye that includes the vitreous and the retina (Fig. 1.1). The treatment of VR diseases is a challenge as they are located at the back of the eye.

A few of the critical VR diseases include uveitis, proliferative vitreoretinopathy (PVR), age related macular degeneration (AMD), cytomegalovirus retinitis (CMV), diabetic retinopathy, choroidal neovascularization (CNV), retinitis pigmentosa (RP) and primary intraocular lymphoma (PIOL). According to the 2010 census records of the National Eye Institute, 4 million Americans suffer from vision impairment due to VR diseases.
Current drug administration protocols for the VR diseases include systemic drug administration, topical administration, periocular injections, intravitreal injections, and implantation of a slow-release implant containing a limited type of drugs. VR diseases are often refractory to the present drug administration techniques as they do not show therapeutic efficacy in the posterior segment due to the: a) ocular physiological barriers like the blood-retina barrier and blood-brain barrier, b) inconsistent drug concentration in the vitreous and c) rapid clearance of the administered drugs.

A sustained drug delivery approach is more appropriate for treating VR diseases such as CMV, AMD, PIOL and uveitis, where the local drug concentration is to be maintained over a long duration of time for therapeutic efficacy. A sustained drug delivery device can also be beneficial for diseases like PVR, diabetic retinopathy and CNV, where ocular complications and re-occurrence is observed post-surgery.

### 1.2 Challenges in treating vitreoretinal diseases

Conventional drug administration techniques such as topical eye drops and systemic administrations are ineffective to provide a therapeutic concentration of administered drugs over a long period of time in the VR domain. Topical administration (Fig. 1.2 A) of drugs is favorable for diseases related to the anterior segment, but is unfavorable for VR diseases as there is restricted penetration (< 5%) of the drugs in the VR domain and rapid drug clearance due to tear washout and aqueous humor flow [1]. It is reported that the drug concentration in the vitreous is several orders of magnitude lower than the drug concentration in the cornea when the drug is administered in the form of eye drops [2, 3]. Systemic administration (Fig. 1.2 B) is associated with limited drug penetration due to the blood-retina barrier. The blood-retina barrier is comprised of
two structures, outer and inner blood-retina barriers made up of retinal pigment epithelium (RPE) and the retinal capillary walls [3]. The blood-retina barrier obstructs transport of hydrophilic and large molecule drugs from the systemic blood to the VR domain and vice versa. Since only a small fraction of the drug can reach the VR domain from systemic and topical administrations, higher doses of drugs are administered to achieve and maintain a therapeutic concentration in the VR domain. This often induces unwanted systemic toxicity to collateral tissues.

Figure 1.2 Modes of administration – A) Topical – eye drops; B) Systemic; and C) Intravitreal injection
Intravitreal injections are employed to bypass the blood-retina barrier and administer the drugs directly in the VR domain (Fig. 1.2 C). The issues with intravitreal injections are, by definition: a) highly variable intravitreal drug concentrations over time, b) potential retinal toxicity, optic-disc injury, c) supra- and sub-therapeutic concentrations at target tissues over time, and d) potential for intraocular infection (Fig. 1.3). It is reported that intravitreal injections of hydrophilic drugs such as the test drug of this study, methotrexate (MTX), have short half-lives (~ 14.3 hours) and are associated with rapid clearance in the aqueous vitreous medium [4]. Repetitive administrations of intravitreal injections of hydrophilic drugs are therefore required to maintain the therapeutic efficacy. This often induces patient discomfort and causes escalated chances of collateral ocular complications like rhegmatogenous retinal detachment, cataracts, trauma, vitreous hemorrhage, endophthalmitis and ocular infection [1].

A sustained-release micro-implant that can be implanted by minimally invasive surgery is an attractive alternative to the repetitive intravitreal injections for treatment of selected VR diseases (Fig. 1.3).

Figure 1.3 Comparison of drug release profile between intravitreal MTX injection and a sustained release MTX micro-implant
The target disease of this study is PIOL. PIOL, a subset of primary central nervous system lymphoma (PCNSL), is a non-Hodgkin’s lymphoma of B-cell origin[5, 6]. PIOL provides a therapeutic challenge because of its diverse clinical presentations and variable clinical course. At present, repetitive intravitreal MTX injections are being used to treat many patients with PIOL, especially those with active CNS involvement. The drawbacks of this treatment are that it requires repetitive administrations to maintain effective MTX concentration in the vitreous, which are inconvenient, expensive, potentially painful and toxic to ocular tissues. There is no long-term intraocular sustained drug delivery device available for MTX to treat PIOL.

The model drug of this study, MTX, is a hydrophilic chemotherapeutic agent used to treat PIOL. It is a well-known anticancer drug which destroys cancer cells by obstructing an enzyme required for cell growth. Based on a prior numerical study, it has been determined that a sustained drug delivery device that can administer a therapeutic MTX concentration of 0.2-2.0 µg/day or 0.1-1 µM, for a period of a month or more is desired for improved treatment of PIOL (Fig. 1.3) [7]. If shown to be both safe and effective, such a sustained drug delivery device can also be used to treat other diseases such as uveitis and PVR, where MTX is used.

At present, the FDA-approved micro-implants in the market are used for sustained release of lipophilic drugs. These micro-implants use lipophilic materials such as poly-lactic acid (PLA), poly-(lactic-co-glycolic) acid (PLGA) and silicones to blend and administer the lipophilic drugs [8]. Such sustained-release intravitreal implants, which are presently FDA-approved, are Retisert™ (Bausch & Lomb) and Ozurdex™ (Allergan). The shortcoming is that there are no devices for sustained release of hydrophilic drugs in the VR domain. There is a need for a sustained release drug delivery system (micro-implant) to maintain therapeutic dosage of hydrophilic drugs (MTX) over a prolonged time period while avoiding toxicity.
In this study we have fabricated a novel intravitreal micro-implant for sustained release of hydrophilic drugs such as MTX for a period of >1 month. The micro-implant is based on biodegradable and biocompatible materials – chitosan (CS), poly-lactic acid (PLA) and poly-(lactic-co-glycolic) acid (PLGA), that have been tested extensively in ophthalmic human and animal studies. This micro-implant platform is expected to have widespread clinical application as it can be used for sustained release of other hydrophilic drugs like Ranibizumab and Afibercept, used to treat diseases like wet-AMD, which thereby makes this study significant.

1.3 Objective

The objective of the presented research was to develop a mechanically stable, biocompatible, biodegradable, non-toxic, easily implantable micro-implant that will provide sustained release of hydrophilic drugs such as MTX over a month or more in the vitreoretinal domain for possible improved treatment of VR diseases.

1.4 Hypothesis

The hypothesis of the current study was that the micro-implant will allow a sustained release of hydrophilic drugs near the retina, thus enhancing the efficacy of the drug for treating VR diseases.

1.5 Specific aims

To test the hypothesis, the specific aims of this study are as follows.

Specific aim 1: Fabricate a mechanically stable yet biocompatible and biodegradable intraocular micro-implant and quantify the release rate of hydrophilic drugs such as MTX in vitro.
Specific aim 2: Assess the *in vivo* drug release kinetics, toxicity and safety of the micro-implant and it’s implantation in a rabbit model using minimally invasive surgery.

Specific aim 3: Assess the *in vivo* toxicity and safety of the micro-implant using non-invasive techniques.

Specific aim 4: Improve the formulation and design of the micro-implant by studying the influence of the lipophilic surface modification on the sustained release of hydrophilic drugs.

1.6 Outline of thesis

The thesis consists of 8 chapters.

Chapter 1 provides an introduction to the VR diseases that are potential therapeutic targets of MTX-releasing micro-implants, and the challenges involved in treating the VR diseases. Chapter 1 also includes the objective, hypothesis and specific aims of the study.

Chapter 2 presents the background and literature review of target intravitreal diseases, present treatment methods, the issues with present treatment methods, sustained drug delivery approaches in VR diseases, commercially available sustained drug delivery products and their drawbacks, the challenge in administering *hydrophilic* active agents (MTX) and biodegradable polymer-based intraocular drug delivery systems.

Chapter 3 provides the methodologies that include details of the fabrication protocol of the micro-implant device, rationale of material selection, *in vitro* characterization of the micro-implant (structure, morphology, drug release study, and release kinetics analysis), *in vivo* characterization of the micro-implant using minimally invasive surgery and simultaneous assessment of toxicity using non-invasive methods.
Chapter 4 discusses the results obtained in the *in vitro* study of the MTX micro-implant. It details the results of the materials’ characterizations and drug release analysis.

Chapter 5 mentions the results obtained in the *in vivo* characterization of the MTX micro-implant in rabbit eyes using minimally invasive surgery. The pharmacokinetics analysis and toxicity evaluation using histopathology are provided in this chapter.

Chapter 6 discusses the results obtained from the non-invasive assessment of potential retinal toxicity of the micro-implant using electroretinography (ERG) and ultrasonography (US).

Chapter 7 provides the results obtained in the *in vitro* characterization of the improved CS-MTX intravitreal micro-implant by altering the lipophilic surface modification. The influence of the lipophilic coating on drug release kinetics and swelling of the micro-implant is discussed in this chapter.

Chapter 8 summarizes the work presented in this dissertation and discusses the future scope of this research.
Chapter 2. Background and Literature review

In this chapter, the background and a detailed literature review of the study is presented. First, the target vitreoretinal (VR) diseases of this study and the present treatment methods being employed to treat these diseases are mentioned. All issues with the existing treatment protocols are discussed thereafter. Further, a summary on the commercially available intraocular sustained drug delivery devices are mentioned followed by their limitations in administering hydrophilic drugs. The challenge to administer hydrophilic drugs in a sustained release manner in the eye is subsequently discussed. Lastly, a rationale of material selection for a device to cause sustained release of hydrophilic drugs in the eye is mentioned.

2.1 Target vitreoretinal (VR) diseases

As mentioned in Chapter 1, the VR diseases which are within the scope of this study include PIOL, AMD, uveitis, PVR or any VR diseases which can be treated with hydrophilic drugs. However, the target VR disease of this study is primary intraocular lymphoma (PIOL). In a recent report by the American Cancer Society, PIOL has been rated as the second most common primary intraocular cancer after intraocular melanoma.

Lymphoma is regarded as a type of cancer which is initiated in the immune system cells known as lymphocytes. There are 2 types of lymphocytes – B lymphocytes (B cells) and T lymphocytes (T cells). Lymphoma is caused by the restricted clonal proliferation of the B and / or T lymphocytes. The lymph nodes, which are bean size aggregates of lymphocytes, are where lymphoma is most frequently observed. Lymphoma is further categorized as 2 types: Hodgkin lymphoma and non-Hodgkin lymphoma (named after Dr. Thomas Hodgkin who described it).
According to a 2014 report of the American Cancer Society, B-cell lymphoma accounts for 85% of non-Hodgkin lymphoma (NHL) in the United States. The American Cancer Society also reports that 70,800 people will be diagnosed with NHL in the United States and 18,990 will die from this cancer in 2014.

PIOL, a non-Hodgkin lymphoma (NHL) of B-cell origin, is a subset of primary lymphoma of the brain known as the primary central nervous system lymphoma (PCNSL) [4, 7, 9-11]. It has been reported that PIOL occurs in approximately 25% of PCNSL patients [6, 12]. It is estimated that out of 1,927 PCNSL cases in 2006, 480 cases had IOL involvement [13]. Although PIOL is rare, the frequency of its occurrence has increased in the last few years in immunosuppressed and immunocompetent subjects [14]. Some cases of PIOL are associated with improper defective immune systems such as acquired immunodeficiency syndrome (AIDS). It is reported that the risk of developing PCNSL increases $3,600 \text{fold}$ in AIDS patients compared to normal subjects [15].

PIOL refers to the nonmetastatic malignant lymphoid neoplasia that develops primarily inside the eye [16]. It is distinguished from intraocular malignant lymphoid tumors and infiltrates that develop metastatically from systemic non-Hodgkin lymphoma [16]. Approximately 100 new cases of PIOL were diagnosed in the United States between 2000 and 2005 [7, 17, 18]. PIOL is manifested in 3 major clinical forms as follows, 1) primary vitreo-retinal lymphoma (PVRL), 2) primary uveal lymphoma, and 3) PIOL with overlapping vitreo-retinal and uveal features [19]. PVRL, which accounts for 85% of PIOL cases in the United States, is characterized by the accumulation of pale lymphoid cells in the vitreous, development of tiny dot-like to extensive geographic pale subretinal infiltrates, frequent bilaterality, and common association with independent primary lymphoid infiltrates in the brain (primary central nervous system lympho-
Due to the diverse clinical presentations of PIOL and its variable course, the diagnosis and management of PIOL is a *therapeutic challenge*.

### 2.2 Present treatment methods

The detailed anatomy of the eye is provided in Fig. 2.1 showing the aqueous humor, cornea, iris, conjunctiva and the lens form the anterior segment and the choroid, vitreous humor, retina form the posterior segment. The different routes of intraocular drug administration with respect to the ocular structures are shown in Fig. 2.2.

![Figure 2.1 Detailed anatomy of the eye](Adapted from source: National Eye Institute, National Institutes of Health)
Figure 2.2 Various routes of drug administration in the eye [20]

The most prevalent treatment methods include the systemic administration and the topical administration. However, depending on the type of VR disorder and the drug used to treat it,
these methods may lack therapeutic efficacy for diseases related to the posterior segment. The systemic administration of drugs is usually given orally or intravenously. The drug enters the bloodstream and is distributed throughout the body, but may fail to cross the lipophilic blood-retina barrier in sufficient amounts to attain an effective therapeutic concentration in the posterior ocular segment as shown in Fig. 2.1. Further, the blood retina barrier being lipophilic in nature causes a particular obstacle for the transport of hydrophilic drugs into the posterior segment. Since there is limited ocular penetration via the systemic route, higher and more frequent dosages are required for maintaining therapeutic concentration in the posterior segment. This often causes troublesome side effects of possible toxicity to other tissues in the body.

Topical administration, as shown in Fig. 2.2A, is the most common mode of drug administration to the eye. It is also the least invasive. This mode of administration also has its drawbacks when used to treat diseases in the posterior segment. Drugs applied topically frequently fail to cross the cornea (which is made up of corneal epithelium, stroma and corneal endothelium as shown in Fig 2.1) in sufficient amounts to attain a satisfactory intraocular concentration even in the aqueous humor. In addition to this barrier, the majority of the drug (95%) is washed out by the tears. Further, other factors like drug binding to anterior segment structures, drug metabolism, and the diffusion distance to the posterior segment cause a limited uptake of the drug in the posterior segment with a short acting therapeutic efficacy.

In order to avoid the drug wastage and improve the uptake of the drug at the target site of the posterior segment, intravitreal injections and sustained release devices are employed to administer drugs locally in the posterior segment.

Intravitreal injection as shown in Fig. 2.2G is an invasive procedure. This procedure administers the drug directly into the vitreous, bypassing all the physical physiological barriers. How-
ever, it has drawbacks especially while administering hydrophilic drugs such as MTX. The composition of the vitreous humor comprises 98% water. Therefore, hydrophilic drugs when administered using intravitreal injection have a short half life as they are rapidly washed away from the vitreous. Repetitive injections are required to maintain therapeutic concentrations of the drug in the target issues, which may cause collateral ocular toxicity and other complications as reported later.

Sustained release devices facilitate achieving a therapeutic concentration of the drug at the target site over a long duration. A variety of sustained release device routes are shown in Fig. 2.2. A particular route of a sustained release device is determined based on the site of the posterior segment disease. Sustained release devices require surgery to be administered. There are two categories of sustained release devices: 1) non-biodegradable devices, which must be removed after drug administration is completed, and 2) biodegradable devices, which do not need to be removed after the completion of drug administration. Sustained release devices for intraocular drug administration are discussed in detail later.

Current treatment of PIOL consists of a) systemic multi-drug chemotherapy [10, 11], a treatment in which only a limited quantity of the intravenously administered drugs cross the blood-retinal barrier and reaches the eye; b) fractionated external beam radiation therapy, a treatment which has been proven to be fruitful in the remission of tumors but can be associated with complications such as radiation-induced superficial punctuate keratopathy, keratoconjunctivitis sicca, radiation induced cataract (in phakic eyes) and radiation induced retinopathy [9-11, 21]; and c) multiple intravitreal injections of the hydrophilic drug methotrexate (MTX) [9-11].
2.3 Issues with present treatment methods

At present, one of the accepted treatments of PIOL is repeated intravitreal injections of MTX [4, 22-24]. Although MTX injections have proven to be an effective treatment for PIOL, Velez et al. [4] reported that 400 µg intravitreal dose of MTX provided a therapeutic level of the drug (> 0.5µM) for only about 48-72 hours in non-victrectomized rabbit eyes. MTX intravitreal injection has a short half life (t½) of 14.3 hours [7]. Because of this, repetitive administration of MTX intravitreal (IVIT) is required to maintain cytotoxic concentrations of the drug over a sufficiently long duration to eradicate PIOL.

Frenkel et al. [23] reported that 44 eyes in 26 patients involved with PIOL developed conjunctival hyperaemia and some form of keratopathy when treated with intravitreal MTX injections. The nature of keratopathy, noticed after the 3rd injection, varied from diffuse punctuate keratopathy to severe epitheliopathy. These complications subsided when the frequency of injections were reduced to monthly injections from weekly injections.

In another study by Smith et al. [24], the efficacy and safety of intravitreal MTX to treat PCNSL involving the eye was conducted. The complications which occurred during treatment and follow up included cataract (73% of 26 eyes), corneal epitheliopathy (58% of 26 eyes), maculopathy (42% of 26 eyes), vitreous hemorrhage (8% of 26 eyes), optic atrophy (4% of 26 eyes) and sterile endophthalmitis (4% of 26 eyes). However, in the review of Rajagopal et al. [9], it is reported that these complications are reduced in recent times as more experience with the MTX injection has been gathered.

Shortcomings of currently advocated IVIT protocols for intravitreal MTX injections for PIOL include a) the need for multiple injections at relatively shorter intervals; b) the likelihood
of sub-therapeutic intravitreal concentration of MTX between injections; and c) the increased risk of avoidable ocular complications with multiple injections.

### 2.4 Sustained drug delivery approach in vitreoretinal diseases

There is a need for a sustained release drug delivery system (micro-implant) for maintaining the therapeutic dosage of MTX over a prolonged time period while avoiding unwanted systemic and local toxicity, if possible. In the most recent study of our group by Palakurthi et al.[7], the retinal permeability of MTX in rabbit and human eyes was evaluated. The pharmacokinetics of MTX in the human eye following MTX intravitreal injection versus micro-implant implantation was evaluated. Our conceptual goal was to develop a MTX intravitreal micro-implant that would deliver MTX within the therapeutic window of 0.2-2.0 µg/day or 0.1 – 1 µM for a period of a month or more. Sustained release of MTX is also preferred as its time effect (sensitivity of the cells to MTX increases with time) is reported to be greater than its dose effect [25].

The challenges for administering hydrophilic drugs such as MTX in the vitreous environment include: a) preventing rapid release of MTX from the micro-implant matrix and b) finding a polymer matrix which blends well with MTX. The key factors for choosing the components of a polymer matrix for administering hydrophilic drugs are a) their nature (lipophilic or hydrophilic type), b) molecular weights, c) glass transition temperature (Tg), d) their biocompatibility, e) their biodegradability, and f) their mutual compatibility.

As mentioned before, ocular sustained drug delivery devices are classified as a non-biodegradable and a biodegradable device. The non-biodegradable devices are mainly reservoir type devices where the drug is present in the core of the device and is gradually released across a semi-permeable non-biodegradable polymer barrier. It is reported in the review of Lee et al.
[8] that the drug release mechanism from the reservoir type matrices follows zero order kinetics after a steady state concentration is achieved. The drug release is nearly constant and independent of the drug concentration in the core, as long as the drug is present in the core of the device. The non-biodegradable materials used to make reservoir type devices are silicones and a combination of polymers such as polyvinyl alcohol (PVA) and ethylene vinyl acetate (EVA) [1, 8]. The drawbacks of the non-biodegradable devices include: a) their relatively large size, b) their persistence after the entire drug has been released and c) the surgical removal of the device after the completion of drug release, which causes discomfort.

The biodegradable devices are matrix type devices in which the drug and the binding polymer are blended to form a homogeneous structure in the shape of a pellet, fiber, plug, implant, etc. The polymers used to blend and bind the drug are of a biodegradable nature, which are metabolized by enzymatic or non-enzymatic degradation to form biocompatible products. The most commonly used biodegradable polymers for intraocular sustained drug delivery devices are polyglycolic acid (PGA), polylactic acid (PLA) and poly-(lactic-co-glycolic) acid (PLGA). The degraded products of these polymers are metabolized to produce carbon dioxide and water. The PGA, PLA and PLGA based intravitreal implants show a tri-phasic drug release profile [1, 26]. An initial burst release is noticed in the first few days followed by a steady diffusion release and lastly, a late sudden burst is observed due to the disintegration of the polymer matrix. An example of a tri-phasic profile is shown in Fig. 2.3 when PLA-based ganciclovir (GCV) scleral implant was used to treat CMV in the study of Kunou et al. [26].
PLA is composed of two enantiomeric monomers, which are D-Lactic acid and L-Lactic acid, as shown in Fig. 2.4 [27]. As a result, PLA exists in two enantiomeric forms. They are poly D-lactic acid (PDLA) and poly L-lactic acid (PLLA).
The physicochemical and mechanical properties of the PLA are influenced by the respective contribution of each enantiomeric form of PLA (PDLA or PLLA) in the PLA structure. The structure of PLA has a hydroxyl group in each of the enantiomers, which is oriented in opposite directions with respect to the main backbone structure of the PLA. This difference of orientation of the hydroxyl group governs the physicochemical and mechanical properties of PLA. The contribution of the respective enantiomeric forms in the PLA structure influences the crystallinity of the polymer. It is expected that the PLA comprising only PLLA or PDLA to be relatively more crystalline than PLA comprising both PLLA and PDLA. This is because in the PLA comprising of only PLLA or PDLA, the polymer chains are oriented in the same direction, yielding a more crystalline structure. In the case of PLA comprising both PLLA and PDLA, the polymer chains are oriented in opposite directions, yielding a more amorphous structure. The degree of crystallinity of the PLA structure is an important factor to be considered while fabricating a drug delivery device because it influences the glass transition temperature (Tg), swelling characteristics, ability to go through hydrolysis and biodegradation of the polymer, as discussed later.

PGA is composed of glycolic acid monomers. Unlike PLA, PGA does not have any methyl group in the structure backbone (Fig. 2.5). The absence of the methyl group in PGA results in a relatively more crystalline structure compared to that of PLA. The presence of the methyl group in a structure directly promotes lipophilicity. Since PGA does not have a methyl group, it is expected that PGA is relatively less lipophilic compared to PLA. Therefore, it can be anticipated that PGA would undergo hydrolysis relatively faster than PLA, leading to a quicker biodegradation.
PLGA is made up of both PLA and PGA. Unless reported, PLGA refers to poly (D,L-lactic-co-glycolic acid), where D- and L-Lactic forms are present in equal contribution [27]. The synthesis of PLGA involves random ring-opening copolymerization of the lactic acid and glycolic acid [8, 26] as shown in Fig. 2.6. PLGA is formed by the ester linkages of lactic acid and glycolic acid monomers.

![Diagram of PLGA synthesis](image)

Figure 2.6 Synthesis of PLGA from PLA and PGA
The physicochemical, mechanical and biodegradation properties of PLGA are governed by the respective contribution of PLA and PGA. An increase in the content of PLA in the PLGA structure would impart increase lipophilicity and molecular weight due to the presence of the methyl group. However, the crystallinity of the PLGA would depend on the crystallinity of the PLA structure. As discussed before, if the PLA is comprised of PLLA or PDLA, it would increase the crystallinity of the PLGA. However, if the PLA is made up of D, L-PLA, then the PLGA structure would be less crystalline. The presence of more PGA in PLGA contributes in an improved crystallinity and reduced lipophilicity of the PLGA structure.

The degree of crystallinity and the molecular weight directly governs the glass transition temperature (Tg) of the polymer. Tg plays a pivotal role in the drug release mechanism. If the Tg of the polymer is less than the physiological temperature (37°C), then the polymer matrix will soften in the *in vivo* environment, causing structural deformation and an increased flux of drug release from the polymer matrix. The Tg of a polymer increases with an increase in the degree of crystallinity and the molecular weight of the polymer. An increase in PGA content in the PLGA structure is expected to improve the crystallinity of PLGA, thereby raising the Tg of PLGA.

The biodegradation of the PGA, PLA and the PLGA polymers is caused by the hydrolysis of the ester linkages present throughout the polymer matrix, causing bulk erosion of the surface. As discussed before, PLA undergoes slower hydrolysis compared to PGA because of the presence of a methyl group. Therefore, an increase in the PLA content in PLGA would cause the PLGA to be more lipophilic, thereby reducing the rate of biodegradation. Therefore, the degradation time of the PLGA based intraocular devices can be altered by varying the copolymer ratio (PLA: PGA).
The critical physicochemical properties of the polymers (PLA, PGA and PLGA) can therefore be altered by varying the molecular weight, degree of crystallinity and the glass transition temperature (Tg) of the polymer. The issue with existing polymer matrices (PLA, PGA and PLGA) is that they are lipophilic in nature and do not blend well with hydrophilic drugs like MTX. Another disadvantage of these hydrophobic (PLA, PGA and PLGA) matrices is that they degrade very slowly even after the drug has been released, causing local toxicity [28].

2.5 Commercially available sustained drug delivery products

Various intravitreal drug delivery systems have been based on non-biodegradable materials like PVA, EVA and silicones, and lipophilic biodegradable polymers like lactic and glycolic acid-based matrices such as PLA, PGA and their co-polymer PLGA [26, 29, 30].

Retisert® is a silicone based disc-shaped non-biodegradable implant of dimensions (3mm x 2mm x 5mm) administering corticosteroid Fluocinolone acetonide (FA) to treat uveitis and diabetes macular edema (DME) over a period of 30 months [8]. Vitrasert® is a PVA and EVA based implant to administer GCV over a period of 5-8 months to treat CMV retinitis.

The focus of this study is to fabricate a biodegradable micro-implant device so that the necessity for the surgical removal of the device post-therapy can be avoided. The summary of the existing biodegradable devices for intraocular drug administration is presented in Table 1.
Table 1. Summary of commercially available and on-going research on biodegradable intraocular devices [8]

<table>
<thead>
<tr>
<th>Biodegradable implants</th>
<th>Manufacturer</th>
<th>Drug / Active agent</th>
<th>Polymer / Delivery vehicle</th>
<th>Duration</th>
<th>Eye diseases</th>
<th>Price per dose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ozurdex</td>
<td>Allergan, Inc</td>
<td>dexamethasone lipophilic</td>
<td>PLGA lipophilic</td>
<td>6 months</td>
<td>uveitis, macular edema</td>
<td>$1295 bi-annually</td>
</tr>
<tr>
<td>Surodex</td>
<td>Allergan, Inc</td>
<td>dexamethasone lipophilic</td>
<td>PLGA lipophilic</td>
<td>7-10 days</td>
<td>post-operative inflammation (cataract surgery)</td>
<td>N/A not approved in USA</td>
</tr>
<tr>
<td>Verisome&lt;sup&gt;TM&lt;/sup&gt;IBI 20089</td>
<td>ICON Bioscience Inc.</td>
<td>triamcinolone acetonide lipophilic</td>
<td>proprietary lipophilic gel</td>
<td>~ 1 year</td>
<td>cystoid macular edema, uveitis</td>
<td>N/A phase II trials</td>
</tr>
</tbody>
</table>

In all of these studies, the drug administered is lipophilic in nature, which binds well with a lipophilic polymer matrix reservoir made of PLA, PLGA or silicones. Also, the lipophilic drugs have a sustained release due to its inherent property of limited diffusivity in the aqueous vitreous medium of the eye. The obvious challenge for administering hydrophilic drugs like MTX to treat PIOL is the choice of polymer matrix, which blends well with hydrophilic compounds and causes sustained release of the same compounds such as MTX.

2.6 Biodegradable polymer based intraocular drug delivery systems for hydrophilic drugs

Chitosan (CS), which is known for biocompatible, biodegradable and non-toxic material, is a potential candidate for drug delivery systems inside the eye [31, 32]. CS has been considered Generally Recognized as Safe (GRAS) material by the US FDA and is also listed as a food additive in countries like Finland, Italy and Japan [33]. CS is a copolymer of N-acetylglucosamine and glucosamine, which is fully or partially a N-deacetylated (DA) derivative of the natural polymer, Chitin as shown in Fig. 2.7.
CS nanoparticles containing 5-Fluorouracil showed no signs of inflammation or irritation when tested in rabbit eyes [35]. In another study by Yang et al. [36], CS was investigated as a tamponade material to treat PVR in rabbit eyes. CS showed no significant effect on the histology of the eye and did not affect the intraocular pressure or exhibit any severe inflammatory responses, which makes CS a promising choice for intravitreal applications. The degradation of CS is primarily related to the degree of deacetylation (DA%) and it is also degraded in the presence of hydrolytic enzymes like lysozymes. The presence of lysozymes in the vitreous has been
reported by Stainer et al. [37]. The degradation of CS by lysozyme leads to the formation of aminosugars, which are readily metabolized without any toxicity development. CS has also been used as a drug delivery vehicle for MTX in many formulations because of its similar hydrophilic nature [25, 38-41] and the presence of the free –NH₂ and –OH groups in their respective structures. CS-gellan based MTX implants showed MTX release for a period of 30 days [25]. A poly (vinyl alcohol)-gellan layer was used for MTX reservoir, which was placed in between two layers of CS. A sustained release of MTX was observed due to the lipophilic poly(vinyl alcohol)-gellan layer. The implants when tested in vivo revealed no inflammation in the implantation site and a good parity of in vivo and in vitro drug release was observed. In the study of Singh et al. [40], MTX loaded CS and chitin microspheres showed better antitumor activity in Ehrlich ascites tumor bearing mice when compared to oral administration of MTX. The drug plasma concentration is also reported to be sustained. Sun et al. used MTX loaded CS microspheres for nasal administration where minor ciliotoxicity was reported [39]. Seo et al. prepared MTX loaded methoxy poly(ethylene glycol)-grafted CS nanoparticles and the sustained anti-proliferation effect of these nanoparticles was observed on the B16F10 melanoma cell line [41]. In a recent study by Trapani et al., MTX loaded CS and glycolchitosan based nanoparticles were fabricated to administer MTX to brain tumors where MTX loaded nanoparticles showed cytotoxic activity against the C6 glioma glial cell line [42].

In this study, a CS and PLA based MTX sustained release intravitreal micro-implant was fabricated that could be implanted intravitreally using minimally invasive surgical methods. Without the PLA coating, the MTX micro-implants are expected to release their MTX rapidly in the vitreous because of the similar hydrophilic nature of both CS and MTX. The CS-MTX micro-implant is therefore coated with a lipophilic coating of PLA for sustained release of MTX. The
hypothesis of this study is that the PLA-coated CS-MTX intravitreal micro-implant will administer MTX within the therapeutic window for a period of over one month, thereby enhancing the efficacy of the drug and improving its prospects for suppression and/or elimination of target VR disorders.

2.7 Summary of the background and literature review

Due to the diverse clinical presentations of the PIOL and its variable course, the diagnosis and management of PIOL is considered a therapeutic challenge. At present the repetitive intravitreal MTX injection is an accepted treatment, which is associated with inconsistent MTX concentrations in the vitreous, potential traumatic injury to the eye, and collateral side effects to the ocular tissues. A sustained release intravitreal MTX micro-implant administering therapeutic concentration of MTX over a period of 1 month is expected to improve the treatment of PIOL. This chapter will serve as the background for subsequent chapters such as Chapter 3, where the methods of in vitro and in vivo studies are reported; Chapter 4, where the results of the in vitro study is discussed; Chapter 5, where the results of the in vivo study is reported; Chapter 6, where the results of the non-invasive assessment of retinal toxicity is mentioned; Chapter 7, where the results of the in vitro study related to the improved CS-MTX micro-implant is presented; and Chapter 8, where the future scope of this research is proposed.
Chapter 3. Materials and Methods

In this chapter, we first describe the fabrication protocol of the micro-implants. Thereafter, the characterization techniques to analyze the in vitro release rate of MTX from the micro-implants are described. Then, methods to evaluate the PLA-coated CS-MTX micro-implants using drug release kinetics (pharmacokinetics) and toxicity analysis (histopathology) in normal rabbit eyes are described. Furthermore, the procedures involving the non-invasive assessment of retinal toxicity using electroretinography (ERG) and micro-implant position and degradation in the in vivo environment using ultrasonography are reported. Lastly, the in vitro characterization of the improved CS-MTX micro-implant with altered lipophilic surface modification is presented.

3.1 Fabrication of the Micro-implant Device

In this section, the rationale behind the materials selection is provided, followed by steps employed to fabricate the sustained release MTX micro-implant is presented. This section is the methodology for the Specific Aim 1 of this study.

3.1.1 Rationale of Material Selection

The micro-implant is developed to administer the hydrophilic drug, MTX, over a period of 1 month in a sustained release manner. As mentioned in Chapter 2, the micro-implant technologies for sustained release of hydrophilic drugs in the VR domain are not commercially available. The commercially available micro-implant technologies are fabricated to administer lipophilic drugs, such as steroids, and are manufactured using FDA approved lipophilic polymers like PLA, PGA and PLGA.
In a prior animal study of our group, PLA-based micro-needle implants were fabricated for sustained release of MTX in normal rabbit eyes [16]. The micro-needle implants were prepared using a blend of D,L-PLA of molecular weights 90,000 and 6,500 in the ratio of 80:20, respectively. It was observed that the PLA did not blend well with MTX. The PLA based MTX micro-needle implant degraded in the *in vivo* environment more rapidly than desired. The rapid degradation was probably caused by the softening of the micro-needle structure of the implant in the physiological temperature due to the low glass transition temperature (Tg) of the PLA blend. No pharmacokinetic data was obtained due to the degradation of the micro-needle implant. However, the micro-needle implant was found to be non-toxic in the rabbit eyes.

The critical factors to be taken into account for fabricating a sustained release micro-implant for a *hydrophilic* drug such as MTX are a) choice of a *hydrophilic* polymer matrix for uniform blending of the *hydrophilic* drug; b) choice of a lipophilic polymer to facilitate sustained release of the *hydrophilic* drug from the *hydrophilic* matrix; and c) material properties of the polymers such as Tg, molecular weight, biocompatibility and biodegradability.

Based on the prior studies involving CS and MTX and their proven biocompatibility (Chapter 2), CS is chosen as the *hydrophilic* polymer matrix for blending MTX. It is expected that MTX will rapidly release in the aqueous vitreous medium, as CS and MTX are both *hydrophilic* in nature (Fig. 3.1).

![Figure 3.1 Formulation of the Chitosan-Methotrexate (CS-MTX) micro-implant](image)

**Figure 3.1 Formulation of the Chitosan-Methotrexate (CS-MTX) micro-implant**
A lipophilic coating on the CS-MTX micro-implant is required for the sustained release of MTX. Being biocompatible and having extensive application in fabrication of ocular implants, high molecular weight PLA (inherent viscosity of 1.16 dL/g in CHCl₃ @ 30°C) is the preferred choice for the lipophilic surface modification of the CS-MTX micro-implant for sustained release of MTX (Fig. 3.2).

![Diagram](image_url)

Figure 3.2 Formulation of the PLA-coated CS-MTX micro-implant

### 3.1.2 Materials and Fabrication Protocol

MTX (MP Biomedical) was mixed with the low molecular weight CS (M.W. 50,000-190,000 and DA% ≥ 75%; Sigma Aldrich) in dilute HCl to make different mixtures of 10%, 25% and 40% w/w drug loadings. These mixtures were then injected into Tygon® tubing (1/16 in I.D). The tubes containing the mixture were lyophilized at a temperature below -40°C and a pressure below 1200 mTorr for 2 hours (Millrock BT48A, Millrock Technology) to obtain CS-MTX fibers. The CS-MTX fibers extracted from the Tygon® tubing were cut into the desired micro-implant lengths using a surgical knife under an optical microscope to ensure accurate dimensions of the micro-implant.

DL-PLA (inherent viscosity of 1.16 dL/g in CHCl₃ @ 30°C) (Lactel Biodegradable Polymers) was mixed in Dichloromethane (Fisher Sci.) to synthesize a 40 mg/ml coating solution. The CS-MTX micro-implants were then dip coated in the PLA coating solution for a lipophilic surface coating. The dip coating protocol was carried out on both longitudinal directions of the
micro-implant to ensure uniform coating on the surface and on the two ends of the micro-implant. Each micro-implant was dipped in the PLA solution for 5 seconds and dried at room temperature for 2 minutes. This dipping process was carried out 3 times in each direction, longitudinally. The manufacturing steps are shown in Fig. 3.3. Subsequently, the micro-implants were dried overnight at room temperature in dark conditions. After the initial drying, the micro-implants were vacuum-dried overnight at 45°C to evaporate the dichloromethane (DCM) from the micro-implant.

The fabrication protocol had been consistent while preparing the micro-implants for the in vitro characterization (section 3.2), in vivo characterization using minimally invasive surgery (section 3.3), and in vivo characterization using non-invasive methods (section 3.4). However, the fabrication protocol was slightly altered for the study on influence of lipophilic surface modification on the MTX release rate and swelling of the micro-implants (section 3.5). Furthermore, only 40% w/w CS-MTX micro-implant was characterized in section 3.3, section 3.4 and section 3.5, as 40% w/w CS-MTX micro-implant contains the same dosage of MTX as administered in 1 dose of intravitreal MTX injection (400µg).

In section 3.5, the polymers used for the lipophilic surface modification were a) different copolymer ratio of poly-(lactic-co-glycolic acid) (PLGA) and b) different molecular weights of DL-PLA. The different types of PLGA investigated were PLGA 5050, PLGA 6535 and PLGA 7525, where the copolymer ratio of PLA:PGA in PLGA were 50:50, 65:35 and 75:25, respectively. The different types of DL-PLA used were DL-PLA with inherent viscosities 0.67 and 1.16 dL/g in CHCl₃ @ 30°C. All the polymers used for the lipophilic surface modification were obtained from Lactel Biodegradable Polymers. Furthermore, another modification was done in
the dip-coating procedure. While the dipping process was carried out 3 times in each direction, longitudinally in the initial micro-implant prototype manufacturing; the dipping process employed in the lipophilic surface modification study involved 4 times in each direction, longitudinally. This ensured a more consistent coating on the two ends of the micro-implants.

Figure 3.3 Flowchart of the fabrication steps involved in making the PLA-coated Chitosan-Methotrexate micro-implant

3.2 *In vitro* Characterization of the Micro-implant

The *in vitro* characterization of the micro-implant involved the structure and materials characterization, and drug release analysis. First, the methods used for structure characterization are discussed followed by the drug release analysis. This section is the methodology for the Specific Aim 1 of this study.

3.2.1 Structure Characterization

In this section the characterization techniques employed to analyze the micro-implant’s material properties are discussed. The characterization techniques that are adopted here are: a) Opt-

*Dimensions and Morphology.* Optical Microscopy (Keyence Digital Microscope, VHX-600) was used to assess the micro-implant’s dimensions and appearance. Scanning Electron Microscopy (SEM) (FEI XL 30-FEG, FEI) was used to assess the microstructure and morphology using an accelerating voltage of 15 KV. The micro-implant samples were sputter-coated prior to the SEM analysis in Argon plasma using an Au-Pd target for 1 minute to make them conductive.

*Lipophilic Modification of the Coated Micro-implant Surface.* Time of Flight-Secondary Ion Mass Spectroscopy (ToF-SIMS) was used to assess the lipophilic modification of the micro-implant’s surface. ToF-SIMS was performed using a ToF-SIMS IV instrument (IONTOF Inc.). Secondary ions are produced from a Ga+ primary ion source at 15 KV accelerating voltage and 1.5 pA current raster over a 200 µm by 200 µm area of the sample. The secondary ions produced are analyzed in high-current bunched mode with analyzer energy of 2 KV. The ion peaks were assigned using SurfaceLab 6 software (IONTOF Inc.). Differential Scanning Calorimetry (DSC) was used to measure thermal properties of the micro-implants at the physiological temperature ~38°C. DSC was performed at the heating rate of 10 °C/min. (DSC6200, Seiko Instruments Inc.).

### 3.2.2 *In vitro* MTX Release Studies

The micro-implants were kept in vials containing 5ml of phosphate buffered saline (PBS; pH 7.4). Each micro-implant weighed ~ 1 mg. The micro-implants containing 40% w/w MTX contained ~ 400 µg of MTX, 25% w/w MTX contained ~ 250 µg of MTX and 10% w/w MTX contained ~ 100µg of MTX. The vials were slowly stirred in a water bath maintained at 38°C. A
sample of 1 ml of release media (PBS) containing MTX was taken out at pre-determined time intervals. To maintain sink conditions, 1 ml of fresh PBS was added. The concentration of MTX present in 1 ml of release media sample was assayed using an UV-Visible Spectrophotometer (Cary 50-Bio UV-Vis Spectrophotometer, Varian) at the characteristic MTX wavelengths (258, 302 and 372 nm) [43]. The calibration of MTX absorbance in the UV-Visible Spectrophotometer was done using MTX standard concentrations in PBS. A calibration curve was derived from the absorbance readings obtained from the MTX standards and the molar absorptivity of MTX was determined. The MTX release rate data was obtained from the absorbance values of the corresponding time points of each sample as explained in Table 2.
Table 2. Flowchart to obtain release rate data from absorbance readings

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Absorbance (a.u.)</th>
<th>Concentration (mM)</th>
<th>Total drug in 5ml of sample release media (mg)</th>
<th>Amount of drug remaining in the vial after 1 ml sample release media taken out (mg)</th>
<th>Release rate (µg/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>$D_{n-1}$</td>
<td>$A_{n-1}$</td>
<td>$C_{n-1}$</td>
<td>$X_{n-1}$</td>
<td>$(Z_{n-1})/5$</td>
<td>$R_{n-1}$</td>
</tr>
<tr>
<td>$D_n$</td>
<td>$A_n$</td>
<td>$C_n$</td>
<td>$X_n$</td>
<td>$(Z_n)/5$</td>
<td>$R_n$</td>
</tr>
<tr>
<td>$D_{n+1}$</td>
<td>$A_{n+1}$</td>
<td>$C_{n+1}$</td>
<td>$X_{n+1}$</td>
<td>$(Z_{n+1})/5$</td>
<td>$R_{n+1}$</td>
</tr>
</tbody>
</table>

1. The predetermined time points are $D_n$.
2. For each time point $D_n$, we obtain the corresponding absorbance values ‘$A_n$’ using UV-Visible spectrophotometer.
3. $C_n$ is the corresponding concentration at time point $D_n$ for absorbance $A_n$. $C_n$ is obtained from the calibration curve.
4. Since we know the concentration, we can calculate the mass of the total drug present in the 5 ml of PBS containing the micro-implant. $X_n$ is the mass of the total drug present in the release media of 5 ml at time point $D_n$.
5. 1 ml of release media is taken out for UV-visible spectrophotometer reading, therefore the amount of drug taken out for sampling is $(X_n)/5$ at time $D_n$.
6. 1 ml of fresh PBS is added to maintain the total volume of 5 ml.
7. Amount of drug remaining in 5 ml of PBS $= X_n - (X_n)/5 = Z_n$.
8. Amount of drug released at time point ‘$D_n$’ $= X_n - Z_{n-1} = R_n$.
9. Release rate at time point ‘$D_n$’ is $P_n = R_n / (D_n - D_{n-1})$.
10. Kindly note at the first time point, $R_n = X_n$. 

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Drug release data of all drug loadings of the *coated* micro-implants were fitted to zero order equation, first order equation, Higuchi model and Korsmeyer-Peppas model in order to analyze the mechanism of drug release and diffusion kinetics. The fitting of each model was evaluated based on correlation coefficient ($R^2$) values.

### 3.3 *In vivo* Characterization of the Micro-implant using Minimally Invasive Surgery

The *in vivo* characterization of the PLA-coated CS-MTX micro-implant includes implantation of such micro-implant in animal study, subsequent pharmacokinetics analysis and toxicity evaluation using histopathology study. This section is the methodology for the Specific Aim 2 of this study. The micro-implant containing 40% w/w MTX was used in this study (~ 400 µg of MTX) so that the pharmacokinetics and safety of the micro-implant could be compared to an intravitreal MTX injection containing the same dosage of 400 µg of MTX, which is the present treatment protocol for PIOL. The PLA-coated CS-MTX micro-implants were manufactured following the same procedure as mentioned in section 3.1.2. The micro-implants were sterilized under UV radiation for 20 minutes before implantation into the rabbit eye.

#### 3.3.1 Design of the *In vivo* Study

Eight immune-competent New Zealand white rabbits, each weighing 2-3 kg, were used in this study. Each rabbit underwent implantation of a sterilized MTX-containing micro-implant in one eye (right eye) and a sterilized micro-implant without MTX in the other eye (left eye) (see Surgical Procedures below). Two rabbits were euthanized on each of the following post-surgical days: day 5, day 12, day 19 and day 33 (Table 3). All rabbit surgery and animal care in this research study were performed in accordance with Institutional Animal Care and Use Committee
(IACUC) protocol # 12-09-13-01 of the University of Cincinnati (approval date 21 November 2012). At each specified time point, both eyes of each animal were removed immediately after death, and a vitreous specimen of both eyes of one of the animals euthanized was obtained immediately thereafter for pharmacokinetics analysis (see section 3.3.5). The eyes of the other animal were fixed in the neutral buffered formalin solution and processed for histopathological analysis (see section 3.3.6). The methodology employed for ERG and ultrasound study assessment is discussed in section 3.4.
Table 3. Summary of *in vivo* experiment

<table>
<thead>
<tr>
<th>Days</th>
<th>Rabbit #</th>
<th>Experiment conditions</th>
<th>Pharmacokinetics</th>
<th>Histopathology</th>
<th>ERG</th>
<th>Ultrasound</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Right eye *</td>
<td>Left eye **</td>
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* Right eye received MTX micro-implant  ** Left eye received placebo micro-implant
3.3.2 Surgical Procedures

The vital signs (heart rate, respiration rate, temperature) of each animal were evaluated prior to surgery and verified to be within normal limits. A 22-gauge catheter (Surflash) was inserted into a marginal vein for intravenous administration of drugs and intravenous fluids. Each rabbit was anesthetized with a mixture of xylazine hydrochloride (5 mg/kg) and ketamine hydrochloride (30 mg/kg) by intramuscular injection. Buprenorphine hydrochloride 0.04 mg/kg was administered subcutaneously as an analgesic. Isoflurane 1-2.5% was administered as needed to deepen the anesthesia. The anesthetized rabbit was prepped and draped in the usual fashion for a procedure of both eyes. Tetracaine hydrochloride 0.05% eye drops were administered topically to the eyes to supplement the anesthesia. One drop of moxifloxacin 0.5% was also administered to each eye prior to the procedure. An eyelid speculum was used to expose the right eye. Using a 1.1mm side port paracentesis surgical knife, a full thickness eye wall incision was created parallel to the limbus at a measured 6 mm from the limbus in the superotemporal quadrant. A MTX-loaded micro-implant was passed through the eye wall incision using a McPherson forceps. The conjunctival-scleral wound was immediately closed with a mattress suture of 7-0 polyglactin 910 (suture material: Ethicon, Cincinnati, OH, USA). Then, the procedure was repeated on the left eye, with the only difference being implantation of a non MTX-loaded micro-implant (placebo implant) in this eye. The PLA-coated CS-MTX micro-implant just before insertion into the right eye of a rabbit is shown in Fig. 3.4A. The PLA-coated CS-MTX micro-implant being inserted into the right eye of a rabbit is shown in Fig. 3.4.B.

Immediately following the procedures, 2 drops of admixed neomycin sulfate (3.5 mg/ml)-polymyxin B (10,000 units/ml)-dexamethasone (1 mg/ml) were applied to each eye.
After the procedure, the animals were given yohimbine 0.2 mg/kg intravenously and monitored in standard fashion until satisfactory post-anesthesia recovery was documented. They were then returned to their cages. An e-collar was placed on each of the animals post-surgery to prevent them from scratching their eyes. The intravenous access catheters were retained until the animals were judged to be stable systemically and then removed. In addition to standard animal care, each animal was given one drop of admixed neomycin sulfate (3.5 mg/ml)-polymyxin B (10,000 units/ml)-dexamethasone (1 mg/ml) eye drops to each eye twice daily for the first five post-surgical days.

3.3.3 Euthanasia

Two rabbits were euthanized on each of the 4 post-surgical days specified above. The euthanasia procedure consisted of administration of sodium pentobarbital (Pentobarbital 120-200 mg/kg) intravenously.

3.3.4 Procurement of Ocular Specimens

Both eyes of each animal that was euthanized were enucleated immediately following death. Each eye was inspected to identify the micro-implant insertion site and any evident abnormalities of that site. The position of the PLA-coated CS-MTX micro-implant with respect to other intraocular features after enucleation of the globe is shown in Fig. 3.4.C. Both eyes of one of the animals euthanized on a specified post-surgical day were opened under sterile conditions for micro-implant recovery and vitreous procurement. The micro-implant was grasped gently with forceps and removed from the vitreous, and the vitreous (approximately 1 ml) was then expressed manually from the eye and collected in a sterile transport vial. The vials containing the vitreous spec-
imens were placed immediately in a Styrofoam box containing dry ice and then kept frozen until the date of pharmacokinetic testing. The recovered micro-implants were washed briefly in sterile balanced salt solution and frozen for future studies. Both eyes of the other animal euthanized on a specified post-surgical day were immersed immediately in 10% neutral buffered formalin for subsequent pathological processing and histopathological analysis.

![Micro-implant before insertion into the eye; B. Micro-implant being inserted into the eye; and C. Micro-implant position with respect to other intraocular features after enucleating the globe](image)

Figure 3.4 A. Micro-implant before insertion into the eye; B. Micro-implant being inserted into the eye; and C. Micro-implant position with respect to other intraocular features after enucleating the globe

### 3.3.5 Pharmacokinetics Evaluation (HPLC)

The vitreous concentration of MTX in the samples obtained from the eyes receiving the MTX micro-implant and the placebo micro-implant for each time point was evaluated by high performance liquid chromatography (HPLC), using the method described in the United States Pharmacopeia assay for MTX [44]. Agilent® 1100 HPLC system with diode array detector was used for the HPLC analysis. The system employed in the HPLC analysis parameters are discussed hereafter. The entire analysis is conducted at 23°C. A C-18 column measuring 150 mm × 4.6 mm with a pore size of 80Å was used. The mobile phase comprised acetonitrile and a phosphate/citrate buffer (pH 6.0) in the ratio of 10:90. The phosphate/citrate buffer (pH 6.0) used in the mobile phase was prepared using 0.2 M potassium hydrogen phosphate and 0.1 M
citric acid in the ratio of 63:37. The flow rate of the mobile phase was 1 mL/min with an injection volume of 10 µL. The MTX concentration was detected at the UV wavelength of 302 nm. Standard MTX concentrations were analyzed to obtain a calibration curve; and then the vitreous samples (one sample from the eye receiving the MTX micro-implant and the other sample from the eye receiving the placebo micro-implant) from each time-point were analyzed in triplicate to obtain the vitreous MTX concentration.

3.3.6 Histopathology Study

The enucleated eyes obtained for pathological analysis were kept in 10% buffered formalin for at least 12 hours prior to being grossed and sectioned to display the micro-implant and surgical wound in pupil-optic nerve (P-ON) sections. The position of the micro-implant relative to the eye wall incision and crystalline lens was evaluated and recorded, and gross photographs showing the micro-implant’s appearance and location were taken at this time, as shown later in Fig. 5.1 of Chapter 5. The micro-implant was removed from the eye with forceps, then photographed next to a millimeter ruler prior to being discarded. The P-ON sections were embedded in paraffin overnight. The embedded tissue was cut in thin sections (4-6 µm thick), and the sections were mounted on glass slides for subsequent staining. Five micro-slides were prepared of each evaluated eye. Three slides were stained with hematoxylin and eosin and two were stained with periodic acid Schiff stain. These slides were evaluated by the ophthalmic pathologist.
3.4 Non-invasive Assessment of Retinal Toxicity Using Electroretinography (ERG) and Ultrasonography

In this section, the procedure employed for the non-invasive assessment of retinal toxicity using electroretinography (ERG) is discussed. Further, the purpose and details of the ultrasonography procedure to assess micro-implant movement and degradation within the \textit{in vivo} environment is provided. This section is the methodology for the Specific Aim 3 of this study.

3.4.1 Introduction to ERG Study

ERG is a \textit{non-invasive} procedure to evaluate retinal damage or toxicity. ERG assesses an electrophysiological response of the ocular structures, such as the retina to a light stimulus. When a light stimulus is applied to the eye, it leads to the transport of ions in the retina, where the positively charged potassium (K\(^{+}\)) and sodium (Na\(^{+}\)) ions are transported due to the polarization (opening) and hyperpolarization (closing) of the cellular pathways in the retina [45]. This transport of ions in the retina causes a waveform which is recorded at the cornea with the use of a corneal contact lens electrode. A typical ERG waveform is shown in Fig. 3.5. The waveform is comprised of an initial negative A-wave followed by a positive B-wave. The time at which the light stimulus is applied and the waveform is initiated is called the flash point. The amplitude and the time at the flash point are considered the baselines for the measurements of subsequent A-wave and B-wave parameters.

A-wave is the first negative component of a typical ERG waveform. The difference between the potential of the trough of the A-wave and the baseline amplitude (0 \(\mu V\)) is called the A-wave amplitude. The time at which the A-wave trough occurs from the baseline flash point is called the A-wave implicit time. The difference between the potential of the B-wave peak and the A-
wave trough is referred to as the B-wave amplitude. The time at which the B-wave trough occurs from the baseline flash point is called the B-wave implicit time. The B-wave and the A-wave are formed as a result of different retinal micro-structural responses to a light stimulus.

The retina is comprised of two fundamental structures – the rods, which are responsible for night vision and the cones, which are responsible for color vision. The A-wave is caused by the rods and cones present in the outer photoreceptor layer of the retina [46, 47]. The B-wave is generated by the Muller cells and the bipolar cells in the retina [46, 47].

![Figure 3.5 A typical ERG waveform.](image)

The activity of the rods and cones are essential for the normal function of the retina. If the retina is damaged, it can be assessed non-invasively by a deviation in the amplitude or implicit time of the B-wave and the A-wave obtained in normal conditions. Further, the ratio of the B-wave amplitude to the A-wave amplitude is considered as an important parameter which indi-
cates the functional integrity of the retina [46, 47]. It is often referred as the B/A ratio. It is used as an index to compare the function of the inner retinal cells to the outer retinal [46, 47].

In order to evaluate the rods and cones individually, certain protocols are followed to condition the eye prior to an ERG study. The rods are responsible for the night vision. In order to assess the rods, the test eye is dark-adapted for at least 25-30 minutes before the response to a series of light stimuli of increasing intensities are recorded. The cones enable the color vision. In order to evaluate the cone activity, the test eye is light adapted for at least 10 minutes before the response to a series of light stimuli of increasing intensities are recorded. The protocol conducted after the dark-adaptation is called the scotopic evaluation and the protocol conducted after the light-adaptation is called the photopic evaluation. The details of the scotopic and the photopic protocols employed in this study are discussed later in this chapter.

For both the B-wave and the A-wave, using any of the protocols, the amplitudes are expected to increase with an increasing intensity of the light stimulus. Also, the B-wave and the A-wave implicit times are expected to decrease with an increasing intensity of the light stimulus. This is the result of a stronger light stimulus causing increased transport of ions in the retinal structures, creating a greater potential difference in a shorter reaction or latency time, which is also known as the implicit time.

ERG is used in this study to evaluate the changes in the retinal function because of the PLA-coated CS-MTX micro-implant. If any toxicity is induced by the PLA-coated CS-MTX micro-implant, it is expected to be revealed in the non-invasive assessment of the ERG recordings by comparing the A-wave and the B-wave amplitudes and implicit times obtained before and after the implantation of the PLA-coated CS-MTX micro-implant. It has been noticed before that the amplitudes of the B-wave and the A-wave are reduced and the implicit times of the B-wave and
the A-wave are increased in the case of an impaired retina. The study protocol of the pilot ERG study conducted is described hereafter.

3.4.2 ERG Study Protocol

The ERG analysis was conducted on the same 8 rabbits used in the in vivo evaluation study of the PLA-coated CS-MTX micro-implant. The study plan of the ERG analysis was concurrent with that of the in vivo study. The detailed study plan of the ERG study is provided in Table 3.

ERG Analysis Set-up and Recording. The ERG study was conducted using a portable ERG machine (HMserg system, Ocuscience LLC, Henderson, Nevada). Animal preparation involved anesthetizing the animals, and then electrode connections were established between the rabbit and the HMserg system. Isoflurane (1 - 2.5%) was used for the anesthetizing the animals during the ERG procedure. Animals were occasionally kept on a heated water bath to maintain their body temperature at 37°C. A droplet of 2.5% hypromellose ophthalmic demulcent solution (Goniovisc™, Hub Pharma., Rancho Cucamonga, CA) was applied on the concave side of the ERG-Jet contact lens electrode (Fabrinal SA, La Chaux-de-Fonds, Switzerland), and then the contact lens electrode was placed on the cornea as shown in Fig. 3.6A. A stainless steel needle electrode was inserted subcutaneously at the base of the ear, which was the reference electrode. Furthermore, another stainless steel needle electrode was inserted subcutaneously on top of the forehead (midline), which served as the ground electrode. The electrodes were secured with surgical tape after the connections were made. The connections of all the electrodes with respect to the ERG instrument are shown in Fig. 3.6B.
Figure 3.6 A. Contact lens eye electrode position with respect to the ERG instrument; B. Electrode connections with respect to the ERG instrument.

ERG analysis at each condition i.e., prior to surgery (PS) and prior to euthanasia (PE), involved recordings of two protocols on each eye: scotopic protocol primarily representing the activity of the rods and the photopic protocol primarily representing the activity of the cones in the eye. The HMsERG machine has preset programs, which provide the required light stimulus for the scotopic (dark adaptation) and the photopic (light adaptation) protocols.

The eyes required 30 minutes of dark adaptation (completely dark room, without any light) prior to the scotopic protocol recording. The preset scotopic protocol in the HMsERG provided 6 stimulus intensities in an ascending order at an interval of 60 seconds between two subsequent intensities. The intensities of the light stimulus during the scotopic protocol were presented in the following sequence: 100, 300, 1000, 3000, 10000, and 25000 mcd.s/m² (Table 4). In the case of photopic protocol recording, the eyes were exposed to 10 minutes of light adaptation at 30,000 mcd.s/m² by the HMsERG unit. The preset photopic protocol in the HMsERG provided 8 stimulus intensities in an ascending order at an interval of 0.5 seconds between two subsequent intensities. The intensities of the light stimulus during the photopic protocol were presented in
the following sequence: 10, 30, 100, 300, 1000, 3000, 10000, and 25000 mcd.s/m² (Table 5). An ophthalmic ointment of Bacitracin Zinc and Polymyxin B sulfate was applied topically to each eye after the ERG procedure.

Table 4. Data acquisition chart for the scotopic protocol for each time point

<table>
<thead>
<tr>
<th>flash intensity (mcd.s/m²)</th>
<th>A-wave amplitude (uV)</th>
<th>A-wave implicit time (mS)</th>
<th>B-wave amplitude (uV)</th>
<th>B-wave implicit time (mS)</th>
<th>B/A ratio</th>
<th>A-wave amplitude (uV)</th>
<th>A-wave implicit time (mS)</th>
<th>B-wave amplitude (uV)</th>
<th>B-wave implicit time (mS)</th>
<th>B/A ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>Prior to surgery (PS)</td>
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<tr>
<td>Prior to euthanasia (PE)</td>
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</table>

Table 5. Data acquisition chart for the photopic protocol for each time point

<table>
<thead>
<tr>
<th>flash intensity (mcd.s/m²)</th>
<th>A-wave amplitude (uV)</th>
<th>A-wave implicit time (mS)</th>
<th>B-wave amplitude (uV)</th>
<th>B-wave implicit time (mS)</th>
<th>B/A ratio</th>
<th>A-wave amplitude (uV)</th>
<th>A-wave implicit time (mS)</th>
<th>B-wave amplitude (uV)</th>
<th>B-wave implicit time (mS)</th>
<th>B/A ratio</th>
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<tbody>
<tr>
<td>Prior to surgery (PS)</td>
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Statistical Analysis of the ERG Data. For both scotopic and photopic protocols, the amplitude and the implicit time of the A-wave and the B-wave for each intensity were recorded for both the prior to surgery (PS) and prior to euthanasia (PE) conditions. As reported in prior studies, the ratio of the B-wave amplitude to the A-wave amplitude, also known as the B/A ratio, is often used as an indicator of the retinal functional integrity [45, 47]. The B/A ratio for each intensity was also recorded for both the PS and PE conditions. The mean values of all parameters for each intensity (A-wave amplitude, A-wave implicit time, B-wave amplitude, B-wave implicit time and B/A ratio) were obtained for each time point (PE conditions: day 5, 12, 19 and 33; n = 2 for each time point) for both scotopic and photopic protocols. These mean values were compared with the mean values of the respective parameter for the corresponding intensity of the PS condition (n = 8, day 0).

Relative B/A Ratio \((B/A)_{rel}\) Analysis. For each of the protocols, at every time point and for each of the intensities, the relative B/A ratio was computed for each rabbit. The relative B/A ratio \((B/A)_{rel}\) is defined as:

\[
(B/A)_{rel} = \frac{(B/A \text{ ratio prior to euthanasia})}{(B/A \text{ ratio prior to surgery})} \tag{Eq 1}
\]

Subsequently, the effect of the protocols, the intensities, the days (observation time points) and their interaction on the \((B/A)_{rel}\) was studied using a three-way analysis of variance (ANOVA) model (Eq 2). The three–way ANOVA model is described as,

\[
((B/A)_{rel})_{ijkl} = \mu + I_i + D_j + P_k + (ID)_{ij} + (IP)_{ik} + (DP)_{jk} + \epsilon_{ijkl} \tag{Eq 2}
\]

where, \(i = 3000, 10000, \text{and} 25000 \text{ mcd.s/m}^2; j = \text{day 5, day 9, day 12 and day 33}; k = \text{scotopic and photopic protocols}; l = 1, 2 \text{ rabbits (replication number)}; \mu = \text{over-all effect}; I_i = \text{effect of the } i^{th} \text{ level of intensity}; D_j = \text{effect of the } j^{th} \text{ level of day}; P_k = \text{effect of the } k^{th} \text{ level of protocol};
(ID)\(_{ij}\) = interaction between the \(i^{th}\) level of intensity and \(j^{th}\) level of days; (IP)\(_{ik}\) = interaction between the \(i^{th}\) level of intensity and \(k^{th}\) level of protocol; (DP)\(_{jk}\) = interaction between the \(j^{th}\) level of days and \(k^{th}\) level of protocol; \(\varepsilon_{ijkl}\) = random error \(\sim N(0, \sigma)\). The responses to the intensities \(\leq\) 1000 mcd.s/ m\(^2\) were ignored for both the protocols as the light stimulus of low intensities did not yield significant measurable A-wave responses.

The \(p\)-value > 0.05 is indicative of the statistically insignificant effect of the intensities, days, protocols and their interaction on the \((B/A)_{rel}\). The interactions between days and protocol \((DP)_{jk}\) (\(p\)-value = 0.02) and also between intensity and days \((ID)_{ij}\) (\(p\)-value = 0.02) were found to be statistically significant where the linear model fit was moderate with \(r = 0.74\); \(p\)-value = 0.0002.

Since the scotopic and the photopic protocols are treated independently, the effect of protocol on intensity and days (observation time points) can be eliminated. Therefore, we examined the significance of days and intensities along with their interaction \textit{within} each protocol using a two-way ANOVA model (Eq 3). The two-way ANOVA model is described as,

\[
((B/A)_{rel})_{ijk} = \mu + I_i + D_j + (ID)_{ij} + \varepsilon_{ijk}
\]

(Eq 3)

where, \(i = 3000, 10000,\) and \(25000\) mcd.s/m\(^2\); \(j = \text{day 5, day 9, day 12 and day 33}; k = 1, 2; \mu = \) over-all effect; \(I_i = \) effect of the \(i^{th}\) level of intensity; \(D_j = \) effect of the \(j^{th}\) level of days; \((ID)_{ij} = \) interaction between the \(i^{th}\) level of intensity and \(j^{th}\) level of days; \(\varepsilon_{ijk} = \) random error \(\sim N(0, \sigma)\);

\(k = \) replication number.

The \(p\)-value > 0.05 is indicative of the statistically insignificant effect of the intensities, days and their interaction on the \((B/A)_{rel}\) for the scotopic and the photopic protocols. If the \(p\)-value for the interaction parameter is observed to be greater than 0.05, then the above two-way ANOVA
model is conducted without the interaction parameter \((ID)_{ij}\) to study the influence of the main effects – intensity and days, on the \((B/A)_{rel}\) for the scotopic and the photopic protocols (Eq 4).

\[
((B/A)_{rel})_{ijk} = \mu + I_i + D_j + \varepsilon_{ijk}
\]  
(Eq 4)

Subsequently, the mean \((B/A)_{rel}\) is computed over all intensities for each day (day 5, day 12, day 19 and day 33) and compared between each combination of protocol (scotopic and photopic) and eye (right eyes receiving the MTX micro-implant and the left eyes receiving the placebo micro-implant). Also, the mean \((B/A)_{rel}\) is computed over all days for each intensity (3000, 10000, and 25000 mcd.s/m\(^2\)) and compared between each combination of protocol and eye.

Furthermore, for each protocol, the group mean \((B/A)_{rel}\) computed over all intensities for each day (day 5, day 12, day 19 and day 33) is compared between each eye (right eyes receiving the MTX micro-implant and the left eyes receiving the placebo micro-implant) using two tailed student’s \(t\)-test \((p < 0.05, n = 6,\) three \((B/A)_{rel}\) obtained for each of the intensities: 3000, 10000, and 25000 mcd.s/m\(^2\), for each eye from two rabbits at each time point). Similarly, for each protocol, the mean \((B/A)_{rel}\) computed over all days for each intensity (3000, 10000, and 25000 mcd.s/m\(^2\)) is compared between the right eye and the left eye using two tailed student’s \(t\)-test \((p < 0.05, n = 8,\) four \((B/A)_{rel}\) obtained for each of the time points: day 5, day 12, day 19 and day 33, for each eye from two rabbits for each intensity). The retinal function was further verified by fitting the B-wave amplitude data to the Naka-Rushton equation [45, 48, 49].

**Naka Rushton Analysis.** The generic Naka-Rushton equation is defined as follows:

\[
\frac{V}{V_{max}} = \frac{I^n}{(I^n + K^n)}
\]  
(Eq 5)

\(V\) is the B-wave amplitude, \(V_{max}\) is the maximum B-wave amplitude, \(I\) is the intensity of the light stimulus, \(K\) is the intensity of light necessary for the B-wave to reach its half of \(V_{max}\) [45], and ‘n’
is the slope when \( I \) is equal to \( K \). As reported in the analysis of Aylward [48], Eq. 5 can be re-written as:

\[
\log \left[ \frac{V/V_{max}}{(1 - V/V_{max})} \right] = n \log I - n \log K \quad \text{ (Eq 6)}
\]

The ‘\( n \)’ values and the ‘- \( \log K \)’ values obtained by fitting the ERG data to the Naka-Rushton equation (Eq. 6) were used as indices to assess retinal sensitivity [45, 50]. The value of ‘\( n \)’ and the value of ‘- \( \log K \)’ (log mcd.s/m\(^2\)) has been reported to be approximately 1 [45] and approximately 3 [50], respectively, for a normal retina.

At each time point, \( V \) is the mean B-wave amplitude (\( n = 2 \), mean of the B-wave amplitudes from two rabbits) obtained for each intensity \( I \). Using equation 6, the ‘\( n \)’ and ‘- \( n \log K \)’ are subsequently obtained as the slope and the \( y \) axis-intercept from the linear regression fit of ‘log \([V/V_{max}] / (1-V/V_{max})\)’ versus ‘log \( I \)’ for each time point. Such fits are obtained independently for either eye (right eyes receiving the MTX micro-implant and left eyes receiving the placebo micro-implant) and for each protocol (scotopic and photopic). Thereafter, to assess the deviation of the state of the retina from the normal condition, the group mean values of ‘\( n \)’ and ‘- \( \log K \)’ of all time points (\( m = 4 \)) are compared between the prior to surgery (PS) and the prior to euthanasia (PE) conditions for both eyes and for both the scotopic and the photopic protocols.

3.4.3 Methodology for Ultrasonography Assessment

Light cannot be used to visually assess the conditions of ocular structures in the posterior segment and around the globe as it does not penetrate through the physiological barriers. However, ultrasonography is a safe and non-invasive method to evaluate any intraocular disorders at the posterior segment. Ultrasound is able to penetrate the blood cells due to its high energy, and therefore can be used to image the posterior segment of the eye.
There are two types of ultrasound scanning modes. They are known as the A-scan mode and the B-scan mode. A-scan mode is used to measure the dimensions of ocular features and assess any movement of the ocular structures. The ‘A’ of the A-scan mode stands for ‘Amplitude’ and the A-scan recording is in the form of peaks and spikes of different heights [51]. In an echogram of an A-scan mode recording, the x-axis denotes the depth of the reflecting ocular structure from the ultrasound scanning probe and the y-axis consists of peaks of varying heights from the respective reflecting ocular structure. The heights of the peaks and spikes signify the strength of the signal from different structures. The biometric measurements are obtained by converting the parameters like the sound velocity in tissues, time of the peak occurrences into distance and length measurements.

In the B-scan mode, the ‘B’ refers to ‘Brightness’. In a B-scan mode recording, a 2D image is obtained. The brightness of an image is dependent on the reflected energy of the signal response from an ocular structure reaching the ultrasound scanning probe [51]. Therefore, the ocular structures at a particular plane reflect the same amount of energy to the scanning probe and form its 2D image of consistent brightness. The ocular structures are not in the same plane and are situated in varying distances relative to the ultrasound scanning probe. Due to the varying distances from the scanning probe, the ocular structures could be distinguished from one another as they form their respective 2D image of relative brightness.

Ultrasonography Data Recording. In this study, B-scan ultrasonography (US) was performed using a Linscan 12 MHz veterinary ophthalmic ultrasound unit (Ocuscience LLC, Rolla, Missouri). A drop of 2.5% hypromellose ophthalmic demulcent solution (GonioviscTM, Hub Pharma, CA) was applied to the tip of the ultrasound probe to provide probe-cornea coupling and avoid corneal abrasion. The tip of the ultrasound probe was brought into contact with the topical-
ly anesthetized cornea for image acquisition. Both antero-posterior and transverse images of the sector of the peripheral fundus where the micro-implant was located were obtained during each US session. All rabbits were imaged bilaterally immediately following insertion of the micro-implants, and two rabbits were reimaged bilaterally on each of the following post-insertion days: day 5, day 12, day 19 and day 33 (Table 3). For each rabbit and each eye, the US images showing the micro-implant immediately post-insertion were compared with corresponding US images acquired on the specified post-insertion days. The specific aspects of the images that were assessed in this study were (1) the cross-sectional size and shape of the micro-implant, (2) the position of the implant relative to the crystalline lens and peripheral retina, and (3) the appearance of the peripheral vitreous around the micro-implant and peripheral retina near the micro-implant’s position.
3.5 *In vitro* characterization of the improved CS-MTX micro-implant with altered lipophilic surface modification

In this section, the procedure employed for the *in vitro* characterization of the improved CS-MTX intravitreal micro-implant was obtained by altering the *lipophilic surface modification* is discussed. Further, the purpose and details of the altered lipophilic surface modification procedure is provided. This section is the methodology for the Specific Aim 4 of this study. The procedures employed in this study are similar to fabrication methods reported in section 3.1 and characterization methods discussed in section 3.2.

The *purpose* of this study was to characterize the influence of a) PLA:PGA copolymer ratio in poly(lactic-co-glycolic) acid (PLGA) and b) the molecular weight of PLA and PLGA on the structural stability of the micro-implant, MTX release rate and swelling of the micro-implants. A summary of various techniques used to characterize the lipophilic surface modification of the CS-MTX micro-implants is provided in Table 6. The methodologies employed for dimension, morphology and microstructure (optical microscopy and SEM) and glass transition temperature (DSC) have been discussed in section 3.2.1. The *in vitro* MTX release studies (UV-visible spectrophotometer) along with *in vitro* MTX release kinetics analysis have been discussed in section 3.2.2. The detailed methodologies for Gel permeation chromatography (GPC), Fourier transform infrared spectroscopy (FTIR) and swelling analysis are discussed hereafter.
Table 6. Summary of techniques used to characterize the *lipophilic surface modification* of the CS-MTX micro-implants

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Method</th>
</tr>
</thead>
<tbody>
<tr>
<td>Molecular weight of the coating polymer</td>
<td>Gel permeation chromatography (GPC)</td>
</tr>
<tr>
<td>Dimension, Morphology and Microstructure</td>
<td><strong>Optical microscopy</strong> and SEM - <em>same process</em> as discussed in section 3.2.1</td>
</tr>
<tr>
<td>Chemical bonding</td>
<td>Fourier transform infrared spectroscopy (FTIR)</td>
</tr>
<tr>
<td>Glass transition temperature (Tg)</td>
<td>Differential scanning calorimetry (DSC) - <em>same process</em> as discussed in section 3.2.1 (different instrument - NETZSCH DSC 200F3)</td>
</tr>
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</table>

*In vitro* MTX release studies

*In vitro* MTX release kinetics analysis

Swelling analysis

<table>
<thead>
<tr>
<th></th>
<th>Characteristic <strong>Korsmeyer-Peppas, Zero order, First order</strong> and Higuchi models - <em>same process</em> as discussed in 3.2.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Weight measurement</strong> at pre-determined time points with respect to the weight prior to the start of the experiment</td>
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</table>

(n = 3 micro-implants, per coating type)
3.5.1 Gel Permeation Chromatography (GPC)

Molecular weights and polydispersities of PLGA 5050, PLGA 6535, PLGA 7525 and DL-PLA with an inherent viscosity of 0.67 dL/g and 1.16 dL/g were determined by GPC. Samples were weighed on an analytical balance and placed in sample vials where they were diluted to a concentration of 5-15 mg/mL using dimethyl formamide (DMF) with 0.1% LiBr w/v and 0.05% toluene v/v as a flow rate marker. Samples where then placed on a shaker table for 72 hours to dissolve and then filtered through a Whatman Anotop 25 syringe filter with a 0.2 µm porosity prior to injection.

The samples were characterized with an Agilent 1200 series HPLC equipped with a PSS Gram (10 µm) guard column and 2 PSS Gram columns (10 µm) (linear range of MW = 100 – 1x10^6 g/mol). A mobile phase of DMF with 0.1% LiBr w/v was used at a flow rate of 0.5 mL/min at 60°C. Optilab rEX differential refractometer (light source = 658 nm) detector was used and calibrated against poly(methyl methacrylate) standards (850 Da – 2,000,000 Da). ASTRA software v. 5.3.4 was used to determine polymer characteristic values.

3.5.2 Fourier transform infrared spectroscopy (FTIR)

Reflectance (%R) FTIR spectra was acquired using the Thermo Fisher Scientific Nicolet 6700 machine with a Smart orbit ATR module for a frequency range of 4000 cm\(^{-1}\) – 400 cm\(^{-1}\). The samples analyzed were pure CS powder, pure MTX powder, pure PLGA and PLA crystals used for lipophilic coating, uncoated CS-MTX micro-implant and PLA /PLGA-coated CS-MTX micro-implant. The samples were investigated for any possible chemical bonding between the lipophilic coating with the CS-MTX matrix of the micro-implant.
3.5.3 Swelling analysis

Swelling experiments were conducted by placing samples of PLA / PLGA-coated CS-MTX micro-implants (n = 3, for each type of lipophilic coating) in vials containing 5ml PBS (pH 7.4). The vials were slowly stirred in a water bath maintained at 38°C, which is similar to the in vitro MTX release analysis (section 3.2.2). At pre-determined time points, the samples were removed from vials and subsequently the PBS on the surface of the samples was gently wiped using Kimwipes (Kimtech Science Brand, Kimberly Clark). Thereafter, the samples were re-weighed for each pre-determined time point. The swelling index (Swelling index %) for each PLA / PLGA-coated CS-MTX micro-implant is obtained using the following equation,

\[
\text{Swelling index \%} = \left( \frac{W_t - W_0}{W_0} \right) \times 100
\]  

(Eq 7)

where \( W_0 \) is the initial weight of the micro-implant at the start of the experiment and \( W_t \) is the weight of the micro-implant at time ‘t’.

In the next chapter, the results of the in vitro characterization of the PLA and CS-based MTX intravitreal micro-implant are presented (Chapter 4). In Chapter 5, the results of the in vivo characterization of the PLA and CS-based intravitreal micro-implants in rabbit eyes using minimally invasive surgery are presented. Subsequently, the findings of the non-invasive assessment of retinal toxicity using electroretinography (ERG) and ultrasonography is reported in Chapter 6. Lastly, the findings of the in vitro characterization of the improved CS-MTX intravitreal micro-implant by altered lipophilic surface modification are presented in Chapter 7.
Chapter 4. Results: In vitro characterization of the Chitosan and Polylactic acid-based Methotrexate intravitreal micro-implant

In this chapter, the results of the in vitro characterization of the CS and PLA-based MTX intravitreal micro-implant (Specific Aim 1) are reported. The material characterization is first discussed, followed by the release rate study of the micro-implants. Evaluation of drug release is addressed using the characteristic drug release model fitting technique. Results are presented as mean ± standard deviation (SD) unless otherwise mentioned.

4.1 Structure Characterization of the Micro-implant

Optical microscopy and scanning electron microscopy (SEM) techniques were utilized to assess the micro-implant appearance, dimensions and microstructure morphology. Lipophilicity of the PLA coating was evaluated using time of flight-secondary ions mass spectroscopy (ToF-SIMS) and differential scanning calorimetry (DSC) studies.

4.1.1 Dimensions and Appearance

For micro-implant samples (n = 9; 3 samples and 3 readings per sample), the dimensions of the uncoated type and the PLA-coated type were measured using an optical microscope. The length and cross-sectional diameter of the uncoated micro-implant were 4 ± 0.04 mm and 0.7 ± 0.03 mm, respectively. The length and cross-sectional diameter of the PLA-coated micro-implant were 4.2 ± 0.03 mm and 0.9 ± 0.04 mm, respectively.

The optical microscopy images of surfaces of the PLA-coated and the uncoated micro-implants are shown in Fig. 4.1A and 4.1B, respectively. Comparing Fig. 4.1A and 4.1B, it can be seen that the surface of the PLA-coated micro-implant was relatively smoother and more uni-
form compared to that of the uncoated micro-implant. The optical microscopy images of the cross-sectional view of the PLA-coated and uncoated micro-implants are shown in Fig. 4.1C and Fig. 4.1D, respectively. A 100 µm PLA coating can be noticed in the PLA-coated micro-implant in Fig. 4.1C, which is absent in the uncoated micro-implant in Fig. 4.1D. The micro-implants were a yellow color, signifying uniform distribution of MTX throughout the CS polymer matrix. Thus, optical microscopy images reveal uniform coating of PLA on the surface of the PLA-coated micro-implants.

![Figure 4.1 A. Longitudinal view of PLA-coated micro-implant; B. Longitudinal view of uncoated micro-implant; C. Cross sectional view of PLA-coated micro-implant showing PLA coating on the edge; D. Cross sectional view of uncoated micro-implant. (Scale Bar = 500 µm)](image-url)
4.1.2 Morphology and Microstructure

SEM images showing the longitudinal view of the surface of the uncoated and PLA-coated micro-implants are shown in Fig. 4.2. From the SEM images, the porous and irregular CS surface of the uncoated micro-implant can be seen. By coating the micro-implants with PLA, the porous surface gets filled up with PLA and results in a smoother non-porous surface as shown in the SEM images of the coated micro-implant. SEM images of the cross-section of the uncoated and the PLA-coated micro-implant are shown in Fig. 4.3. The cross-sectional diameter of the uncoated (0.706 mm) and the PLA-coated (0.878) micro-implants are shown in Fig. 4.3A and 4.3D, respectively. They are consistent (~ 2.4% difference) with the results of optical microscopy, as shown in Fig. 4.1. In Fig. 4.3B and 4.3C, the porous internal CS matrix of the uncoated micro-implant is shown. In Fig. 4.3D and 4.3E, it is visible that the PLA deposition takes place in the internal voids of the coated micro-implant, resulting in a denser internal matrix with reduced porosity. The internal deposition of PLA also plays an important role in the reduction of swelling of the CS matrix and restricting the MTX release.

SEM images showing the longitudinal view of the surface of the uncoated micro-implants with an increasing drug loading are shown in Fig. 4.4. From the SEM images, it can be concluded that with an increase in drug loading, there is an increase in the quantity of loosely bound drug particles on the surface of the micro-implant. SEM images showing the cross-section of the uncoated micro-implants with an increasing drug loading are shown in Fig. 4.5. It can be seen that the voids of the CS matrix get more filled up with the drug particles with an increase in drug loading.
Figure 4.2 SEM Images of longitudinal view showing the surface of  A. Uncoated micro-implant at 26x; B. Uncoated micro-implant at 80x; C. Uncoated micro-implant at 200x; D. Coated micro-implant at 26x; E. Coated micro-implant at 80x; F. Coated micro-implant at 200x
Figure 4.3 SEM Images of the cross-section of  A. Uncoated micro-implant at 80x; B. Uncoated micro-implant at 200x; C. Uncoated micro-implant at 500x; D. PLA-coated micro-implant at 80x; E. PLA-coated micro-implant at 200x; F. PLA-coated micro-implant at 500x
Figure 4.4 SEM Images of the longitudinal view of uncoated micro-implants A. Placebo at 50x; B. 10% MTX at 50x; C. 25% MTX at 50x; D. 40% MTX at 50x; E. Placebo at 500x; F. 10% MTX at 500x; G. 25% MTX at 500x; H. 40% MTX at 500x
Figure 4.5 SEM Images of the cross-section of uncoated micro-implants A. Placebo at 80x; B. 10% MTX at 80x; C. 25% MTX at 80x; D. 40% MTX at 80x; E. Placebo at 500x; F. 10% MTX at 500x; G. 25% MTX at 500x; H. 40% MTX at 500x
4.1.3 Time of Flight-Secondary Ion Mass Spectroscopy (ToF-SIMS)

ToF-SIMS was used to evaluate the surface chemistry of the coated micro-implants. ToF-SIMS spectra of PLA (MW 150,000) (blue), PLA-coated 40% CS-MTX micro-implant surface (red) and uncoated 40% CS-MTX micro-implant surface (green) is reported in Fig. 4.6. Figure 4.6 shows the characteristic peaks (blue color) of pure PLA mass fragments (43 [C$_2$H$_3$O$^+$], 56 [C$_3$H$_4$O$^+$], 71 [C$_3$H$_5$O$_2^+$], 73 [C$_3$H$_5$O$_2^+$], 127 [C$_6$H$_7$O$_3^+$], 128 [C$_6$H$_8$O$_3^+$], 129 [C$_6$H$_9$O$_3^+$], 143 [C$_6$H$_7$O$_4^+$] and 145 [C$_6$H$_9$O$_4^+$]) match with that of the PLA-coated micro-implant (red color) with similar intensities. The characteristic peaks of pure PLA mass fragments (blue color) and PLA-coated micro-implant (red color) match with the previous study [52].

The spectrum of the uncoated micro-implants (green color) does not show the same characteristic peaks (56 [C$_3$H$_4$O$^+$], 71 [C$_3$H$_5$O$_2^+$], 73 [C$_3$H$_5$O$_2^+$], 127 [C$_6$H$_7$O$_3^+$], 128 [C$_6$H$_8$O$_3^+$], 129 [C$_6$H$_9$O$_3^+$], 143 [C$_6$H$_7$O$_4^+$] and 145 [C$_6$H$_9$O$_4^+$]) as that of pure PLA mass fragments (blue color) and PLA-coated micro-implant (red color). However, in the spectrum of uncoated micro-implants (green color), there is a match with the spectra of pure PLA mass fragments (blue color) and PLA-coated micro-implants (red color) at mass fragment 43 [C$_2$H$_3$O$^+$], but with a much higher relative intensity than the spectra of the pure PLA mass fragments (blue color) and PLA-coated micro-implant (red color). The higher relative intensity from the uncoated micro-implants is probably due to the mass fragment 43 [C$_2$H$_3$O$^+$] being generated from the CS and MTX present on the surface of the uncoated micro-implants. Therefore, we can qualitatively confirm the successful coating of PLA on the surface of the coated micro-implant based on the spectra of Fig. 4.6.
Figure 4.6 TOF-SIMS spectra of PLA (MW 150,000) (blue), PLA-coated 40% CS-MTX micro-implant (red) and uncoated 40% CS-MTX micro-implant (green)
4.1.4 Glass Transition Temperature (Tg)

The purpose of the DSC study was to assess the stability of the constituents of the coated micro-implant in physiological temperature. The DSC study of the PLA coating was done because the Tg of PLA could be close to physiological temperature depending on the molecular weight. If the coating polymer PLA undergoes glass transition in the physiological conditions, then the PLA coating would soften, affecting the structural properties of the micro-implant, thus leading to faster drug release [53]. A DSC plot of one of the PLA-coated micro-implants is shown in Fig. 4.7. Tg is the point where the slope of the endotherm changes. The Tg values of the PLA coating obtained for the 10%, 25% and 40% PLA-coated micro-implants (n = 4) are 50.2 ± 1.3, 51.3 ± 1.1 and 51.9 ± 2.8 °C, respectively.

![Figure 4.7 Characteristic DSC curve of a PLA-coated micro-implant showing the Tg around 50°C](image-url)
The Tg values obtained in this study are consistent with previous studies [54]. The DSC study confirms that the PLA coating will not experience glass transition or soften in the physiological temperature (~37°C) inside the intraocular domain. The DSC study of the uncoated CS-MTX micro-implants are not conducted because the decomposition temperature of MTX and Tg of CS is much higher than the physiological temperature and thus would not undergo decomposition and glass transition, respectively. Chadha et al. [55] reported that MTX has no melting transition and decomposes around ~245°C, and the Tg of CS is reported to be around 140-150°C by Dong et al. [56].

4.2 In vitro MTX release studies

The details of the drug release rate studies are reported in this section. The calibration of MTX is described followed by the discussion of MTX release rate profiles from the coated and uncoated micro-implants. The drug release rate data is fitted to pharmacokinetic models to interpret the drug diffusion kinetics.

4.2.1 Calibration of MTX

Figure 4.8 describes the calibration procedure for MTX. Characteristic MTX spectra for different concentrations are shown in Fig. 4.8A. The characteristic MTX peaks are at 258 nm, 302 nm and 372 nm and the calibration curves for the 258 nm peak, 302 nm peak and 372 nm peak are shown in Fig. 4.8B, 4.8C and 4.8D, respectively. The calibration curve of each peak is obtained by linear regression fitting of the UV-absorbance values for different MTX concentrations. The linear regression is based on terms of correlation coefficient (R²) values. The 258 nm peak of the MTX spectra is used for the release rate experiments as it provides a sharper deflection compared to the others.
Figure 4.8 A. Characteristic MTX UV-Vis Spectra for different concentrations; B. Calibration curve for MTX peak at 258 nm; C. Calibration curve of MTX peak at 302 nm; D. Calibration curve for MTX peak at 372 nm

4.2.2 Release Rate Profiles

Release rate profiles of MTX from the uncoated micro-implants are shown in Fig. 4.9A. Figure 4.9B shows release rate profiles of MTX from the uncoated micro-implants in the therapeutic window (0.2-2.0µg/day). Cumulative release profiles of MTX from the uncoated micro-implants are shown in Fig. 4.9C. Release rate profiles of MTX from the PLA-coated micro-implants are shown in Fig. 4.10A. Figure 4.10B shows release rate profiles of MTX from the PLA-coated micro-implants in the therapeutic window. Cumulative release profiles of MTX from the PLA-coated micro-implants are shown in Fig. 4.10C. The mean profile of each type of
drug loading is plotted along with the standard error. The summary of release rate characteristics for the uncoated and coated micro-implants for different drug loadings is provided in Tables 7 and 8, respectively.

Figure 4.9 A. Release rate curves from uncoated CS-MTX micro-implants with different drug loadings; B. Release rate curves from uncoated CS-MTX micro-implants with different drug loadings in the therapeutic window (shaded region); C. Cumulative drug release profile from uncoated CS-MTX micro-implants
Figure 4.10 A. Release rate curves from PLA-coated CS-MTX micro-implants with different drug loadings; B. Release rate curves from PLA-coated CS-MTX micro-implants with different drug loadings in the therapeutic window (shaded region); C. Cumulative drug release profile from PLA-coated CS-MTX micro-implants
Table 7. Summary of release rate characteristics of uncoated CS-MTX micro-implants (n = 3)

<table>
<thead>
<tr>
<th>Implant Drug loading (w/w)</th>
<th>Mean Release Rate ± Standard Error (µg/day)</th>
<th>Total Release Duration (hours)</th>
<th>Time of Peak Release Rate (hours)</th>
<th>Peak Release Rate ± Standard Error (µg/day)</th>
<th>Start time of release rate within therapeutic limits (hour)</th>
<th>Drug released before therapeutic release rate starts ± Standard Error (%)</th>
<th>End time of release rate within therapeutic limits (hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>88.9 ± 4.8</td>
<td>19</td>
<td>0.5</td>
<td>1414 ± 66</td>
<td>~12</td>
<td>99.2 ± 0.2</td>
<td>~19</td>
</tr>
<tr>
<td>25</td>
<td>188.0 ± 7.9</td>
<td>29</td>
<td>0.5</td>
<td>4314 ± 221</td>
<td>~22</td>
<td>98.8 ± 0.3</td>
<td>~29</td>
</tr>
<tr>
<td>40</td>
<td>372.6 ± 7.5</td>
<td>32</td>
<td>0.5</td>
<td>5041 ± 311</td>
<td>~25</td>
<td>98.7 ± 0.4</td>
<td>~32</td>
</tr>
</tbody>
</table>

Table 8. Summary of release rate characteristics of PLA-coated CS MTX micro-implants (n = 3)

<table>
<thead>
<tr>
<th>Implant Drug loading (w/w)</th>
<th>Mean Release Rate ± Standard Error (µg/day)</th>
<th>Total Release Duration (days)</th>
<th>Time of Peak Release Rate (days)</th>
<th>Peak Release Rate ± Standard Error (µg/day)</th>
<th>Start time of release rate within therapeutic limits (day)</th>
<th>Drug released before therapeutic release rate starts ± Standard Error (%)</th>
<th>End time of release rate within therapeutic limits (day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.8 ± 0.4</td>
<td>58</td>
<td>4</td>
<td>11.2 ± 6.0</td>
<td>10</td>
<td>62.7 ± 5.3</td>
<td>~58</td>
</tr>
<tr>
<td>25</td>
<td>3.2 ± 0.1</td>
<td>74</td>
<td>4</td>
<td>21.6 ± 4.3</td>
<td>18</td>
<td>82.3 ± 1.5</td>
<td>~74</td>
</tr>
<tr>
<td>40</td>
<td>6.6 ± 0.3</td>
<td>66</td>
<td>3</td>
<td>60.4 ± 14.1</td>
<td>14</td>
<td>88.5 ± 1.8</td>
<td>~66</td>
</tr>
</tbody>
</table>
4.2.3 Release Rate Study of the Uncoated Micro-implant

The mean release rate of the uncoated CS-MTX micro-implants was 88.9 ± 4.8 µg/day, 188.0 ± 7.9 µg/day and 372.6 ± 7.5 µg/day for the 10%, 25% and 40% w/w drug loadings, respectively, as mentioned in Table 7. The total release duration is defined as the duration from the start of drug release till the time it remains in the therapeutic window. The total release duration for 10%, 25% and 40% w/w CS-MTX micro-implants was 19, 29 and 32 hours, respectively. The 10% w/w, 25% w/w and the 40% w/w micro-implants remained in the therapeutic window between the 12th to 19th hour, 22nd to 29th hour and 25th to 32nd hour, respectively (Fig. 4.9B).

4.2.4 Release Rate Study of the PLA-coated Micro-implant

The mean release rate of the PLA-coated CS-MTX micro-implants was 1.8 ± 0.4 µg/day, 3.2 ± 0.1 µg/day and 6.6 ± 0.3 µg/day for the 10%, 25% and 40% w/w drug loadings, respectively, as mentioned in Table 8. The total release duration for 10%, 25% and 40% w/w PLA-coated CS-MTX micro-implants was 58, 74 and 66 days, respectively.

For the 10% coated MTX micro-implant, there was an initial burst release on the 4th day (Fig. 4.10A), then a small secondary burst between the 10th and 20th day and a final burst near the 50th day (Fig. 4.10B). The 10% w/w coated micro-implants exhibited a release rate in the therapeutic window from the 10th day onward up to the 58th day, as shown in Fig. 4.10B.

For the 25% coated MTX micro-implant, an initial burst release was seen on the 3rd day (Fig. 4.10A). Although there was no prominent secondary burst, there were a couple of bursts between the 20th and 40th day, followed by a major burst between the 40th and 50th day before a final burst around the 70th day (Fig. 4.10B). The 25% w/w coated micro-implants showed a release rate in the therapeutic window from the 18th day onward up to the 74th day.
In the case of 40% coated micro-implant, a significant initial burst release was noticed on the 3rd day (Fig. 4.10A), and then a secondary burst was observed between the 30th and 40th day (Fig. 4.10B). There was no prominent final burst noticed in the release profile of the 40% coated micro-implant. The 40% w/w micro-implants maintained the release rate in the *therapeutic window* from the 14th day onward up to the 66th day.

Thus, the drug administration was achieved in the desired *therapeutic window* for all types of coated micro-implants (10%, 25% and 40% w/w MTX) for a period of 8 to 10 weeks.

### 4.3 *In vitro* MTX Release Kinetics Analysis

Drug release data of all drug loadings of the *coated* micro-implants were fitted to zero order equation, first order equation, Higuchi model and Korsmeyer-Peppas model in order to analyze the mechanism of drug release and diffusion kinetics. The fitting of each model was evaluated based on correlation coefficient (R²) values. The R² values of each model fitting are reported in Table 9.

<table>
<thead>
<tr>
<th>MTX loading w/w % (N = 3)</th>
<th>First 8 days (60% drug release during initial burst)</th>
<th>10th day – end of therapeutic release (drug release in therapeutic window)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Korsmeyer Peppas R²</td>
<td>Zero Order R²</td>
</tr>
<tr>
<td>10%</td>
<td>0.99</td>
<td>1.22</td>
</tr>
<tr>
<td>25%</td>
<td>0.99</td>
<td>1.24</td>
</tr>
<tr>
<td>40%</td>
<td>0.99</td>
<td>1.24</td>
</tr>
</tbody>
</table>

*The half-life (t₁/₂) obtained from the first order kinetics for the whole range of drug release is ~ 10 days*
4.3.1 Korsmeyer-Peppas model

The Korsmeyer-Peppas model provides an insight into the type of drug release mechanism taking place from swellable polymeric devices [57]. The ‘n’ of the Korsmeyer Peppas model was estimated from the linear regression fit of the logarithmic release rate data. \( n \geq 1 \) suggests super case II transport relaxational release and also indicates zero order kinetics [58]. The generic equation for the Korsmeyer Peppas model is as follows;

\[
F = \left( \frac{M_t}{M_0} \right) = K_{kp} \ t^n \quad \text{(Eq 8)}
\]

where \( M_0 \) is the initial amount of drug, \( M_t \) is the amount of drug released in time \( t \), \( F \) is the fraction of drug released at time \( t \), \( K_{kp} \) is the Korsmeyer Peppas release constant and \( n \) is estimated from linear regression of \( \log F \) versus \( \log t \); \( n \) suggests the type of diffusion. We obtained consistent \( R^2 \) values \( \sim 0.99 \) and ‘n’ values \( \sim 1.2 \) by fitting our first 60% of drug release rate data to the Korsmeyer Peppas model (Fig. 4.11A), which suggests that the first 60% of the drug release is influenced by swelling and relaxation phenomena of the polymer matrix. The 60% of the drug release takes place in the first 8 days out of the total drug release duration. It may be noted that if we fit the whole range of drug release data to the Korsmeyer Peppas model, then the \( R^2 \) values reduces to 0.82 - 0.89 and the ‘n’ values vary between 0.62 - 0.73.

4.3.2 Zero order equation

The zero order release equation represents a process when the release rate of the drug is independent of the concentration of the drug in the system and the generic equation for the zero order equation is as follows;

\[
M_t = M_0 + K_0 t \quad \text{(Eq 9)}
\]
where $M_0$ is the initial amount of the drug, $M_t$ is the amount of the drug released in time $t$, $K_0$ is the zero order release constant. The range of $R^2$ values is between 0.02 and 0.49 when the whole range of drug release data is fit to the zero order equation. $R^2$ values improve to ~ 0.9 when the initial 60% drug release data is fit to the zero order equation (Table 9). Therefore the drug release from the coated micro-implants follows zero order equation for the first 60% of the drug release.

Figure 4.11 Fitting of MTX release from the coated micro-implants using A. Korsmeyer Peppas equation (for the first 60% of drug release); B) First order equation (from the 10th day to the end of therapeutic drug release)
4.3.3 First order equation

The first order release equation represents a system where the release rate of the drug is dependent on the concentration of the drug in the system and the generic equation for the first order equation is as follows;

$$\log M_t = \log M_0 + K_1 \left(\frac{t}{2.303}\right)$$  \hspace{1cm} (Eq 10)

where $M_0$ is the initial amount of drug, $M_t$ is the amount of drug released in time $t$ and $K_1$ is the first order release constant. The $R^2$ values are $\sim 0.9$ when the whole range of drug release data is fit to the first order equation. However, by fitting the drug release data to the first order equation from the 10th day to the end of the drug release ($\sim 60$ days) provides the $R^2$ values of 0.83, 0.94 and 0.98 for 10%, 25% and 40% coated micro-implants, respectively (Fig. 4.11B). This implies the drug release rate from the coated micro-implants in the therapeutic window, after the 10th day (post-initial burst), is primarily governed by first order kinetics and is dependent on the concentration of the drug in the coated micro-implants. The half life ($t_{1/2}$) of MTX release from an intravitreal injection is reported to be $\sim 14.3$ hours [1], whereas the $t_{1/2}$ of MTX release from the coated micro-implants for the whole range of data is $\sim 240$ hours (10 days) (Table 9).

4.3.4 Higuchi model

The Higuchi release equation [59] predicts that the drug release is caused primarily by diffusion mechanism and the generic equation for the Higuchi model is as follows;

$$M_t = K_H t^{1/2}$$  \hspace{1cm} (Eq 11)

where $M_t$ is the amount of drug released in time $t$ and $K_H$ is the Higuchi constant. The range of $R^2$ values is between 0.7 and 0.91 when the whole range of drug release data is fitted to the Higuchi model. However, fitting the drug release data to the Higuchi model from the 10th day to
the end of drug release (~ 60 days) provides the R² values of 0.98, 0.96 and 0.95 for 10%, 25% and 40% coated micro-implants, respectively (Table 9). This implies the drug release from the coated micro-implants, after the 10th day (post-initial burst), was primarily governed by diffusion kinetics.

Therefore, it can be concluded that the drug release mechanism primarily followed i) Korsmeyer Peppas model, and zero order model for the first ~ 8 days where the initial burst took place and 60% of the drug was released due to swelling of the polymer matrix; and ii) first order and Higuchi model from the 10th day till the end of drug release signifying the drug release mechanism being concentration dependent and was primarily caused by diffusion mechanism.

4.4 Discussion

In this study, the in-vitro analysis of a unique CS and PLA based MTX intravitreal micro-implant is reported. It is expected that such a micro-implant will improve the treatment of PIOL in the future. Each of the PLA-coated micro-implants exhibited sustained release of MTX within the therapeutic window for a period of more than 50 days. This micro-implant can be administered using minimally invasive surgical procedures. The existing treatment method for PIOL, the influence of PLA coating and drug loading on the sustained MTX release, toxicity from the micro-implants, the assumptions and the limitations of this study are discussed in detail in the following sections.

4.4.1 Existing Treatment Method for PIOL

PIOL provides a therapeutic challenge. The present protocol of intravitreal injections of MTX to treat PIOL has several drawbacks. The MTX intravitreal injection has a short half life (t
\(\frac{1}{h}\) of 14.3 hours and therefore leads to an uncertainty in the effective duration of therapeutic concentration. Repetitive administrations are required to maintain therapeutic dosage over a period of time, which can also be associated with ocular complications as mentioned before. A sustained release drug delivery system (micro-implant) that maintains the therapeutic dosage of MTX over a prolonged time period may prove to be more effective and safer treatment method than multiple intravitreal injections. However, as mentioned before, there are no devices currently present for sustained release of MTX or other hydrophilic drugs in the intravitreal domain. The PLA-coated CS-MTX micro-implant can be potentially used for improved treatment of PIOL.

4.4.2 Influence of PLA Coating

PLA coating played an important role in sustained release administration of MTX and also influenced the initial burst release or the peak release rate of MTX. PLA coating imparted lipophilicity to the surface of the micro-implant. The PLA coating prevented the entry of PBS into the CS matrix, thereby reducing the rate of swelling of the CS matrix and subsequent MTX release. The sustained release of MTX from the PLA-coated micro-implants, as reported earlier, can be attributed to the degradation rate of PLA coating and of the micro-implant.

The peak release rate (initial burst) of 10% w/w, 25% w/w and 40% w/w uncoated micro-implants were 1414 ± 66, 4314 ± 221 and 5041 ± 311 µg/day, respectively (Table 7), and the peak release rate (initial burst) of 10% w/w, 25% w/w and 40% w/w coated micro-implants were 11.2 ± 6.0, 21.6 ± 4.3 and 60.4 ± 14.1 µg/day, respectively (Table 8). Therefore, the PLA coating reduced the initial burst of the drug from the micro-implants and influenced the sustained release of MTX.
4.4.3 Influence of Drug Loading

The peak release rate and release duration were governed by the drug loading. The peak release rate of the 40% w/w micro-implants was the highest and that of the 10% w/w micro-implants was the lowest in both coated and uncoated micro-implants (Tables 7 and 8). The high peak release rate indicated an initial burst which was directly related to the quantity of loosely bound MTX present in the micro-implant matrix, which again depended on the drug loading.

As mentioned in a review article by Fu et al. [60], a burst is defined as an enhanced dissolution of drug from the polymer matrix, which is due to the rapid release of the surface associated drug molecules. In this study, a burst was qualitatively characterized by a ‘bell curve’ shaped profile in the release rate plot indicating enhanced rate of drug release (Fig. 4.10A and 4.10B).

The initial burst observed in the first ~ 8 days (Fig. 4.10A) was proportional to the loosely bound drug present on the surface of the micro-implant, which was again proportional to the drug loading. Therefore, higher drug loading resulted in a higher burst release. The increasing presence of loosely bound drug molecules on the surface with increasing drug loading is shown in the SEM images (Figs. 4.4 and 4.5).

Bursts were also caused due to polymer swelling, degradation, and erosion of the polymer matrix. Swelling was an important factor that influenced the initial burst, whereas the subsequent bursts were usually due to the degradation and erosion of the polymer matrix as mentioned in other previous study [30] and our previous study [7]. The initial burst from all three types of coated micro-implants can be explained using the Korsmeyer-Peppas and the zero order models, where 60% of the drug was rapidly released due to the swelling of the polymers. The subsequent bursts after day 10 were probably due to the degradation of the polymer matrix with time, which could not be explained with the models used. However, the final burst in the 10% coated micro-
implant around the 50th day and a major burst in the 25% coated micro-implant between the 40th and 50th day could be inferred from the change in slope of the cumulative release profile of the respective coated micro-implant (Fig. 4.10C).

The coated 10%, 25% and 40% w/w micro-implants produced therapeutic release rate administration until 58, 74 and 66 days, respectively. Since the coated 40% w/w micro-implant contained more quantity of MTX, it is expected that the 40% w/w micro-implant would provide longer drug release duration than the 25% w/w coated micro-implant. However, the 40% coated micro-implant provided shorter release duration of 66 days when compared to 74 days of the 25% coated micro-implant. The optimized polymer to drug (CS: MTX) ratio is expected to influence the maximum duration of the therapeutic release rate. The duration of the release depends on i) drug loading and ii) the polymer to drug ratio. Drug loading is responsible for the total amount of the drug that can be released. Polymer to drug ratio determines how much polymer binding site is available to bind the drug. Higher drug loading results in low polymer to drug ratio, which results in less binding sites and a more loosely bound drug. This led to a higher initial burst, as seen in 40% w/w micro-implant. A higher polymer to drug ratio implies relatively more polymer binding site available to bind the drug as in 10% w/w micro-implant.

Comparing the 25% w/w and 10% w/w micro-implant, the 25% contained higher drug loading and a lower polymer to drug ratio than the 10%. Although the polymer to drug ratio in the 10% (9:1) was higher than the 25% (3:1), the drug loading in the 10% was not sufficient to provide a longer duration of therapeutic release. This is because the 10% micro-implant had relatively more available binding sites and lower availability of the drug. On the contrary, in the 40% micro-implant, the binding sites were all used up, thus leading to more unbound drug. This resulted in most loosely bound MTX in the micro-implant’s CS-MTX matrix in the 40% micro-
implant as compared to that in 25% micro-implant and 10% micro-implant. As a result, the 40% coated micro-implants showed the highest mean release rate and the highest peak release rate, which eventually reduced the overall drug release duration compared to the 25% coated micro-implants. Thus, 25% MTX loading had the optimal polymer to drug ratio or binding site to available drug ratio in order to obtain maximum therapeutic release duration.

4.4.4 Toxicity from the Micro-implants

Each uncoated micro-implant weighed ~ 1 mg. The weight of the MTX (drug) present in the 10%, 25% and 40% w/w micro-implant was ~ 100, 250 and 400 µg, respectively. It is reported that an intravitreal MTX injection containing 400 µg MTX provides a therapeutic level of the drug without toxicity for about 48-72 hours in both pre-clinical setting [4] and in clinical setting [21, 61]. In our study, the micro-implant that had the maximum drug loading (400 µg of MTX) was the 40% w/w MTX which is comparable to the dosage of the intravitreal MTX injection (400 µg of MTX). This 400 µg of MTX was released over a longer duration (> 50 days) from the micro-implant compared to the injection. Therefore, it is expected that the micro-implants containing 40% w/w MTX (400 µg of MTX), 25% w/w MTX (250 µg of MTX) and 10% w/w MTX (100 µg of MTX) would not cause any toxicity despite the burst release in the initial time points.

In our previous study [6, 7] as well as other studies [4], it is mentioned that a release rate of 0.2 - 2.0 µg/ day or a concentration of 0.1 – 1µM would be considered as therapeutic. The micro-implants fabricated in this study were able to administer MTX within the therapeutic window (0.2 - 2.0 µg/day) for a period of more than 50 days, and therefore should not cause any toxicity.
4.4.5 Assumptions of the Study

The lipophilic PLA coating on the surface of the coated micro-implants played an important role in sustained release of MTX. The PLA coating was carried out using dip coating method following a manual protocol of predetermined dipping and drying steps. It was assumed that the PLA coating obtained was consistent in every micro-implant. An automated process of dip coating would yield more consistent coating, thereby minimizing the variability of the pharmacokinetics of MTX.

Further during the coating procedure, DCM was used as a solvent for PLA. DCM is often reported to have toxic effects. Vacuum drying the coated micro-implants ensured evaporation of the DCM. Energy Dispersive Spectroscopy (EDS) studies of the surface of the micro-implants was performed before and after vacuum drying. EDS studies revealed 0.5% w/w chlorine content in vacuum dried coated micro-implants as compared to 5% w/w chlorine in non-vacuum dried coated micro-implants.

The DSC study of the PLA coating was performed at the heating rate of 10°C/min. The influence of any other heating rate on the Tg was not assessed in this study. The motivation of this study was to confirm that the Tg of the PLA coating is greater than physiological temperature. This, in turn, will avoid softening of PLA coating under physiological conditions. Though the heating rate is expected to have some influence on the resolution of the endotherm peak showing the Tg, the values obtained in our experiment are consistent with previous work [54]. Thus, it was assumed that the moderate variation in heating rate may not have a significant influence on the Tg of PLA.

Lastly, the fitting of drug release data in the Higuchi model assumes that i) initial drug concentration in the matrix was much higher than the drug solubility in the polymer matrix; ii) drug
diffusion was taking place from one dimension as one side of the micro-implant was in contact with the vial surface; iii) drug diffusivity was constant; iv) sink conditions were maintained and v) size of the drug particles were much smaller than the thickness of the system.

### 4.4.6 Limitations of the In vitro Study

In the set-up of our *in-vitro* release rate analysis, PBS was used to simulate the volume and conditions of the vitreous fluid in the eye. The composition of the vitreous fluid was not exactly replicated by the PBS fluid which, in turn, could have affected the pharmacokinetics. The positioning of the micro-implant in the intravitreal domain of the eye could have somewhat influenced the drug distribution, which was not assessed in this study. Also, CS is supposed to degrade in the presence of lysozyme. The presence of lysozyme is reported in the vitreous fluid by Stainer *et al.* [37]. This study did not take into account the effect of lysozymic degradation of CS, which could impact the pharmacokinetics of MTX.

The molecular weight of CS influences the internal structure [39, 62, 63]. Higher molecular weight yields stronger and tighter structures due to intramolecular and intermolecular linkages. It therefore directly impacts the swelling properties and drug release mechanisms. Similar influences in structural properties are also noticed by different molecular weights of PLA [30]. This study can be further improved by analyzing the influence of different molecular weights of PLA and CS on the drug release mechanism.

ToF-SIMS analysis does not provide a *quantitative* measure of the chemical composition. It is a *qualitative* measure of the mass fragments generated by the surface when a Ga+ primary ion source at 15 KV accelerating voltage and 1.5 pA current rasters over a 200 µm by 200 µm surface area of the sample. The spectra of CS and MTX were ignored, as it was not expected to be on the surface of the coated micro-implant. However, with an increase in drug loading the rela-
tive intensity of the characteristic mass fragments of MTX present on the surface of the uncoated micro-implant is expected to increase.

4.5 Conclusions

In this *in vitro* study, a unique CS and PLA based MTX micro-implant was fabricated to improve treatment of PIOL. The uncoated CS-MTX implants were able to administer the drug for approximately 1 day. The PLA-coated CS-MTX micro-implants were able to administer the therapeutic release rate of 0.2-2.0 µg/day of MTX for more than 50 days. The PLA-coated CS-MTX micro-implant is expected to improve the bioavailability of MTX in the intravitreal domain, as there are no intravitreal sustained MTX delivery devices.

The PLA coating influenced the initial burst and the mean release rate of MTX from the micro-implants. The amount of drug loading influenced the initial burst and the release duration of MTX from the micro-implants. The release kinetics of MTX from the coated micro-implants is explained by a) the Korsmeyer Peppas and zero order model fit ($R^2 \sim 0.9$) of the first 60% of the drug release, which indicates the swelling of polymer and initial burst release of the drug from the coated micro-implant; and b) the first order and Higuchi model fit ($R^2 \sim 0.9$) from the 10th day to the end of drug release, implying MTX release in the therapeutic window depends on its concentration and follows diffusion kinetics. The micro-implants fabricated in this study can be used as a possible alternative to MTX intravitreal injection for better tolerance and improved efficacy of MTX. Also, this device would produce minimal toxicity and can be administered using minimally invasive methods.
The PLA-coated CS-MTX micro-implants has been tested *in vivo* in rabbit eyes to assess the pharmacokinetics and toxicity caused by the micro-implants. The results and the feasibility of this *in vivo* study are presented in Chapter 5.
Chapter 5. Results: In vivo characterization of the Chitosan and Polylactic acid-based Methotrexate intravitreal micro-implants in rabbit eyes: A pilot study

In this chapter, the results of the in vivo characterization of the CS and PLA-based MTX intravitreal micro-implants in rabbit eyes (Specific Aim 2) are reported. The qualitative assessment of the surgical methods is first discussed, followed by the pharmacokinetics study. The MTX concentration in the vitreous, as obtained in this study, is compared to that of the previous experimentally determined MTX concentrations in human and rabbit eyes. Lastly, the histopathology results are reported.

5.1 Surgical Procedure

The surgical procedure was uncomplicated in both eyes of 7 of the 8 animals. One animal proved difficult to anesthetize fully and moved abruptly during the micro-implant implantation procedure on each eye; this resulted in inadvertent focal physical injury to the crystalline lens during the implantation procedure in each eye and a limited vitreous hemorrhage at the eye wall incision site in one eye.

Gross inspection of the eye wall incision site immediately following enucleation of the eyes showed no instance of wound dehiscence or suppuration in any animal in this series. The position and gross appearance of the inserted micro-implant in the 8 eyes processed for pathological study are shown in Fig. 5.1. None of the micro-implants appeared to have migrated away from its initial implantation site. The gross appearance of the micro-implant and the vitre-
ous surrounding the implant was not appreciably different in the eyes that received a MTX-containing micro-implant and those that received a placebo micro-implant.

**Right eyes - MTX micro-implants**

![Images of right eyes on different days](image)

**Left eyes - Placebo micro-implants**

![Images of left eyes on different days](image)

Figure 5.1 Clear vitreous indicating no toxicity caused by MTX micro-implant and placebo micro-implant on 5th day (A and E, respectively); on 12th day (B and F, respectively); on 19th day (C and G, respectively); and on 33rd day (D and H, respectively)

The gross appearance of the paired micro-implants removed from the eyes opened immediately after enucleation for procurement of vitreous for pharmacokinetics study is shown in Fig. 5.2. The physical size and shape of the paired micro-implants obtained on the specified post-surgical days appeared quite similar. However, both micro-implants obtained on post-surgical day 19 appeared slightly swollen compared with those obtained on other post-surgical days and both micro-implants obtained on post-surgical day 33 appeared slightly shrunken compared with those obtained on post-surgical days 5, 12 and 19. Small pores appeared on the surface of the micro-implants as result of the biodegradation process giving it a more swollen configuration and allowing vitreous fluid to enter the micro-implant causing release of MTX, as observed on day 19. The shrinkage of the micro-implant structure, as observed on day 33, is attributed to the
constant biodegradation process in conjunction with the release of MTX over a period of more
than 1 month.

Figure 5.2 Micro-implants retrieved at the time point of histopathologic evaluation. The top row
contains micro-implants containing MTX micro-implants and bottom row contains placebo mi-
cro-implants on A. 5th day; B. 12th day; C. 19th day and D. 33rd day. Notice the swelling of micro-
implants at 19th and 33rd day. (Scale shown: 1cm)

5.2 Pharmacokinetics Study

The concentration profile of MTX in the vitreous of the eye on 5th day, 12th day, 19th day and
33rd day is shown in Fig. 5.3. The drug concentration is within the therapeutic window (0.1 – 1
µM) during the 12th, 19th and 33rd day time points. There is no MTX concentration detected by
the HPLC in the vitreous samples obtained from the eyes containing the placebo micro-implants.
The MTX concentration data of our study is compared to the clinical study results [22] and the
pre-clinical study results [4] obtained from a 400 µg MTX intravitreal injection and also to our
prior in vitro study in Fig. 5.3.

The MTX release profile obtained from the clinical study [22] and the pre-clinical study [4]
showed a peak concentration of 360 µM and 400 µM, respectively, and lasted for 2-6 days. The
MTX release profile obtained from our in vivo study showed a peak concentration of 11.5 µM on
the 5\textsuperscript{th} day and subsequently lasted in the therapeutic window for more than 1 month. The MTX concentration obtained from our prior \textit{in vitro} study [5] is comparatively more than that of the MTX concentration observed the \textit{in vivo} study. This is probably due to the lack of (1) accurate \textit{sink conditions} in the \textit{in vitro} study; and (2) the realistic convective-diffusive condition of the vitreous in the \textit{in vitro} environment, as discussed later.

The concentration of MTX in the vitreous is fitted to the characteristic first order equation model. A regression value of $R^2 \sim 0.88$ is obtained indicating that the \textit{in vivo} drug release mechanism from the micro-implants follows a \textit{first order kinetics}. The half-life ($t_{1/2}$) of the MTX release is $\sim 4.8$ days.
Figure 5.3 Comparison of concentration of MTX in the vitreous (therapeutic window – shaded region)

First order fit of the in vivo data (MTX micro-implant)

\[ y = 12.321e^{-0.146x} \]

\[ R^2 = 0.8803 \]
5.3 Histopathology Study

Histopathological analysis of the stained micro-slides of the eyes with MTX micro-implant showed (1) no evidence of contamination and bacterial infection of the eye wall incision site or micro-implant in any case; (2) any appreciable structural abnormalities of the cornea, iris, ciliary body, choroid and retina (Fig. 5.4 A: retina of the right eye receiving the MTX micro-implant on 19th day time point; Fig. 5.4 B: retina of the right eye receiving the MTX micro-implant on 33rd day time point) in any case.

![Figure 5.4](image1.png)

Figure 5.4 Histopathological examination showing normal retina of rabbit eyes containing MTX micro-implant indicating no toxicity. Photomicrographs of A) retina of the right eye on 19th day time point (H&E-60X) and B) retina of the right eye on the 33rd day time point (H&E- 15X) showing no toxicity.

Focal cataract corresponding to the previously mentioned micro-implant implantation procedures associated with focal lens trauma was evident in the paired eyes of one animal. Both eyes of all pathologically evaluated animals showed localized inflammation characterized by accumulation of lymphocytes and occasional plasma cells in the eye wall incision, around the incisional...
suture, and in the peripheral vitreous where the micro-implant had been located (Fig. 5.5 A: eye receiving the placebo micro-implant on the 12\textsuperscript{th} day time point; Fig. 5.5 B: eye receiving the placebo micro-implant on the 33\textsuperscript{rd} day time point). The severity of the inflammation was similar in the paired eyes that had contained a MTX implant versus a placebo implant. The vitreous appeared slightly condensed and contained a slight excess of fibroblasts in each eye. However, there were no evident vitreoretinal membranes in any eye.

Figure 5.5 Photomicrographs showing histopathological examination of a) the surgical wound, suture and the inflammation around the placebo micro-implant position on the 12\textsuperscript{th} day time point (H&E-6X); b) the inflammation and vitreous traction surrounding the placebo micro-implant on the 33\textsuperscript{rd} day time point (H&E-6X).

5.4 Discussion

This study showed the cylindrical biodegradable CS and PLA-based MTX micro-implants, fabricated for this study, to be stable physically, able to be implanted via a full-thickness eye wall incision into the peripheral vitreous without difficulty, well tolerated by the evaluated eyes and capable of delivering a sufficient daily dose of MTX to sustain a therapeutic concentration in the vitreous of rabbit eyes for over 30 days. This study did not show evidence of histological
retinal deterioration at any evaluated post-surgical time point related either to the placebo micro-implants or MTX-containing micro-implants.

5.4.1 Issues with sustained release of hydrophilic drugs (MTX) in the intravitreal domain

Our attempts to develop a biodegradable slow-release MTX device for intraocular use were prompted by our desire to have an alternative clinical option to repeated intraocular injections of MTX in appropriately selected eyes of patients with primary intraocular (vitreoretinal) lymphoma. What we learned early on was that all currently approved commercially available intraocular implants for treating eye diseases contain a hydrophobic drug (e.g., corticosteroids, gancyclovir, dexamethasone, fluocinolone acetonide) [15]. In contrast to hydrophilic drugs such as methotrexate, hydrophobic drugs blend readily with FDA-approved hydrophobic or lipophilic matrices such as silicones, polylactic acid (PLA) and polylactic co-glycolic PLGA. They also exhibit inherently limited diffusibility in the vitreous that results in sustained drug released for relatively long time intervals.

In a prior animal study by our group, the toxicity of PLA based MTX micro-needle implants were evaluated in rabbit eyes [16]. The constituents of the micro-implants were well tolerated without any signs of inflammation; however, the devices prepared for and used in that study were unstable physically (they softened unacceptably when heated to the rabbit’s physiological body temperature), were implanted into a lamellar scleral pocket rather than into the vitreous, and degraded substantially faster than anticipated.
5.4.2 Factors affecting the pharmacokinetics

Our recently developed 40% PLA-coated CS-MTX micro-implant containing approximately 400 µg of MTX has shown to deliver a therapeutic concentration of MTX over a period of 1 month in vivo as compared to >2 months in vitro [5] (Fig. 5.3). The difference between the MTX concentrations obtained in the in vivo and the in vitro conditions can be attributed to the reasons such as (1) lack of accurate sink conditions during the in vitro study nearly approximates the true MTX concentration in the vitreous of the rabbit eye at each time point; and (2) lack of the realistic convective-diffusive condition of the vitreous in the in vitro environment. The use of phosphate buffered saline (PBS) of pH 7.4 at ~38 ºC in the in vitro study does not replicate the solubility, diffusivity and convective clearance of MTX in the in vivo vitreous.

Further, the material (PLA and CS) degradation influences the swelling of the micro-implant matrix, which subsequently alters the MTX release mechanism and kinetics. The PLA coating imparts hydrophobicity to the surface of the CS-MTX micro-implant. The hydrophobic PLA coating restricts the vitreous from entering the hydrophilic core of the micro-implant comprised of CS and MTX and prevents rapid MTX release. As expected, the in vivo degradation of CS and PLA by physiological pathways cannot be exactly replicated in the in vitro study. In the in vivo study, PLA is metabolized under physiological conditions to produce carbon dioxide and water, and CS is expected to be degraded by lysozyme, an enzyme present in the vitreous to form aminosugars [37]. Therefore, it is expected that PLA-coated CS-MTX micro-implant will undergo relatively more degradation in the in vivo study as compared to the in vitro study, which probably causes reduced MTX release duration of approximately 1 month in vivo as compared to > 2 months in vitro.
It is observed that the MTX release in both *in vitro* and *in vivo* conditions follows first-order kinetics \( (R^2 \approx 0.9) \) [5]. The consistency in first-order kinetics under both conditions reassures us that the MTX release mechanism depends on the MTX concentration present in the micro-implant. However, due to the limited time points we studied, it was not feasible to fit our *in vivo* drug release data either to the Korsmeyer-Peppas, the zero-order kinetics or the Higuchi models as done in our prior *in vitro* study. We recognize that, fitting drug release data at additional time points to these models could have provided added insight into the contribution of swelling and diffusion in the drug release kinetics analysis.

### 5.4.3 Toxicity and ocular complications

Knowing that the intravitreal injection of MTX (400 µg MTX) is reported to provide a therapeutic concentration of the drug for about 2 – 6 days in both clinical practice [18,19,14] and prior animal studies [11] without significant toxicity, our choice was to use a 40% w/w PLA-coated CS-MTX micro-implant, which also contains similar dosage (400 µg) of MTX, as present in the intravitreal MTX injection.

The peak MTX concentration (11.5 µM) in the vitreous is observed on the 5th day time point when administered using the currently developed micro-implant. The peak MTX concentration obtained from the PLA-coated CS-MTX micro-implant is lower (~ 97%) than the peak MTX concentration obtained with intravitreal injections (360 – 400 µM) [14]. In this study, MTX concentration in the vitreous was within the therapeutic window (0.1 – 1 µM) at the 12th, 19th and 33rd day time points. Since the PLA-coated CS-MTX micro-implant releases the same quantity of MTX (400 µg) over a duration of 30 days as compared to 2 - 3 days when released from the injection, we anticipate that the 40% PLA-coated CS-MTX micro-implant would show sus-
tained release of MTX without causing any significant drug related toxicity despite the burst release as noticed on 5th day time point. The burst release can be further reduced by selecting different combinations of molecular weight and type of polymers.

The histopathology assessment of the enucleated globes revealed no signs of toxicity or microbial infection. Limited inflammation was present, however, adjacent to the micro-implants at later time points (19th and 33rd day time points). Further, some localized granulomatous inflammation was probably caused by foreign bodies such as CS, the polymer used to bind the MTX, and DCM, the solvent used in the PLA coating solution. Although the micro-implants were vacuum-dried after the PLA coating procedure as a precaution to evaporate the DCM [12], we cannot be completely certain that no residual DCM is present on the surface of the micro-implant even after vacuum drying. The inflammatory response, development of scar tissue, and biocompatibility of the micro-implants constituents such as CS and DCM need to be investigated in detail in future studies. However, our findings can also be attributed to the suture material used and the enhanced immunity of the rabbit eye. The animals received a minimal dose of topical steroids for 5 days only. Further, the inflammatory response was very localized and did not cause other ocular issues. At this time, the limited inflammation should not impact future research on these micro-implants because the constituents of our micro-implants such as PLA and PLGA are FDA approved polymers [15], and CS is recognized as a GRAS (Generally Recognized as Safe) material by the FDA [20]. In addition, MTX has anti-inflammatory properties which are expected to reduce post-implantation intraocular inflammation.
5.4.4 Limitations and future directions of the study

In this study, gross examination of the recovered micro-implants failed to demonstrate any obvious disintegration (biodegradation) of these micro-implants through the longest follow-up time in this study (33 days post implantation). The disintegration of the micro-implant must be investigated in future studies as it may be associated with late burst MTX release, vitreous condensation, unanticipated late toxicities or induced structural abnormalities within the eye. Further, the MTX may be washed out of the micro-implant prior to structural disintegration of the micro-implant. It has been reported earlier that biocompatible lipophilic polymers such as poly-lactic acid (PLA), poly-glycolic acid (PGA) and the copolymer of PLA and PGA, poly-(lactic-co-glycolic) acid (PLGA) have been used as matrices to fabricate intravitreal drug delivery devices in both pre-clinical and clinical studies [21-23,15]. In these studies referred above, PLA is reported to have longer biodegradation time than PGA, which subsequently causes longer duration of drug release. PGA is reported to biodegrade faster and cause shorter duration of drug release.

In the study of Kunou et al. [21], PLGA was the preferred polymer instead of pure PLA or PGA in the pre-clinical trials involving intraocular ganciclovir implants. PLGA, which is comprised of both PLA and PGA showed faster biodegradation than pure PLA polymer because of the presence of PGA. The micro-implants assessed in this study had only PLA as the coating polymer. The effect of PLGA as the coating polymer on the sustained release of MTX and the biodegradation of the micro-implant are required to be investigated. A subsequent pre-clinical trial is required to be conducted involving PLGA as the coating polymer in order to better control the biodegradation process, such that another micro-implant can be administered as soon as the previous micro-implant has simultaneously released the drug (MTX) and disintegrated. Further,
this pilot study is somewhat limited by the number of animals used. Another pre-clinical trial with larger sample size and extended time-points will be necessary to better evaluate the pharmacokinetics and potential ocular side-effects of this micro-implant.

5.5 Conclusions

Based on our findings, we conclude that this PLA-coated CS-MTX micro-implant proved to be effective in delivering therapeutic concentrations of MTX in rabbit eyes for over 1 month. The micro-implant appears to be relatively safe and did not cause any serious adverse intraocular reaction such as bleeding, allergic reaction, severe inflammation or tissue damage. This PLA-coated CS-MTX micro-implant could be potentially used as a therapeutic alternative to the present treatment protocol of intravitreal MTX injections in the management of uveitis and intraocular involvement in PCNSL.

In the next chapter, the results of the non-invasive assessment of the retinal toxicity caused by the PLA-coated CS-MTX micro-implant using electroretinography are reported. Further, the qualitative visual assessment of the micro-implant movement, micro-implant degradation and potential intraocular inflammation using ultrasonography is also presented in Chapter 6.
Chapter 6. Results: Non-invasive assessment of retinal toxicity using electroretinography (ERG) and ultrasonography

In this chapter, the results of the non-invasive assessment of retinal toxicity and safety using electroretinography (ERG) and ultrasonography (Specific Aim 3) are presented. The results of the ERG analysis are first reported. Subsequently, the results of the ultrasonography analysis are presented.

6.1 ERG analysis

The summary of the ERG data obtained prior to surgery (PS) and prior to euthanasia (PE) for all eyes at each time point is reported for both the scotopic (dark adapted) and the photopic (light adapted) protocols. Subsequently, B/A ratios are compared between the PS and PE conditions, for each eye at each time point for both the scotopic and the photopic protocols. Lastly, the results of the Naka-Rushton analysis of the ERG data are reported for both the scotopic and the photopic protocols.

6.1.1 Summary of the ERG data

The summary of the ERG data obtained prior to surgery (PS) and prior to euthanasia (PE) for all eyes at each time point is reported for both the scotopic (dark adapted) and the photopic (light adapted) protocols. Subsequently, B/A ratios have been used to compare between the PS and PE conditions, for each eye at each time point for both the scotopic and the photopic protocols.

The representative ERG plots shown in Fig. 6.1 provide a comparison between the scotopic ERG data obtained from PS and PE conditions on the 33rd day time point for the same eye treat-
ed with the MTX micro-implant. Similar ERG plots were obtained for all eyes for both protocols at the pre-determined time points and then compared between the PS and PE conditions.

Figure 6.1. Representative ERG plots of the scotopic protocol obtained from the eye of one rabbit receiving the MTX micro-implant – prior to surgery (day 0) and prior to euthanasia (day 33)

Figure 6.1. Representative ERG plots of the scotopic protocol obtained from the eye of one rabbit receiving the MTX micro-implant – prior to surgery (day 0) and prior to euthanasia (day 33)
Tables 10 and 11 summarize the ERG findings for the scotopic and photopic protocols in our study animals. The values shown in Tables 10 and 11 represent the results obtained for flash intensity of 25000 mcd.s/m². The baseline values for each parameter are obtained from all 8 rabbits used in this study prior to surgery (n = 8, day 0). However, the values for each parameter prior to euthanasia are obtained from only 2 rabbits at each time point (n = 2, day: 5, 12, 19 and 33). The baseline values (PS condition) are reported as mean ± standard deviation (n = 8) while the PE values reported for each time point are mean values only (n = 2).

In both the scotopic and the photopic protocols, the range of values obtained for each parameter during the PE conditions (days: 5, 12, 19 and 33) is similar to the baseline values obtained in the PS condition (day 0). There was some variability from one testing encounter to another but we noted no trend of progressive reduction of ERG amplitudes and/or prolongations of ERG response times in our study animals. This indicates no change to the retinal function as a consequence of the sustained MTX release from the micro-implant.
Table 10. Representative ERG data obtained from the scotopic protocol (Data reported as mean ± standard deviation for Baseline as n = 8; and all other values are reported as mean as n = 2 for days: 5, 12, 19 and 33)

<table>
<thead>
<tr>
<th>Day</th>
<th># of rabbits tested</th>
<th># of eyes tested for each type of micro-implant</th>
<th>Mean A-wave amplitude (µV)</th>
<th>Mean A-wave implicit time (ms)</th>
<th>Mean B-wave Amplitude (µV)</th>
<th>Mean B-wave implicit time (ms)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Right eye</td>
<td>Left eye</td>
<td>Right eye</td>
<td>Left eye</td>
<td></td>
</tr>
<tr>
<td>Baseline (pre-operative)</td>
<td>8</td>
<td>8</td>
<td>68.1 ± 13.4</td>
<td>52.7 ± 22.7</td>
<td>10.4 ± 2.7</td>
<td>10.1 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Day 5 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>93.4 ± 13.4</td>
<td>82.4 ± 22.7</td>
<td>9.2 ± 1.9</td>
<td>8.6 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Day 12 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>64.6 ± 13.4</td>
<td>76.1 ± 22.7</td>
<td>8.2 ± 2.7</td>
<td>9.0 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Day 19 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>81.6 ± 13.4</td>
<td>53.8 ± 22.7</td>
<td>9.0 ± 1.9</td>
<td>9.4 ± 1.9</td>
<td></td>
</tr>
<tr>
<td>Day 33 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>81.9 ± 13.4</td>
<td>92.8 ± 22.7</td>
<td>13.1 ± 1.9</td>
<td>11.8 ± 1.9</td>
<td></td>
</tr>
</tbody>
</table>

For stimulus intensity 25000 mcd.s/m²
Right eye : MTX micro-implant
Left eye: Placebo micro-implant
Table 11. Representative ERG data obtained from the photopic protocol (Data reported as mean ± standard deviation for Baseline as n = 8; and all other values are reported as mean as n = 2 for days: 5, 12, 19 and 33)

<table>
<thead>
<tr>
<th>Day</th>
<th># of rabbits tested</th>
<th># of eyes tested for each type of micro-implant</th>
<th>Mean A-wave amplitude (µV)</th>
<th>Mean A-wave implicit time (ms)</th>
<th>Mean B-wave amplitude (µV)</th>
<th>Mean B-wave implicit time (ms)</th>
<th>B/A</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Right eye</td>
<td>Left eye</td>
<td>Right eye</td>
<td>Left eye</td>
<td></td>
</tr>
<tr>
<td>Baseline (pre-operative)</td>
<td>8</td>
<td>8</td>
<td>18.4 ± 2.8</td>
<td>17.1 ± 6.2</td>
<td>13.4 ± 0.9</td>
<td>14.3 ± 0.8</td>
<td></td>
</tr>
<tr>
<td>Day 5 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>21.1 ± 17.3</td>
<td>14.4 ± 14.1</td>
<td>64.0 ± 6.2</td>
<td>63.9 ± 13.9</td>
<td>2.9 ± 3.5</td>
</tr>
<tr>
<td>Day 12 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>15.4 ± 12.8</td>
<td>12.8 ± 13.5</td>
<td>42.9 ± 12.1</td>
<td>74.1 ± 13.9</td>
<td>3.0 ± 3.7</td>
</tr>
<tr>
<td>Day 19 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>19.1 ± 15.6</td>
<td>14.5 ± 14.5</td>
<td>49.3 ± 4.8</td>
<td>44.8 ± 14.5</td>
<td>2.6 ± 2.2</td>
</tr>
<tr>
<td>Day 33 after micro-implantation</td>
<td>2</td>
<td>2</td>
<td>21.3 ± 28.6</td>
<td>14.4 ± 14.5</td>
<td>74.9 ± 107.3</td>
<td>35.4 ± 31.2</td>
<td>3.7 ± 3.8</td>
</tr>
</tbody>
</table>

For stimulus intensity 25000 mcd.s/m²
Right eye : MTX micro-implant
Left eye: Placebo micro-implant
6.1.2 Relative B/A Ratio [(B/A)\text{rel}] Analysis

The p-values obtained from the two-way ANOVA analysis for each eye and each protocol is reported in Table 12. In the scotopic protocol, the effect of the intensities, days and their interaction on the \((B/A)\text{rel}\) (p-values: 0.60, 0.40, 0.99, respectively) was statistically insignificant (p > 0.05), as observed in the right eye receiving the MTX micro-implant. Moreover, in the scotopic protocol involving the right eye, the effect of the intensities and the days (p-values: 0.48, 0.24, respectively) on the \((B/A)\text{rel}\) excluding their interaction was also statistically insignificant (p > 0.05). Similarly, the effect of the intensities, days on the \((B/A)\text{rel}\) in the left eye receiving the placebo micro-implant was statistically insignificant (p > 0.05), both including as well as excluding their interaction. Furthermore, in the photopic protocol conducted in the right eye, the effect of the intensities and the days on the \((B/A)\text{rel}\) was statistically insignificant (p > 0.05) including their interaction as well as excluding their interaction. However, in the photopic protocol conducted in the left eye receiving placebo micro-implant, the effect of the intensities and the days on the \((B/A)\text{rel}\) was statistically significant (p < 0.05) including their interaction. The discussion section includes additional explanation for significant p-values. Subsequently, the effect of the intensities and the days on the \((B/A)\text{rel}\) was not analyzed using ANOVA excluding their interaction.
Based on the ANOVA analysis, it can be concluded statistically, that there is no effect on the mean \((B/A)_{rel}\) caused by the intensities and the days (observation time points) for the scotopic protocol in both the eyes. Also, it can be inferred statistically, that there is no effect on the mean \((B/A)_{rel}\) caused by the intensities and the days (observation time points) for the photopic protocol in the right eye. However, in the photopic protocol conducted in the left eye, there is an effect of the intensities, days and their interaction on the mean \((B/A)_{rel}\) \((p < 0.05)\).

Subsequently, the comparison of the mean \((B/A)_{rel}\) computed over all intensities for each day (day 5, day 12, day 19 and day 33) is presented in Fig. 6.2A for each combination of protocol (scotopic and photopic) and eye (right eye receiving MTX micro-implant and left eye receiving placebo-micro-implant). Also, the comparison of the mean \((B/A)_{rel}\) computed over all days for each intensity (3000, 10000, and 25000 mcd.s/m²) is presented in Fig. 6.2B, for each combina-

<table>
<thead>
<tr>
<th>Protocol</th>
<th>Right</th>
<th>Left</th>
<th></th>
<th>Right</th>
<th>Left</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>p-values (with interaction)</td>
<td>p-values (without interaction)</td>
<td>p-values (with interaction)</td>
<td>p-values (without interaction)</td>
<td></td>
</tr>
<tr>
<td>Scotopic Right</td>
<td>0.60</td>
<td>0.48</td>
<td>0.14</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Intensity</td>
<td>0.40</td>
<td>0.24</td>
<td>0.88</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td>0.99</td>
<td></td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity + Days (Interaction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scotopic Left</td>
<td>0.14</td>
<td>0.10</td>
<td>0.88</td>
<td>0.86</td>
<td></td>
</tr>
<tr>
<td>Intensity</td>
<td>0.88</td>
<td>0.86</td>
<td>0.70</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity + Days (Interaction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photopic Right</td>
<td>0.92</td>
<td>0.92</td>
<td>0.12</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>Intensity</td>
<td>0.51</td>
<td></td>
<td>0.51</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Days</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Intensity + Days (Interaction)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Photopic Left</td>
<td>0.0024</td>
<td>NA</td>
<td>0.0007</td>
<td>NA</td>
<td></td>
</tr>
<tr>
<td>Intensity</td>
<td>0.0181</td>
<td></td>
<td>0.0181</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
tion of protocol and eye. All data presented in Fig. 6.2 are shown as mean ± 2 x standard error (SE).

Figure 6.2. For each eye and each protocol, A. Mean (B/A)_{rel} computed over all intensities for each day; B. Mean (B/A)_{rel} computed over all days for each intensity. Data shown as mean ± 2 x SE.
The *group mean* values of the \((B/A)_{\text{rel}}\), as obtained for both the scotopic and photopic protocols, have been compared between each eye at each time point and each intensity (Table 13A and Table 13B, respectively). The values are reported as mean ± 2 x SE. The p-value > 0.05 is indicative of the statistically insignificant mean comparisons of the \((B/A)_{\text{rel}}\) between the right and the left eyes.

Table 13A. Summary of \((B/A)_{\text{rel}}\) obtained on each day \((n = 6)\); B. Summary of \((B/A)_{\text{rel}}\) obtained on each intensity \((n = 8)\). Data reported as mean ± 2 x SE

### A

<table>
<thead>
<tr>
<th>Days</th>
<th>((B/A)_{\text{rel}}) obtained on each day (Mean ± 2 x SE)</th>
<th>Scotopic protocol</th>
<th>Photopic protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Right Eye (MTX)</td>
<td>Left Eye (Placebo)</td>
</tr>
<tr>
<td>5</td>
<td>0.9 ± 0.1</td>
<td>1.0 ± 0.6</td>
<td>0.74</td>
</tr>
<tr>
<td>12</td>
<td>1.1 ± 0.3</td>
<td>0.9 ± 0.2</td>
<td>0.50</td>
</tr>
<tr>
<td>19</td>
<td>1.3 ± 0.4</td>
<td>1.3 ± 0.5</td>
<td>0.93</td>
</tr>
<tr>
<td>33</td>
<td>1.3 ± 0.4</td>
<td>1.2 ± 1.2</td>
<td>0.93</td>
</tr>
</tbody>
</table>

### B

<table>
<thead>
<tr>
<th>Intensity (mcd.s/m²)</th>
<th>((B/A)_{\text{rel}}) obtained on each intensity (Mean ± 2 x SE)</th>
<th>Scotopic protocol</th>
<th>Photopic protocol</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Right Eye (MTX)</td>
<td>Left Eye (Placebo)</td>
</tr>
<tr>
<td>3000</td>
<td>1.2 ± 0.3</td>
<td>1.6 ± 0.8</td>
<td>0.36</td>
</tr>
<tr>
<td>10000</td>
<td>1.2 ± 0.4</td>
<td>0.7 ± 0.3</td>
<td>0.06</td>
</tr>
<tr>
<td>25000</td>
<td>1.0 ± 0.2</td>
<td>1.0 ± 0.4</td>
<td>0.94</td>
</tr>
</tbody>
</table>
Mean \((B/A)_{rel}\) values comparison for each day. In the case of \textit{scotopic} protocol, there is an insignificant difference \((p > 0.05)\) in the mean \((B/A)_{rel}\) of the right eye \((0.9 \pm 0.1)\) and the left eye \((1.0 \pm 0.6)\), as obtained on the 5\textsuperscript{th} day of the study. Similarly, the difference between the mean \((B/A)_{rel}\) of the right eye \((1.1 \pm 0.3)\) and the left eye \((0.9 \pm 0.2)\), as obtained on the 12\textsuperscript{th} day of the study, is insignificant \((p > 0.05)\). In addition, on the 19\textsuperscript{th} day of the study, the mean \((B/A)_{rel}\) of the right eye \((1.3 \pm 0.4)\) is not significantly different \((p > 0.05)\) from that of the left eye \((1.3 \pm 0.5)\). Lastly, on the 33\textsuperscript{rd} day of the study, the mean \((B/A)_{rel}\) of the right eye \((1.3 \pm 0.4)\) also does not differ significantly \((p > 0.05)\) from the mean \((B/A)_{rel}\) of the left eye \((1.2 \pm 1.2)\). Similarly, in the case of \textit{photopic} protocol, there was no significant difference \((p > 0.05)\) between the mean \((B/A)_{rel}\) values obtained between the right eyes and the left eyes at all time points.

Mean \((B/A)_{rel}\) values comparison for each intensity. In the case of \textit{scotopic} protocol, there is an insignificant difference \((p > 0.05)\) in the mean \((B/A)_{rel}\) of the right eye \((1.2 \pm 0.3)\) and the left eye \((1.6 \pm 0.8)\), as obtained for 3000 mcd.s/m\(^2\). The difference between the mean \((B/A)_{rel}\) of the right eye \((1.2 \pm 0.4)\) and the left eye \((0.7 \pm 0.3)\), as obtained for 10000 mcd.s/m\(^2\), is also insignificant \((p > 0.05)\). The mean \((B/A)_{rel}\) of the right eye \((1.0 \pm 0.2)\) is not significantly different \((p > 0.05)\) from that of the left eye \((1.0 \pm 0.4)\), as obtained for 25000 mcd.s/m\(^2\). Similarly, in the case of \textit{photopic} protocol, there was no significant difference \((p > 0.05)\) between the mean \((B/A)_{rel}\) values obtained between the right eyes and the left eyes at all intensities. Lastly, the comparison of the group mean values of the \((B/A)_{rel}\), as obtained for both the scotopic and photopic protocols, between each eye at each time point is presented in Fig. 6.3A and Fig. 6.3B, respectively; and for each intensity is presented in Fig. 6.3C and Fig. 6.3D, respectively.
Figure 6.3. Comparison of the group mean values of the \((B/A)_{rel}\), A. between each eye at each time point for scotopic protocol; B. between each eye at each time point for photopic protocol; C. between each eye at each intensity for scotopic protocol; and D. between each eye at each intensity for photopic protocol.
There was no significant difference between the mean \((B/A)_{rel}\) values in any of the eyes at any time point and any intensity for either of the protocols. This indicates no significant change in the functional integrity of the retina due to the presence of the PLA-coated CS-MTX or the placebo micro-implants. On a similar note, the B-wave amplitude data, when fitted to the Naka-Rushton equation \([45, 48, 49]\), also revealed no change in the retinal function, as presented hereafter.

### 6.1.3 Naka Rushton Analysis

The ‘n’ values and the ‘- log K’ values at each time point for different combinations of eyes and protocols are presented in Table 14A. Thereafter, to assess the deviation of the state of the retina from the normal condition, the group mean values of ‘n’ and ‘- log K’ of all time points \((m = 4)\) are compared between the prior to surgery (PS) and the prior to euthanasia (PE) conditions for both eyes and for both the scotopic (Table 14B) and the photopic (Table 14C) protocols.

For the scotopic protocol involving the right eye (MTX micro-implant), there is an insignificant difference \((p > 0.05)\) in the mean ‘n’ values of the PS condition \((0.3 \pm 0.1)\) and PE condition \((0.4 \pm 0.9)\). Similarly, in the left eyes (placebo micro-implant), the difference of the mean ‘n’ values of the PS condition \((0.4 \pm 0.1)\) and the PE condition \((0.4 \pm 0.1)\), is insignificant. Furthermore, in the right eye, there is an insignificant difference in the mean ‘- log K’ values of the PS condition \((2.3 \pm 0.4)\) and the PE condition \((2.2 \pm 0.2)\). Lastly, in the left eye, the difference of the mean ‘- log K’ values of the PS condition \((2.0 \pm 0.1)\) and the PE condition \((1.7 \pm 0.6)\) is insignificant. This indicates no significant change in the retinal sensitivity due to the presence of the PLA-coated CS-MTX micro-implant or the placebo micro-implant. Similar to the scotopic protocol, all comparisons of the photopic protocol are found to be statistically insignificant (Table 14C).
Table 14 A. Summary of Naka Rushton parameters for each time point; B. Summary of Naka Rushton parameters obtained from scotopic protocol (n = 4); C. Summary of Naka Rushton parameters obtained from photopic protocol (n = 4)

### A

<table>
<thead>
<tr>
<th>Days</th>
<th>Micro-implant type</th>
<th>n values</th>
<th>- log K values (log mcd.s/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to surgery</td>
<td>Prior to euthanasia</td>
<td>Prior to surgery</td>
</tr>
<tr>
<td>5</td>
<td>Right eyes (MTX)</td>
<td>0.4</td>
<td>0.5</td>
</tr>
<tr>
<td></td>
<td>Left eyes (Placebo)</td>
<td>0.3</td>
<td>0.4</td>
</tr>
<tr>
<td>12</td>
<td>Right eyes (MTX)</td>
<td>0.3</td>
<td>0.2</td>
</tr>
<tr>
<td></td>
<td>Left eyes (Placebo)</td>
<td>0.2</td>
<td>0.5</td>
</tr>
<tr>
<td>19</td>
<td>Right eyes (MTX)</td>
<td>-0.1</td>
<td>0.7</td>
</tr>
<tr>
<td></td>
<td>Left eyes (Placebo)</td>
<td>0.6</td>
<td>0.1</td>
</tr>
<tr>
<td>33</td>
<td>Right eyes (MTX)</td>
<td>0.5</td>
<td>0.0</td>
</tr>
<tr>
<td></td>
<td>Left eyes (Placebo)</td>
<td>0.3</td>
<td>0.7</td>
</tr>
</tbody>
</table>

### B

Naka Rushton parameters obtained from scotopic protocol (Mean ± Standard Error)

<table>
<thead>
<tr>
<th>Micro-implant type</th>
<th>n values</th>
<th>- log K (log mcd.s/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to surgery</td>
<td>Prior to euthanasia</td>
</tr>
<tr>
<td>Right eyes (MTX micro-implant)</td>
<td>0.3 ± 0.1</td>
<td>0.4 ± 0.9</td>
</tr>
<tr>
<td>Left eyes (Placebo micro-implant)</td>
<td>0.4 ± 0.1</td>
<td>0.4 ± 0.1</td>
</tr>
</tbody>
</table>

*indicates n = 3

### C

Naka Rushton parameters obtained from photopic protocol (Mean ± Standard error)

<table>
<thead>
<tr>
<th>Micro-implant type</th>
<th>n values</th>
<th>- log K (log mcd.s/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Prior to surgery</td>
<td>Prior to euthanasia</td>
</tr>
<tr>
<td>Right eyes (MTX micro-implant)</td>
<td>0.8 ± 0.7</td>
<td>0.8 ± 0.1</td>
</tr>
<tr>
<td>Left eyes (Placebo micro-implant)</td>
<td>0.8 ± 0.1</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>
Prior Naka Rushton Analysis. There have been no prior reports of Naka-Rushton analysis in pre-clinical studies involving intravitreal administrations of MTX. However, research by Oliveira et al.[50] on intravitreal administration of triamcinolone acetonide in normal rabbit eyes reported ‘- log K’ values in the range of 2.82 – 3.37 for both treated and non-treated eyes. The range of ‘- log K’ values obtained in the photopic protocol of the current study (2.92 – 2.97) is similar to the study of Oliveira et al.[50]. Furthermore, the Naka Rushton analysis showed that there is no significant difference in the means of the ‘n’ values and the means of the ‘- log K’ values in any of the eyes for both the scotopic and the photopic protocols, which also implies no significant change in the retinal sensitivity due to the presence of the PLA-coated CS-MTX micro-implant or the placebo micro-implants.

6.2 Ultrasonography study

The 2D ultrasound images of the VR domain, showing the micro-implant location after the implantation procedure, are compared with the micro-implant position after euthanasia at every time point (Days: 5, 12, 19 and 33). The images are assessed to compare: a) any movement of the micro-implant post-implantation with respect to the initial implantation site in the VR domain; b) any visible changes in the intraocular structures indicating inflammation and toxicity; and c) biodegradation of the micro-implant.

Representative corresponding US images obtained immediately post-insertion of the micro-implants and on the specified post-insertion days are shown in Fig. 6.4. As reported before, the PLA-coated CS-MTX micro-implants were implanted in the right eyes. The position of the micro-implant immediately post-surgery is presented in Fig. 6.4A. In Fig. 6.4B, the position of the micro-implant immediately on the 5th day post euthanasia is shown. Similarly, on the 12th day of
the study, the position of the micro-implant post-euthanasia is shown in Fig. 6.4C. Further, the position of the micro-implant post-euthanasia on the 19th and 33rd day of the study is shown in Fig. 6.4D and Fig. 6.4E, respectively.

The PLA-coated CS-MTX micro-implants and placebo micro-implants all appeared stationary in their post-insertion intravitreal position at all time points evaluated in this study. None of them migrated into contact with the crystalline lens or peripheral retina or into the posterior or central vitreous. The size and shape of each of the micro-implants remained stable throughout the study. No evident abnormalities of the peripheral vitreous adjacent to the micro-implant or of the peripheral retina near the micro-implant were identified in any of the evaluated eyes. Similar results were also observed for the placebo micro-implants, which were implanted in the left eyes.

Therefore, based on the preliminary assessment of the ultrasound images it can be assumed that the micro-implants remain stationary at the implanted position and are safe to use in the VR domain.
Figure 6.4 Ultrasound images of the micro-implant: A) Post surgery; B) Post euthanasia (5 days); C) Post euthanasia (12 days); D) Post euthanasia (19 days); and E) Post euthanasia (33 days)
6.3 Discussion

This non-invasive study presented here is the first attempt to analyze the ocular toxicity effects of a PLA-coated CS-MTX micro-implant in normal rabbit eyes using ERG and ultrasonography. The ERG and the ultrasound evaluation conducted in this study confirm no toxicity and alterations in the retinal integrity, which is consistent with the findings of our prior in vivo study on the same rabbits (Chapter 5). Based on these findings, it is expected that the PLA-coated CS-MTX micro-implant is safe for future administration in humans for sustained release of MTX. The prior ERG evaluations involving MTX administrations, comparison of toxicity assessment with histopathology results and the advantages of using ERG and ultrasonography to evaluate retinal toxicity are discussed in detail in the following sections.

6.3.1 Prior ERG evaluations involving MTX administrations

There have been limited studies in the past involving ERG analysis following MTX administration. Velez et al. reported that an intravitreal MTX injection (400 µg of MTX provided therapeutic concentration (> 0.5 µM) of MTX for a period of 2 – 3 days in a pre-clinical setting involving rabbit eyes [4]. ERG analysis conducted in this study showed no statistically-significant difference in the mean of the B- and A-wave amplitudes between the eyes receiving MTX injection and control eyes (placebo) after 162 days (p = 0.11, n = 10 rabbits / 20 eyes) [4]. Also, the difference in the ratio of the B- and the A-wave amplitudes was not statistically significant between the treated eyes and the control eyes (p > 0.20).

The PLA-coated CS-MTX micro-implant that was used in this study contained the same drug loading as administered in an intravitreal MTX injection reported by Velez et al. [4] (400 µg of
MTX). The findings concerning the ERG analysis of retinal toxicity in this study are consistent with that of Velez et al. [4].

### 6.3.2 Comparison of Toxicity with Histopathology Results

As reported in section 5.3, histopathological findings showed no major structural abnormalities between the right and the left eyes for all time points. Histology of all eyes revealed normal retina as seen on Figs. 5.4A and 5.4B. The ANOVA analysis indicates that there is no effect of the intensities and days on the mean \((B/A)_{rel}\) in the right eye receiving the MTX micro-implant, as observed in both the scotopic and the photopic protocols. This indicates that the MTX micro-implant is safe and does not alter the functional integrity of the retina. The \(p\)-value observed in the left eyes (eyes receiving the placebo micro-implant) for the scotopic protocol was statistically insignificant \((> 0.05)\). However, the \(p\)-value observed in the left eyes for the photopic protocol was statistically significant \((< 0.05)\). This indicates changes in the functional integrity of the retina of the left eyes receiving the placebo micro-implant. These changes could possibly be caused by: a) improper light and dark adaptation of the rabbit eyes while recording the ERG data, and b) the toxicity and inflammation induced by the constituents of the placebo micro-implant, such as CS and DCM. On the contrary in the right eyes, which received the MTX micro-implant, the inflammation and toxicity which may have been caused by the micro-implant constituents is neutralized by the anti-inflammatory action of the drug, MTX. As a precaution, we have evaporated the DCM during the manufacturing process of the micro-implant [5]. However, there could be traces of DCM in the placebo micro-implant causing minimal toxicity and inflammation in the left eyes, as reflected by the ANOVA analysis. Nonetheless, the range of values observed for each ERG parameter, such as A-wave amplitude, A-wave implicit time, B-wave amplitude, B-
wave implicit time and the B/A ratio for the PE conditions, is similar to the values observed for the respective parameter in the PS condition. Overall, the ERG analysis supports the findings of our recent histopathology study [64] and assures that there is no major retinal toxicity caused by the micro-implants.

6.3.3 Advantages of using ERG and Ultrasound to Analyze Toxicity

The ERG and ultrasound are convenient non-invasive methods to analyze retinal toxicity and intravitreal abnormalities, respectively. The conditions of the different retinal structures can be evaluated from different ERG components as discussed in section 3.4.1. If the retinal structures are impaired due to a specific treatment, it can be determined by a deviation in the ERG parameter values (amplitudes, implicit times of the B-wave and the A-wave). The ERG analysis provides the status of the retinal structures during the course of a treatment without requiring the need of any histopathology analysis of the retinal tissues. Similarly, ultrasonography is a beneficial tool, which provides an insight into the status of the various intraocular structures of the posterior segment without the need of any surgery. Any disorder in the posterior segment can be visually assessed using the ultrasound technique. The ERG and ultrasound analysis are important techniques which are currently being employed in the field of ophthalmology to analyze impaired retinal and intravitreal conditions. Lastly, the patient comfort is not compromised, as both these methods are non-invasive in nature.

6.4 Limitations

As discussed in the limitations of Chapter 5, this study involved two rabbits per time point. A more detailed study is required to perform the statistical evaluation of the ERG analysis. Fur-
thermore, in the B/A ratio analysis of this current study, the ERG responses obtained for intensities ≤ 1000 mcd.s/ m² were ignored for both the protocols as the light stimulus of low intensities yield immeasurable A-wave amplitude, which subsequently inflates the B/A ratio values. A larger pre-clinical trial involving more animals and time points would be required to obtain more robust statistical comparison of the ERG results.

The ERG responses may be affected by the ERG machine set-up in relation to rabbit eye. The ERG results are influenced by the contact lens electrode, which is required to be in contact with the cornea throughout the duration of the study. However, if there is any movement of the eye, it may be possible that the contact lens remains in partial contact instead of complete contact with the cornea, which is expected to somewhat alter the ERG reading.

The ERG responses are further governed by the dark and light adaptation, pupil diameter and the level of anesthesia attained during the course of the ERG procedure. The consistency of the pupil diameter, dark and light adaptation and level of anesthesia is a challenge to implement. The dark adaptation of the left eye of one of the rabbits of the 5th day time point was momentarily compromised during the ERG recording prior to euthanasia. This inadvertent light exposure caused improper adaptation and may have resulted in inaccurate ERG data recording prior to euthanasia for the 5th day time point. Furthermore, the pupil diameter can potentially vary depending on the drug administered for dilation. If the pupil diameter changes by an order of magnitude, then the effective stimulus intensity on the retina photoreceptor changes by a couple of log units.

Lastly in the US study, we have not followed study animals long enough after intravitreal insertion to document complete biodegradation of the micro-implants in the eye. It is possible that toxicity related to terminal release of MTX or to degradation products of the CS, PLA, or both
would become evident as the micro-implant disintegrates. Furthermore, we have implanted our
devices only into healthy vitreous in non-vitrectomized eyes. The micro-implants may or may
not remain stable in position in the peripheral vitreous in vitrectomized human eyes.

6.5 Conclusions

In this study, ERG and ultrasound were employed for non-invasive evaluation of the retinal
toxicity and safety of the PLA-coated CS-MTX micro-implants in normal rabbit eyes. This
ERG study of rabbit eyes receiving a PLA-coated CS-MTX micro-implant or a placebo micro-
implant showed no evident functional bioelectrical toxicity to the retina during the course of the
study. These findings are consistent with the observations of our prior histopathological evalu-
ation.

Further, it can be concluded from the ultrasound study that the PLA-coated CS-MTX micro-
implant would remain immobile at the implanted position and would not cause any collateral
damage to the ocular structures in the VR domain.

As the PLA-coated CS-MTX micro-implant was determined to be non-toxic and well tolerat-
ed in rabbit eyes, the CS-MTX micro-implant prototype was further investigated for different
lipophilic surface modifications to achieve improved release rate profile and better prediction of
biodegradation. In the next chapter, the results of the in vitro characterization of the improved
CS-MTX intravitreal micro-implant by altered lipophilic surface coating (Specific Aim 4) are
reported.
Chapter 7. Results: *In vitro* characterization of the improved Chitosan-Methotrexate intravitreal micro-implant by altering the lipophilic surface coating

In this chapter, the results of the *in vitro* characterization of the improved CS-MTX intravitreal micro-implant by altering the lipophilic surface coating (Specific Aim 4) are reported. The material characterization is first presented, followed by the release rate study and swelling analysis of the micro-implants. Evaluation of drug release is reported using the model fitting technique for the characteristic drug release. Results are presented as mean ± standard deviation (SD) unless otherwise mentioned.

7.1 Structure Characterization of the Micro-implant

Gel permeation chromatography (GPC) was used to characterize the molecular weight of the polymers used for lipophilic surface modification. Optical microscopy and scanning electron microscopy (SEM) techniques were utilized to assess the micro-implant appearance, dimensions and microstructure morphology. The lipophilic surface modification was evaluated by Fourier Transform Infra-red Spectroscopy (FTIR) and Differential scanning calorimetry (DSC) studies.

7.1.1 Gel Permeation Chromatography

The molecular weight of the different polymers used for lipophilic surface modification, as obtained from the GPC analysis, is presented in Table 15. The number averaged molecular weight ($M_n$) and the weight averaged molecular weight ($M_w$) of all the polymers were in direct proportion of the reported inherent viscosity of the polymers, as provided by the manufacturer.
Furthermore, the polydispersity index (PDI) provides a measure of the distribution of the molecular weight for each polymer used for lipophilic coating. The PDI of all the polymers, except that of PLGA 5050, were in the range of ~ 1.5 -1.6. The high PDI of PLGA 5050 (2.78) is indicative of a wide range of molecular weight distribution in the polymer, which is expected to influence the degradation of the polymer coating in the simulated vitreous conditions [65]. DL-PLA with an inherent viscosity of 0.67 dL/g and 1.16 dL/g will be referred as PLA-100 and PLA-250, respectively, for the remainder of this chapter.

<table>
<thead>
<tr>
<th>Polymer used for coating</th>
<th>Inherent viscosity (dL/g) reported by Lactel® biodegradable polymers</th>
<th>Number averaged molecular weight (Mₙ x 1000)</th>
<th>Weight averaged molecular weight (Mₘ x 1000)</th>
<th>Polydispersity index (PDI = Mₘ/Mₙ)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 5050</td>
<td>0.82 in HFIP* at 30°C</td>
<td>103</td>
<td>287</td>
<td>2.78</td>
</tr>
<tr>
<td>PLGA 6535</td>
<td>0.63 in HFIP at 30°C</td>
<td>54.4</td>
<td>84.8</td>
<td>1.56</td>
</tr>
<tr>
<td>PLGA 7525</td>
<td>0.67 in HFIP at 30°C</td>
<td>92.8</td>
<td>141</td>
<td>1.52</td>
</tr>
<tr>
<td>DL-PLA</td>
<td>0.67 in CHCl₃** at 30°C</td>
<td>102</td>
<td>149</td>
<td>1.46</td>
</tr>
<tr>
<td>DL-PLA</td>
<td>1.16 in CHCl₃ at 30°C</td>
<td>257</td>
<td>411</td>
<td>1.6</td>
</tr>
</tbody>
</table>

HFIP* – Hexafluoroisopropanol; CHCl₃** - Chloroform

7.1.2 Dimension, Morphology and Microstructure

The optical microscopy images showing the top view of the CS-MTX micro-implants coated with PLGA 5050, PLGA 6535, PLGA 7525, PLA 100 and PLA 250 are shown in Figs. 7.1A, 7.1C, 7.1E, 7.1G and 7.1I, respectively. The SEM images showing the magnified morphology of the top surface of the CS-MTX micro-implants coated with PLGA 5050, PLGA 6535, PLGA 7525, PLA 100 and PLA 250 are shown in Figs. 7.1B, 7.1D, 7.1F, 7.1H and 7.1J, respectively.
The length and cross-sectional diameter of the PLA / PLGA-coated CS-MTX micro-implants were approximately 4.3 mm and 1.2 mm, respectively. The micro-implants were of uniform yellowish in color, signifying uniform distribution of MTX throughout the CS polymer matrix. Thus, optical microscopy images reveal uniform coating of PLA / PLGA on the surface of the PLA-coated micro-implants.

Comparing Figs. 7.1B, 7.1D and 7.1F, it can be observed that the smoothness of the surface of the PLGA-coated micro-implants improved with an increasing ratio of PLA content in the PLGA polymer. Furthermore, comparing Figs. 7.1H and 7.1J, it is also noticed that the smoothness of the surface of the PLA-coated micro-implants improved with an increasing molecular weight of the PLA polymer. By coating the micro-implants with PLA / PLGA, the porous surface of the uncoated CS-MTX micro-implant gets filled up with PLA / PLGA and results in a smoother non-porous surface as shown in the SEM images of the coated micro-implants.
Figure 7.1 Optical microscopy and SEM images of the top view of the CS-MTX micro-implants coated by PLGA 5050 (A. and B., respectively); PLGA 6535 (C. and D., respectively); PLGA 7525 (E. and F., respectively); PLA 100 (G. and H., respectively); and PLA 250 (I. and J., respectively).
The optical microscopy images showing the cross-sectional view of the CS-MTX micro-implants coated with PLGA 5050, PLGA 6535, PLGA 7525, PLA 100 and PLA 250 are shown in Figs. 7.2A, 7.2 C, 7.2E, 7.2G and 7.2I, respectively. The SEM images showing the magnified morphology of the cross-sectional matrix of the CS-MTX micro-implants coated with PLGA 5050, PLGA 6535, PLGA 7525, PLA 100 and PLA 250 are shown in Figs. 7.2B, 7.2 D, 7.2F, 7.2H and 7.2J, respectively. An approximate coating thickness of 0.2 mm was observed in PLA / PLGA-coated CS-MTX micro-implants.

Comparing Figs. 7.2B, 7.2 D and 7.2F, it can be observed that the porosity of the internal CS matrix of the PLGA-coated micro-implants reduced with an increasing ratio of PLA content in the PLGA polymer. Furthermore, comparing Figs. 7.2H and 7.2J, it is also observed that the porosity of the internal CS matrix of the PLA-coated micro-implants reduced with an increasing molecular weight of the PLA polymer. In Figs. 7.2B, 7.2 D, 7.2F, 7.2H and 7.2J, it is visible that the PLA / PLGA deposition takes place in the internal voids of the coated micro-implant, resulting in a denser internal matrix with reduced porosity. The internal deposition of PLA / PLGA is important for reduction of swelling of the CS matrix and restricting the MTX release.
Figure 7.2 Optical microscopy and SEM images of the cross-sectional view of the CS-MTX micro-implants coated by PLGA 5050 (A. and B., respectively); PLGA 6535 (C. and D., respectively); PLGA 7525 (E. and F., respectively); PLA 100 (G. and H., respectively); and PLA 250 (I. and J., respectively)


7.1.3 Fourier Transform Infra-red Spectroscopy (FTIR)

FTIR was used to evaluate the bonding between the CS-MTX matrix and the lipophilic coating obtained from PLA and PLGA. The characteristic IR bands for CS (Fig. 7.3) appeared around 3355 cm\(^{-1}\) and 3284 cm\(^{-1}\) (O-H and N-H stretching vibrations), 2871 cm\(^{-1}\) (alkyl C-H stretching vibrations), 1638 cm\(^{-1}\) (amide C=O stretching vibrations), 1584 cm\(^{-1}\) (amine N-H bending vibrations) and 1022 cm\(^{-1}\) (C=O stretching vibrations). The characteristic IR bands for MTX (Fig. 7.3) were observed around 3358 cm\(^{-1}\) (O-H stretching vibrations), 1638 cm\(^{-1}\) (amide C=O stretching vibrations) and 1598 cm\(^{-1}\) (amine N-H bending vibrations). The characteristic bands observed for CS are consistent with that reported by Lopez et al. [66] and El-Hefian et al. [67]; and the characteristic bands seen in MTX is similar to that observed by Kohler et al. [68]. In the uncoated CS-MTX micro-implant, the characteristic IR bands observed were around 3290 cm\(^{-1}\) (O-H stretching vibrations), 2871 cm\(^{-1}\) (alkyl C-H stretching vibrations), 1604 cm\(^{-1}\) (amine N-H bending vibrations) and 1022 cm\(^{-1}\) (C=O stretching vibrations), which are similar to the characteristic IR bands of CS and MTX. This indicates there was no chemical bonding or complex formations between CS and MTX.

The characteristic IR spectra for all the combinations of PLGA and PLA showed the IR bands around 2996 cm\(^{-1}\) and 2943 cm\(^{-1}\) (symmetrical and asymmetrical stretchings of alkyl groups, respectively), 1747 cm\(^{-1}\) (carbonyl C=O stretching vibrations) and 1180 cm\(^{-1}\) (C-O stretching vibrations). The IR spectra obtained for PLA and PLGA polymers were consistent with the observations of Marques et al. [69]. In the IR spectra of the PLA / PLGA-coated CS-MTX micro-implant, the IR bands observed are around 2996 cm\(^{-1}\) and 2943 cm\(^{-1}\) (symmetrical and asymmetrical stretchings of alkyl groups, respectively), 1747 cm\(^{-1}\) (carbonyl C=O stretching vibrations) and 1180 cm\(^{-1}\) (C-O stretching vibrations), which represent the lipophilic PLA /
PLGA coating of the CS-MTX micro-implant. Furthermore, in PLA / PLGA-coated CS-MTX micro-implant, an IR band around 1602 cm$^{-1}$ (amine N-H bending vibrations) is also observed, which represents the CS-MTX matrix of the micro-implant. Therefore, in the IR spectra of the PLA / PLGA-coated CS-MTX micro-implant, the characteristic bands of both the coating polymers and the uncoated CS-MTX micro-implant remain unchanged, which *qualitatively* indicates that the lipophilic coating of PLA / PLGA does not have any chemical bonding with the CS-MTX matrix.
Figure 7.3 FTIR spectra of CS, MTX, uncoated CS-MTX micro-implant, PLGA/PLA, and PLGA/PLA-coated CS-MTX micro-implant
7.1.4 Glass Transition Temperature (Tg)

As discussed in section 4.1.4, the purpose of the DSC study was to assess the glass transition temperature (Tg) of the coating polymers used for the lipophilic surface modification. The Tg of the coating polymer is an important parameter which governs the physical stability of the micro-implant. If the Tg of the coating polymer is less than the physiological temperature (37°C), then the coating will soften, which would deteriorate the structural properties of the micro-implant, causing an accelerated drug release from the micro-implant. A representative DSC plot of one of the PLA-coated micro-implants is shown in Fig. 7.4. Tg is the point where the slope of the endotherm changes. The Tg values of the CS-MTX micro-implants coated with PLGA 5050, PLGA 6535, PLGA 7525, PLA 100 and PLA 250 (n = 4) were 40 ± 4.7 °C, 41.5 ± 3.2 °C, 45.4 ± 8.2 °C, 45.2 ± 4.7 °C and 44.3 ± 10.2 °C, respectively. It can be observed, that with an increase in the ratio of PLA content in PLGA, the Tg rises. The DSC study confirms that the PLA or the PLGA coating will not experience glass transition or soften in the physiological temperature (~37°C) inside the intraocular domain, which is consistent with the findings of section 4.1.4.

![Characteristic DSC curve of a PLA-coated micro-implant showing the Tg ~ 40°C](image)

Figure 7.4 Characteristic DSC curve of a PLA-coated micro-implant showing the Tg ~ 40°C
7.2 *In vitro* MTX release studies

Similar to section 4.2, the details of the drug release rate studies are reported in this section. The MTX release profiles from the coated micro-implants are first described, followed by fitting the drug release data to different pharmacokinetic models to interpret the drug diffusion kinetics.

7.2.1 Release Rate Profiles

A calibration curve for MTX is first obtained using a similar calibration curve as demonstrated in section 4.2.1, and thereafter the 258 nm peak of the MTX spectra is used for the release rate experiments. Release rate profiles of MTX from the PLGA-coated micro-implants and PLA-coated micro-implants containing 40% w/w of MTX, are shown in Fig. 7.5. The release profile of MTX from the coated micro-implants in the therapeutic window (0.2-2.0 µg/day) is shown in the shaded region. The cumulative release profiles of MTX from the coated micro-implants are shown in Fig. 7.6. The mean release profile from each type of coated micro-implant is plotted along with the standard error in Figs 7.5 and 7.6. The summary of release rate characteristics for the PLGA-coated CS-MTX micro-implants and PLA-coated CS-MTX micro-implants is provided in Tables 16 and 17, respectively.
Figure 7.5 Release rate profiles of MTX from the PLGA-coated micro-implants and PLA-coated micro-implants.
Figure 7.6 Cumulative release rate profiles of MTX from the PLGA-coated micro-implants and PLA-coated micro-implants
Table 16. Summary of release rate characteristics of PLGA-coated CS-MTX micro-implants (n = 3)

<table>
<thead>
<tr>
<th>Coating polymer</th>
<th>Mean Release Rate ± Standard Deviation (µg/day)</th>
<th>Total Release Duration (days)</th>
<th>Time of Peak Release Rate (days)</th>
<th>Peak Release Rate ± Standard Error (µg/day)</th>
<th>Start time of release rate within therapeutic limits (days)</th>
<th>Drug released before therapeutic release rate starts ± Standard Error (%)</th>
<th>End time of release rate within therapeutic limits (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLGA 5050</td>
<td>5.4 ± 0.1</td>
<td>82</td>
<td>4</td>
<td>48.6 ± 20.1</td>
<td>~22</td>
<td>89.1 ± 1.2</td>
<td>~82</td>
</tr>
<tr>
<td>PLGA 6535</td>
<td>5.7 ± 0.5</td>
<td>82</td>
<td>8</td>
<td>31.4 ± 3.5</td>
<td>~26</td>
<td>86.9 ± 1</td>
<td>~82</td>
</tr>
<tr>
<td>PLGA 7525</td>
<td>3.4 ± 0.6</td>
<td>138</td>
<td>14</td>
<td>15.5 ± 12</td>
<td>~42</td>
<td>53.8 ± 15.5</td>
<td>~138</td>
</tr>
</tbody>
</table>

Table 17. Summary of release rate characteristics of PLA-coated CS-MTX micro-implants (n = 3)

<table>
<thead>
<tr>
<th>Coating polymer</th>
<th>Mean Release Rate ± Standard Deviation (µg/day)</th>
<th>Total Release Duration (days)</th>
<th>Time of Peak Release Rate (days)</th>
<th>Peak Release Rate ± Standard Error (µg/day)</th>
<th>Start time of release rate within therapeutic limits (days)</th>
<th>Drug released before therapeutic release rate starts ± Standard Error (%)</th>
<th>End time of release rate within therapeutic limits (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PLA 100</td>
<td>3.3 ± 0.3</td>
<td>138</td>
<td>8</td>
<td>15.5 ± 5</td>
<td>~42</td>
<td>83.9 ± 6.4</td>
<td>~138</td>
</tr>
<tr>
<td>PLA 250</td>
<td>1.8 ± 0.1</td>
<td>N/R*</td>
<td>86</td>
<td>3.4 ± 0.5</td>
<td>~ whole duration</td>
<td>N/R**</td>
<td>~N/R*</td>
</tr>
</tbody>
</table>

N/R* – Not Registered as the study was truncated before the entire release of MTX from PLA 250-coated micro-implant could be determined
N/R** - Not Registered as the MTX release from PLA 250-coated micro-implant was approximately in the therapeutic window range for the whole duration of study
7.2.2 Release Rate Study of the PLGA-coated Micro-implant

The mean release rate of the PLGA-coated CS-MTX micro-implants was 5.4 ± 0.1 µg/day (PLGA 5050), 5.7 ± 0.5 µg/day (PLGA 6535) and 3.4 ± 0.6 µg/day (PLGA 7525), as reported in Table 16. Furthermore, the peak release rate of MTX observed from the PLGA-coated micro-implants was 48.6 ± 20.1 µg/day (PLGA 5050, 4th day of release), 31.4 ± 3.5 µg/day (PLGA 6535, 8th day of release) and 15.5 ± 12 µg/day (PLGA 7525, 14th day of release). The total release duration is defined as the duration from the start of drug release till the time it remains in the therapeutic window. The total release duration of MTX from the PLGA-coated CS-MTX micro-implants was 82 days (PLGA 5050), 82 days (PLGA 6535) and 138 days (PLGA 7525).

The MTX release from the PLGA 5050-coated CS-MTX micro-implant remained in the therapeutic window from 22nd day to 82nd day time-point. Furthermore, PLGA 6535-coated CS-MTX micro-implant exhibited therapeutic release of MTX from 26th day onward up to 82nd day time-point. Lastly, a therapeutic release of MTX from the PLGA 7525-coated CS-MTX micro-implant was observed from 42nd day up to 138th day time-point. It was observed, that with an increase in the ratio of PLA content in PLGA: a) the mean release rate and the peak release rate of MTX from the micro-implants reduced and b) the total duration of MTX release along with the duration of therapeutic release of MTX from the micro-implants increased.

7.2.3 Release Rate Study of the PLA-coated Micro-implant

The mean release rate of the PLA-coated CS-MTX micro-implants was 3.3 ± 0.3 µg/day (PLA 100) and 1.8 ± 0.1 µg/day (PLA 250), as reported in Table 17. Furthermore, the peak release rate of MTX observed from the PLA-coated micro-implants was 15.5 ± 5 µg/day (PLA 100, 8th day of release) and 3.4 ± 0.5 µg/day (PLA 250, 86th day of release). The total release
duration of MTX from the PLA 100-coated CS-MTX micro-implant is 138 days. The total release duration of MTX from the PLA 250-coated CS-MTX micro-implant could not be obtained as the study was truncated before the entire release of MTX from PLA 250-coated micro-implant could be determined.

The MTX release from the PLA 100-coated CS-MTX micro-implant remained in the therapeutic window from 42\textsuperscript{nd} day to 138\textsuperscript{th} day time-point. Furthermore, PLA 250-coated CS-MTX micro-implant exhibited therapeutic release of MTX for the entire duration of study. It was observed, that with an increase in the molecular weight of PLA: a) the mean release rate and the peak release rate of MTX from the micro-implants reduced, and b) the total duration of MTX release along with the duration of therapeutic release of MTX from the micro-implants increased.

Thus, the drug administration from all the PLGA and PLA-coated micro-implants was achieved in the desired \textit{therapeutic window} for a period between 2 - 5 months.

\textbf{7.3 \textit{In vitro} MTX Release Kinetics Analysis}

Similar to section 4.3, MTX release obtained from all the \textit{coated} micro-implants were fitted to zero order equation, first order equation, Higuchi model and Korsmeyer-Peppas model in order to analyze the mechanism of drug release and diffusion kinetics. The fitting of each model was evaluated based on correlation coefficient ($R^2$) values. The $R^2$ values of each model fitting are reported in Table 18.
Table 18. *In vitro* release kinetic values of MTX from PLA / PLGA-coated CS-MTX micro-implants

<table>
<thead>
<tr>
<th>Coating polymer (N = 3)</th>
<th>Initial 60% of MTX release</th>
<th>Post initial 60% of MTX release</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Time for 60% drug release (days)</td>
<td>Korsmeyer Peppas R²</td>
</tr>
<tr>
<td>PLGA 5050</td>
<td>8</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA 6535</td>
<td>12</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLGA 7525</td>
<td>54</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLA 100</td>
<td>26</td>
<td>0.97</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PLA 250</td>
<td>130</td>
<td>0.99</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

WR indicates R² values obtained for the Whole Range of time points
7.3.1 Korsmeyer-Peppas model

As discussed in section 4.3.1, the Korsmeyer-Peppas model provides an insight into the type of drug release mechanism taking place from swellable polymeric devices [57]. The ‘n’ of the Korsmeyer Peppas model (section 4.3.1, equation 8) was estimated from the linear regression fit of the logarithmic cumulative release rate data against log (time). The n>1 suggests super case II transport relaxational release and also indicates zero order kinetics [58]. On fitting the first 60% of MTX release rate data from all the PLA / PLGA-coated CS-MTX micro-implants to the Korsmeyer Peppas model, a consistent R² values ~ 0.99 and ‘n’ values ≥ 1.2 (Table 18), which suggests that the first 60% of the drug release is influenced by swelling and relaxation phenomena of the polymer matrix.

7.3.2 Zero order equation

The zero order release equation represents a process when the release rate of the drug is independent of the concentration of the drug in the system (section 4.3.2, equation 9). When the MTX release data is fitted to the zero order equation, the R² values obtained for the whole range of drug release is -0.06 (PLGA 5050), 0.47 (PLGA 6535), 0.92 (PLGA 7525), 0.52 (PLA 100) and 0.99 (PLGA 250). The R² values improve to ~ 0.9 when the initial 60% drug release data from all the coated micro-implants is fit to the zero order equation (Table 18). Therefore, the drug release from the coated micro-implants consistently follows zero order equation for the first 60% of the drug release. It is to be further noted when MTX release data for the whole range is fitted to the zero order equation, R² values increase when: a) the ratio of PLA content increases in PLGA and b) the molecular weight of PLA increases. This indicates that the content and the molecular weight of PLA can be altered to obtain a system for zero order drug release kinetics.
7.3.3 First order equation

The first order release equation (section 4.3.3, equation 10) represents a system where the release rate of the drug is dependent on the concentration of the drug in the micro-implant system. The $R^2$ values are $\sim 0.9$ when the post initial 60% of MTX release data from the coated micro-implants is fit to the first order equation (Table 18). This is consistent with our prior observation in PLA-coated CS-MTX micro-implants in Chapter 4, section 4.3.3. Furthermore, the $R^2$ values are also $\sim 0.9$ when the whole range of MTX release data from the coated micro-implants is fit to the first order equation. This implies the drug release rate from the coated micro-implants for the whole range is primarily governed by first order kinetics and is dependent on the concentration of the drug in the coated micro-implants. The half life ($t_{1/2}$) of MTX release from an intravitreal injection is reported to be $\sim 14.3$ hours [1], whereas the $t_{1/2}$ of MTX release from the coated micro-implants for the whole range of data is 11.3 days (PLGA 5050), 11.8 days (PLGA 6535), 24.9 days (PLGA 7525), 18.6 days (PLA 100) and 100.3 days (PLA 250).

7.3.4 Higuchi model

The Higuchi release equation (section 4.3.4, equation 11) [59] predicts that the drug release is caused primarily by diffusion mechanism. When the post initial 60% of MTX release data from the coated micro-implants is fit to the Higuchi model, the $R^2$ values increased with an increase in the ratio of PLA content in PLGA and increase in the molecular weight of PLA (Table 18), where the $R^2$ values observed are -5.76 (PLGA 5050), -1.1 (PLGA 6535), 0.92 (PLGA 7525), -1.69 (PLA 100) and 0.78 (PLA 250). However, fitting the drug release data to the Higuchi model for the whole range of data provides the $R^2$ values of 0.73 (PLGA 5050), 0.87 (PLGA 6535), 0.94 (PLGA 7525), 0.89 (PLA 100) and 0.84 (PLA 250). This implies the drug release
from the coated micro-implants was primarily governed by diffusion kinetics for the whole duration of study.

Therefore, it can be concluded that the drug release mechanism from the PLA / PLGA-coated micro-implants followed Korsmeyer Peppas model, and zero order equation for the first 60% of the MTX release when MTX was released due to swelling of the polymer matrix and loosely bound MTX particles on the surface. However, the drug release mechanism for the entire range of drug release can be best rationalized by the first order and Higuchi models’, signifying the drug release mechanism was concentration dependent and was primarily caused by diffusion mechanism.

7.4 Swelling Analysis

The mean swelling profile of the PLGA-coated CS-MTX micro-implants is presented in Figure 7.7A. The data is presented as mean ± standard error. The swelling of the CS-MTX micro-implants when coated with PLGA is more pronounced than when coated with PLA. The peak swelling (n = 3) of the CS-MTX micro-implants coated with PLGA 5050, PLGA 6535 and PLGA 7525 is observed to be 6.2 times (30th day), 7.4 times (82nd day) and 6.2 times (114th day), respectively. Furthermore, the peak swelling (n = 3) of the CS-MTX micro-implants coated with PLA 100 and PLA 250 is observed to be 2.2 times and 2.1 times, respectively, on the 22nd day. It can be observed from Fig. 7.7, that with an increase in the ratio of PLA content in PLGA, the onset of swelling of the micro-implant gets delayed, along with a delayed peak swelling time.

The PLGA 5050-coated CS-MTX micro-implant and the PLGA 6535-coated CS-MTX micro-implant disintegrated after 66th and 106th day, respectively (Fig. 7.7B and Fig. 7.7C, respectively). There was no disintegration observed in CS-MTX micro-implants coated with PLGA.
7525, PLA 100 and PLA 250 (Fig. 7.7D, Fig. 7.7E and Fig. 7.7F, respectively). The high PDI of PLGA 5050 (2.78), which is indicative of a wide range of molecular weight distribution in the polymer, is also expected to influence the degradation of the polymer coating in the simulated vitreous conditions [65]. Both PLA 100 and PLA 250-coated CS-MTX micro-implant showed significantly reduced (~ 1.9 times) swelling compared to that of PLGA-coated CS-MTX micro-implants, which can be attributed to the pure PLA polymer coating. Lastly, the peak swelling of PLA 250 (2.1 times) is lower compared to that of PLA 100 (2.2 times), which could have been caused by a higher molecular weight of the PLA 250.
Figure 7.7 Swelling profile of the PLGA-coated micro-implants and PLA-coated micro-implants
7.5 Discussion

In this study, the prior PLA and CS-based micro-implants were improved by altering the lipophilic surface modification. The purpose of this chapter was to characterize the influence of modified polymer coating involving: a) PLA:PGA copolymer ratio in PLGA and b) the molecular weight of PLA and PLGA on the MTX release rate and c) swelling of the micro-implants. The influence of lipophilic coating on the MTX release characteristics, and on its therapeutic range are discussed in the following sections.

7.5.1 Influence of lipophilic coating

As reported in section 4.4.2, the lipophilic coating of PLA and PLGA is important to prevent the transport of PBS into the CS matrix, thereby reducing the rate of swelling of the CS matrix and associated MTX release. In this study, the lipophilic surface modification improved the sustained release duration of MTX from 2 months to about 5 months. Furthermore, the PLA:PGA copolymer ratio in PLGA and the molecular weight of both PLGA and PLA played an important role in governing the initial burst of MTX release from the micro-implants. The initial burst or the peak release rate is often considered to be toxic as it is above the therapeutic range. The initial burst was better controlled by increasing the ratio of PLA content in the PLGA copolymer. In other words, increasing the molecular weight of PLA resulted in delayed and reduced initial burst.

Furthermore, the swelling characteristics and the biodegradation properties can be regulated by altering the PLA:PGA copolymer ratio in PLGA and the molecular weight distribution of both PLGA and PLA. A coating comprising of higher molecular weight of PLA (or PLGA with an increased ratio of PLA) would be the preferred choice to fabricate a micro-implant for ex-
tended sustained release and reduced initial burst of the drug, delayed swelling and biodegradation of the micro-implant. On the contrary, a coating comprising low molecular weight PLA or PLGA with a high ratio of PGA would be the preferred choice to fabricate a micro-implant for a shorter duration of sustained release of the drug, faster swelling and biodegradation of the micro-implant.

7.5.2 Therapeutic MTX Release

In this study, 40% w/w CS-MTX micro-implant was used, which had a comparable dosage of the intravitreal MTX injection (400 µg of MTX). It has been reported that an intravitreal MTX injection containing 400 µg MTX provides a therapeutic level of the drug without toxicity for about 48-72 hours in both pre-clinical [4] and in clinical settings [21, 61]. In this research, the PLA / PLGA-coated micro-implants released 400 µg of MTX over a longer duration (2 – 5 months) compared to the injection. Therefore, it is expected that the PLA / PLGA-coated micro-implants containing 40% w/w MTX (400 µg of MTX) would not cause toxicity despite the burst release of MTX in the initial time-points. Furthermore, both PLA and PLGA have been approved by the FDA to be used for intraocular domain. Lastly, the PLA and CS-based MTX micro-implant containing the same 400 µg of MTX was characterized and well tolerated in normal rabbit eyes [64] (Chapters 5-6).

FTIR study confirmed that there was no chemical bonding between any constituents of the micro-implants, which implies that the drug will be available unaltered when released from the coated micro-implants. Furthermore, DSC study revealed the Tg of the coating polymers to be around 40°C, which indicates that the lipophilic polymer coating will not soften in the physiological temperature (~37°C). Lastly, it is desirable for the micro-implant to disintegrate after the
completion of drug release. The disintegration of the micro-implant can be predicted based on the swelling and degradation characteristics of the lipophilic coating polymer.

7.6 Conclusions

In this *lipophilic surface modification* research, the PLA / PLGA-coated CS-MTX micro-implants were able to administer the therapeutic release rate of 0.2-2.0 µg/day of hydrophilic drugs, such as MTX, for a period of 2 – 5 months. The release kinetics of MTX from the coated micro-implants is evaluated by a) the Korsmeyer Peppas and zero order model fit ($R^2 \sim 0.9$) of the first 60% of the drug release, which indicates *the swelling of polymer* and *initial burst release* of the drug from the coated micro-implant; and b) the first order and Higuchi model fit ($R^2 \sim 0.9$) for the whole range of drug release, implying MTX release was depending on its *concentration* and followed by *diffusion kinetics*.

It can be observed that with an increase in the PLA content in PLGA and the elevated molecular weight of PLA, a) the initial burst of MTX and the mean release rate of MTX from the micro-implants can be reduced; and b) the swelling and biodegradation of the micro-implants can be delayed. Therefore, the lipophilic surface modification of the CS-MTX micro-implant surface is important for improving and optimizing the release duration of MTX.
Chapter 8. Summary and Future scope

8.1 Summary: Tasks accomplished

In this chapter, the summary of the research conducted in this dissertation are reported. Chapter 4 presents the results of the *in vitro* characterization of the CS and PLA-based MTX intravitreal micro-implant (Specific Aim 1). Chapter 5 presents the results of the *in vivo* characterization of the CS and PLA-based MTX intravitreal micro-implants in rabbit eyes (Specific Aim 2). Chapter 6 presents the findings of the *non-invasive* assessment of retinal toxicity and safety using ERG and US (Specific Aim 3). Lastly, Chapter 7 presents the results of the *in vitro* characterization of the improved CS-MTX intravitreal micro-implant by altering the lipophilic surface modification (Specific Aim 4).

8.1.1 Summary of the *in vitro* characterization of the CS and PLA-based MTX intravitreal micro-implants (Specific Aim 1)

In this *in vitro* study, a unique CS and PLA-based MTX micro-implant was fabricated to improve PIOL management. CS and PLA-based micro-implants were fabricated for different MTX loadings (10%, 25% and 40% w/w). First, CS (M.W. 50,000-190,000 and DA% ≥ 75%) and MTX mixtures were prepared for different drug loadings, and lyophilized in Tygon® tubing to obtain CS-MTX fibers. The fibers were then cut into desired micro-implant lengths and *dip coated* in PLA (inherent viscosity of 1.16 dL/g in CHCl₃ @ 30°C) for a lipophilic surface coating. The micro-implant was characterized using Optical Microscopy, SEM, ToF-SIMS and DSC techniques. The release rate studies were carried out using a UV-Visible Spectrophotometer.
The uncoated CS-MTX implants were able to administer the drug for approximately 1 day. The PLA coated CS-MTX micro-implants were able to administer the therapeutic release rate of 0.2-2.0 µg/day of MTX for more than 50 days. The PLA coated CS-MTX micro-implant is expected to improve the bioavailability of MTX in the intravitreal domain, as there are no intravitreal sustained MTX delivery devices.

The PLA coating influenced the initial burst and the mean release rate of MTX from the micro-implants. The amount of drug loading influenced the initial burst and the release duration of MTX from the micro-implants. The release kinetics of MTX from the coated micro-implants is explained by a) the Korsmeyer Peppas and zero order model fit ($R^2 \sim 0.9$) of the first 60% of the drug release, which indicates the swelling of polymer and initial burst release of the drug from the coated micro-implant; and b) the first order and Higuchi model fit ($R^2 \sim 0.9$) from the 10th day to the end of drug release, implying MTX release in the therapeutic window depends on its concentration and follows diffusion kinetics. In this study, the influence of the lipophilic PLA coating and the drug loading on the release kinetics of MTX were studied in an in vitro environment.

8.1.2 Summary of the in vivo characterization of the CS and PLA-based MTX intravitreal micro-implants in rabbit eyes (Specific Aim 2)

The purpose of this study was to evaluate the pharmacokinetics and toxicity of a CS and PLA based MTX intravitreal micro-implant in an animal model using rabbit eyes. CS and PLA-based micro-implants containing 400 µg of MTX were fabricated using lyophilization and dip-coating techniques as specified in Chapter 3. The micro-implants were surgically implanted in the vitreous of eight New Zealand rabbits employing minimally invasive technique. The PLA-coated
CS-MTX micro-implant was inserted in the right eye and the placebo micro-implant in the left eye of each rabbit. Two rabbits were euthanized at each pre-determined time point (days: 5, 12, 19 and 33) for pharmacokinetics and histopathology evaluation.

A therapeutic concentration of MTX (0.1 – 1.0 µM) in the vitreous was detected in the rabbit eyes studied for 33 days using HPLC. The MTX release from the coated micro-implants followed a first order kinetics ($R^2 \sim 0.88$) implying MTX release depends on the concentration of MTX in the micro-implant. Histopathology analysis of the enucleated eyes failed to show any signs of infection in any of the specimens. Intravitreal hemorrhage was present in eyes receiving the placebo micro-implant. Focal, traumatic cataract was present in two eyes due to micro-implant positioning. There were no signs of direct tissue toxicity in any of the enucleated eyes.

8.1.3 Summary of the non-invasive assessment of retinal toxicity and safety using ERG and US (Specific Aim 3)

The purpose of this section was non-invasive evaluation of the retinal toxicity and safety of the PLA-coated CS-MTX micro-implants in normal rabbit eyes using ERG and US. The ERG analysis and the US study were conducted on the same eight rabbits used in the in vivo evaluation study of the PLA coated CS-MTX micro-implant (Specific Aim 2).

The ERG study was conducted on a portable ERG machine (HMsERG system) on each eye. ERG recordings were obtained for two conditions; 1) prior to the surgery (PS) involving the implantation of the micro-implant and 2) prior to the euthanasia (PE). ERG analysis involved recordings of two protocols on each eye – scotopic protocol, representing the activity of the rods and the photopic protocol, representing the activity of the cones. Statistical analysis of the ERG
data (B/A ratio analysis and Naka-Rushton analysis) was conducted to analyze the change in retinal function due to the PLA-coated CS-MTX micro-implant.

The ultrasound study was conducted employing a B-scan mode recording of the Linscan 12 MHz veterinary ophthalmic ultrasound unit. The purpose of the ultrasound study was to evaluate a) any movement of the micro-implant in the VR domain post-surgery; b) any visible changes in the vitreous or ocular structures indicating inflammation; and c) biodegradation of the micro-implant.

The statistical analysis of the ERG data implied no change in the retinal function due to the PLA-coated CS-MTX micro-implant. This was consistent with the findings of the histopathology study (Specific Aim 2). The ultrasound study further showed that the micro-implants were stationary at the implanted position and did not cause any alterations in the intraocular structures of the posterior segment.

Based on the findings of the ERG study and the ultrasound study it was expected that the PLA-coated CS-MTX micro-implant 1) would not cause any retinal toxicity; 2) would remain stationary at the implanted position; 3) would not cause any collateral damage to the ocular structures in the VR domain; and 4) safe to use for sustained release of MTX in the VR domain.

8.1.4 Summary of the in vitro characterization of the improved CS-MTX intravitreal micro-implant by altering the lipophilic surface modification. (Specific Aim 4)

In this study, the CS-MTX micro-implants, containing ~ 400 µg of MTX, were dip coated in fixed concentrations of different PLGA combinations (PLGA 7525, PLGA 6535 and PLGA 5050, where PLA:PGA – 75:25, 65:35, 50:50, respectively) and DL-PLA of 2 different molecular weights for the lipophilic surface modification. The structure of the micro-implants was
thereafter characterized with optical microscopy and SEM, FTIR and DSC. The influence of the
lipophilic surface modification on the MTX release was characterized by a comparative assess-
ment of a) the MTX release rate and b) the swelling of the different PLA / PLGA-coated CS-
MTX micro-implants. The MTX release rate and the swelling studies are conducted by placing
the PLA / PLGA-coated CS-MTX micro-implants (n = 3, for each type of coating polymer) in a
vial containing 5 ml of phosphate buffered saline (pH 7.4) in a water bath at 38°C. At predeter-
minded time intervals, samples are assayed for a) MTX release rate using UV-Visible Spectropho-
tometer (MTX characteristic peaks-258, 302, 372 nm) (Specific Aim 1) and b) swelling of the
micro-implant.

FTIR analysis revealed there was no chemical bonding between the lipophilic PLA / PLGA
coating and the hydrophilic CS-MTX core of the micro-implant. It was observed from the DSC
study that the Tg of the coated micro-implants was between 40 – 45°C, which indicated that the
PLA / PLGA coating will not soften in the physiological temperature (37°C). Optical micro-
scopy and SEM showed that the porosity of the matrix and the surface of the micro-implant re-
duces with: a) an increase in PLA ratio in PLGA, and b) an increase in molecular weight of PLA.
Furthermore, the initial burst of MTX release, swelling and degradation of the micro-implants
can be reduced by: a) increasing the PLA ratio in PLGA, and b) increasing the molecular weight
of PLA. Therefore, it can be concluded that the lipophilic surface modification is important to
optimize the drug release characteristics, swelling and biodegradation of the micro-implant.
8.2 Future scope

Based on the results obtained in Specific Aims 1, 2 and 3, it can be anticipated that the PLA / PLGA-coated CS-MTX micro-implant can be used as a potential safe alternative to the present treatment protocol of intravitreal MTX injections to treat PIOL and other VR diseases like AMD, PVR and uveitis. This sustained release micro-implant platform can be applied for other hydrophilic drugs like Aflibercept, Ranibizumab, 5-Fluorouracil to treat a variety of intraocular diseases. The key to the formulation of this micro-implant device is the choice of a hydrophilic polymer, such as CS, to blend a hydrophilic drug, such as MTX, and then the subsequent lipophilic surface modification of the micro-implant using a lipophilic polymer, such as PLA / PLGA, to enable sustain release of the hydrophilic drug (MTX). The future scope of this research includes:

a) *In vitro* trials to reduce the size of the micro-implant to pass through a 23G trocar.

b) *In vitro* trials to fabricate the micro-implant with other hydrophilic polymers such that the inner polymer-MTX matrix is less porous compared to CS-MTX micro-implant.

c) *In vitro* trials to characterize the micro-structure and chemical bonding between the lipophilic coating and the hydrophilic matrix core.

d) *Larger* pre-clinical trial for improved assessment of pharmacokinetics and toxicity.

e) *Develop* an automated fabricating device for the micro-implants, to avoid the variability while preparing it manually.
References


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