An injectable ocular biostimuli-degradable, spacer-based, polymer drug conjugate for targeted treatment of posterior segment inflammation

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A dissertation submitted to the Faculty of Health Sciences, University of the Witwatersrand, in fulfilment of the requirements for the degree of Master of Pharmacy

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Johannesburg, 2020

DECLARATION

I, Muhammed Anwary, declare that this Dissertation is my own, unaided work. It is being submitted for the degree of Master of Pharmacy in the Faculty of Health Sciences at the University of the Witwatersrand, Johannesburg, South Africa. It has not been submitted before for any degree or examination at this or any other University.

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Signed this 6th day of August 2020

ANIMAL ETHICS DECLARATION

I hereby confirm that the following study entitled "**An injectable ocular biostimuli-degradable, spacer-based, polymer drug conjugate for targeted treatment of posterior segment inflammation**" had received the approval from the Animal Research Ethics Committee of the University of the Witwatersrand with ethics clearance number 2018/09/39/C (Appendix).

ABSTRACT

The posterior segment of the eye presents a daunting task in relation to effective administration of therapeutic measures, due in part to its relative inaccessibility anteriorly and in part due to its protective blood-ocular barriers which do not allow for a significant amount of drug to be passed through. Posterior segment inflammatory disorders have a range of causes but the potential outcome for most of these disorders, if left untreated, is permanent blindness. Thus, the advantages of intravitreal polymeric drug delivery systems which release drug directly into the posterior segment for varying time periods are numerous.

Current market formulations as well as ongoing research related to injectable, intravitreal injectable devices for posterior segment applications were critically reviewed. A potential way forward was researched: An injectable ocular biostimuli-degradable, spacer-based polymer drug conjugate for targeted treatment of posterior segment inflammation. This dissertation details the outcomes of this research.

Design of stimulus-responsive intravitreal drug delivery technologies are one step further in the right direction as these systems would potentially translate to more controlled drug release mechanisms. Several intravitreal devices have been formulated, however, there are certain drawbacks present such as the lack of biodegradability and the need for relatively complex surgical procedures. This research was focused on the pharmaceutical, rather than the therapeutic, perspective of the development of a biostimuli-responsive, biodegradable and injectable intravitreal drug delivery system for administration in inflammatory posterior segment disorders. The Schiff-base based formulation was synthesized, characterized and tested *in vivo* in a New Zealand White Albino rabbit eye model and evaluated for toxicity and enhanced drug release. The “intelligent” system was designed using a biocompatible methoxy polyethylene glycol polymer with a hydrazide cap; which served as a linker; and dexamethasone, an established anti-inflammatory drug. The product would form a pH-labile hydrazone bond, a type of Schiff base. *In vitro* release studies showed a slight enhancement of drug release in low pH conditions. The system was then moved ahead into the *in vivo* stage of testing.

Post-*in vitro* investigations, the system was tested *in vivo* in a study group of 33 New Zealand White Albino rabbits. The system was tested in physiological inflammatory conditions and normal non-inflammatory physiological conditions in a 10-day study and compared to negative and positive control groups which served as a reference. Results showed an enhanced release of drug during inflammatory (pH~ 5.5) conditions in the posterior segment which also translated to an increased anterior chamber concentration (Inflamed = 0.691µg/mL vs non-inflamed 0.0634µg/mL) and histomorphological analysis determined the formulation to be non-toxic to the ocular environment. Thus, this formulated system has potential to be utilized in posterior segment inflammatory diseases.

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ACKNOWLEDGEMENTS

My deepest gratitude goes to my creator, the One without beginning nor end, without whom everything would be an impossibility, my parents and family for their patience and encouragement, my friends, associates and colleagues, and especially to those who assisted me knowing that there was nothing for them to gain from it apart from, maybe, a bit of happiness.

I would like to express my gratitude to Professor Viness Pillay, Professor Yahya Choonara, Associate Professor Pradeep Kumar, and Associate Professor Lisa du Toit for their patience, guidance, encouragement and useful critique to this research work.

To Zain, Eish! We're still here…

DEDICATIONS

This work is dedicated to all my brothers and sisters around the world who are subject to oppressive rulers. Do not worry, justice will be served.

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CHAPTER 1

Overview, Rationale and Motivation for Study

1.1. Introduction

Uveitis is a broad name for a collection of inflammatory disorders affecting the eye, specifically the uvea (anteriorly or posteriorly), but may also affect surrounding structures such as the retina and vitreous body (Durrani *et al*., 2004). The uvea consists of the iris, the ciliary body and the choroid. It may be of infectious or non-infectious origin (Haghjou *et al*., 2011) and is one of the major treatable causes of blindness (Shenzhen *et al*., 2013). According to Durrani *et al*. (2004) occurrence of uveitis is reportedly at 14-17 per 100 000 people annually. Once the structures of the posterior eye are affected, this is termed as posterior segment uveitis.

Topical application of medicament is not a suitable treatment as it may not provide appreciable penetration into the posterior segment of the eye (Dong *et al*., 2006). Systemic treatment is associated with side-effects (Mohamed Khalil *et al*., 2016). Drugs that have been used systemically include corticosteroids and both new and old immunosuppressive agents including antimetabolites, alkylating agents, calcineurin inhibitors, biologic agents and interferons (Angela *et al*., 2013; Haghjou *et al*., 2011).

Thus, the employment of a local intravitreal route of administration, whether implant or by intraocular injection, would significantly decrease the occurrence of systemic side-effects (Mahlumba *et al*., 2016). Local administration would also increase the efficacy of the drug, as improved bioavailability would be attained. However, local administration does not mean that the formulation would be free from side effects. Local administration of intravitreous corticosteroids do aid in reducing inflammation, but they have also been associated with unwanted effects such as raised intraocular pressure, which is sometimes vision-threatening, and cataract formation (Sugita, 2007). Thus, an immunosuppressive agent would be the alternative for reduction of inflammation (Yeh *et al*., 2008).

In recent years, many different designs of drug delivery systems for posterior segment delivery have been investigated including direct intravitreal injection and various types of implantable intravitreal devices which are bioerodible or non-bioerodible (Choonara *et al*., 2010). Such devices include Vitrasert® - a reservoir ganciclovir insert, Retisert®- a fluocinolone acetonide tablet that is sutured to the sclera, I-vation®- a helical implant containing triamcinolone that

should release drug over a period of two years and Ozurdex®- dexamethasone-loaded polymer pellet formed to the shape of a rod (Christoforidis *et al*., 2012).

This study focused on formulating a spacer-based, biodegradable, biostimuli responsive, hydrogel system (BioRes) for use in the treatment of posterior segment inflammation in the eye. As per the literature, there are currently no such products on the market. This formulation would allow for a more targeted and controlled release of an immunosuppressive agent therefore greatly reducing the side effects, relative to other means of drug delivery, and improving the drug therapy. It would be more viable for patients as the procedure would be less invasive than inserting an implant. Further, the fact that it would be biodegradable indicates that it would not have to be removed once the treatment is complete. This formulation would respond to a certain stimulus within the environment of the eye such as the temperature or pH.

1.2. Rationale and Motivation

Currently there are intravitreal devices available for the treatment of posterior segment uveitis, however, as some of the implants are non-biodegradable, they require major surgery to be removed (Choonara *et al*., 2010). This removal surgery carries the risk of further complications. An intravitreal injection ensures the efficient delivery of drug for different ailments including uveitis (Peyman *et al*., 2009). Designing an intravitreous, spacer-based, biostimuli-responsive and biodegradable injectable hydrogel would ensure better outcomes for patients with the aforementioned condition as it would provide targeted therapy thus reducing the risk of systemic side effects and removal surgery.

This novel system would consist of a spacer-based smart Polymer-drug conjugate that, on being injected intravitreally via a hydrogel drug delivery vehicle into the ocular environment, would slowly start degrading (Qiu & Park, 2001) as depicted in Figure 1 below. During degradation of the spacer, which may be inflammation responsive such as poly(ethylene glycol)-based compounds (Reid *et al*., 2015) or hydrolysable such as hydrolysable esters and acid-sensitive acetals and Schiff bases (imines), there will be release of the immunosuppressive or anti-inflammatory drug, depending on which will be used in the formulation, from the copolymer matrix thus decreasing intraocular inflammation (Mathews *et al*., 2010).

The polymer in question viz. poly(ethylene glycol), and if necessary, poly(lactic-co-glycolic acid) and poly(lactic acid), would impart the attributes of biodegradability and forming a bioresponsive hydrogel. It should be noted that poly(ethylene glycol)-based compounds are responsive to hydroxyl radicles produced during inflammatory processes and Schiff bases are responsive to the change in pH occurring in inflammatory processes (Saito *et al*., 2007). These

poly(ethylene glycol)-based compounds and Schiff bases would act as a spacer to conjugate the drug, dexamethasone, with the copolymer mentioned above.

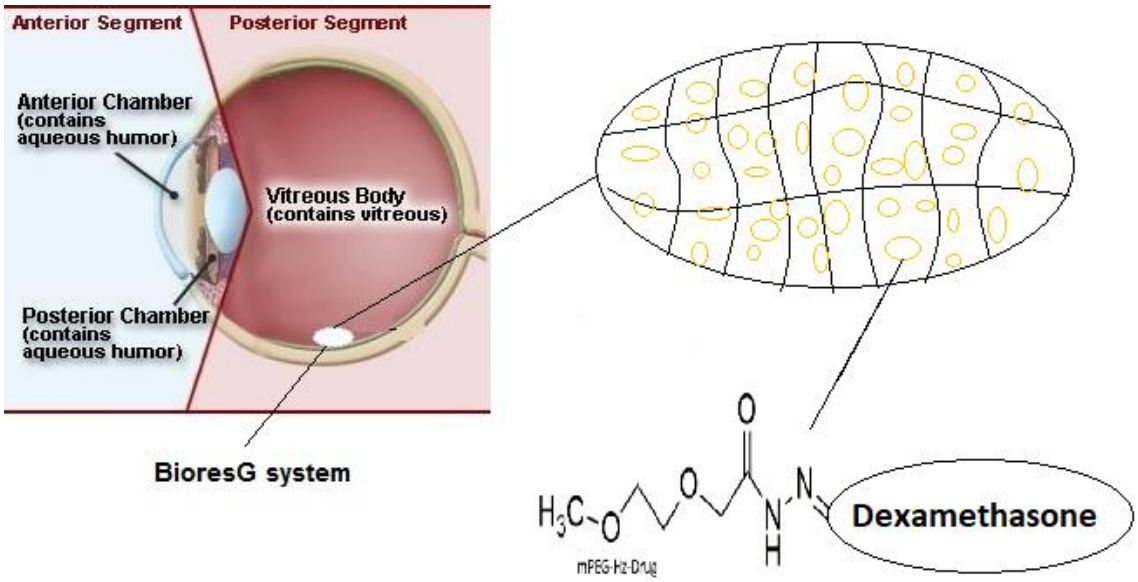

Figure 1: Schematic representation of the BioRes system in-situ and elucidation of structure (Adaptation from University of Minnesota, Fluids in each eye segment, 2016)

1.3. Aim and objectives

The aim of this research was to develop a novel BioRes system for intravitreal administration to treat inflammatory conditions associated with posterior segment uveitis. To this end the following objectives were achieved:

1. To identify and achieve an appropriate hydrogel system for delivery of the system into the eye.
2. To incorporate an appropriate spacer to ensure the binding of the drug and polymer.
3. To optimize the formulation variables with regards to optimal degradation and drug release.
4. To determine the physicochemical properties of the formulation by means of Fourier Transform Infrared Spectroscopy, Differential scanning calorimetry, Nuclear Magnetic Resonance and X-Ray Diffraction.
5. To conduct an *in vitro* evaluation of the formulation to determine drug release and polymer degradation.

6. To perform animal studies to determine the *in vivo* behaviour of the system with regards to the safety of the formulation.

1.4. Overview of dissertation

1.4.1. Chapter 1

This chapter introduced uveitis (an inflammatory posterior segment ocular disease), its causes and impacts of this disease. Drawbacks of topically administered drugs and some current market formulations are briefly discussed. Benefits of local administration, the need for a targeted and controlled release drug delivery system and a means of achieving this type of system is discussed. It also outlined the rationale, aims and objectives of this study.

1.4.2. Chapter 2

This is a published review paper that discussed Polymeric Injectable Intravitreal Hydrogel Devices for Posterior Segment Applications/ Diseases. The various types of polymers, related to their sources, biodegradability, gelation time, biocompatibility, toxicity, *in vitro* studies, *in vivo* studies and drug release durations are critically reviewed and discussed in this chapter. A future prospect for ocular drug delivery is also mentioned here.

1.4.3. Chapter 3

This chapter outlines the preformulation studies that were conducted relating to the synthesis of a thermoresponsive hydrogel consisting of a polyethylene glycol- polycaprolactone (PEG-PCL) copolymer as the vehicle for delivery of BioRes delivery system. The challenges that were experienced in the formulation of these systems are also discussed.

1.4.4. Chapter 4

This paper discusses the design, synthesis, characterization, *in vitro* drug release and *in vivo* toxicity and drug release of the biostimuli-degradable, spacer-based, polymer-drug conjugate an injectable intraocular hydrogel system for delivery of drug in the posterior segment of the eye. Characterization tests including nuclear magnetic resonance, calorimetric and Fourier transform infrared spectroscopy were conducted and analysed. *In vivo* analysis was conducted on New Zealand White Albino rabbits.

1.4.5. Chapter 5

This chapter concluded the dissertation with a summary of the results obtained throughout the study period and a provision of possibilities and recommendations to further improve this drug delivery system.

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CHAPTER 2

Review Article: Polymeric Injectable Intravitreal Hydrogel Devices for Posterior Segment Applications/ Diseases

Peer-reviewed and published as: Muhammed Anwary, Pradeep Kumar, Lisa C. du Toit, Yahya E. Choonara & Viness Pillay (2018) Polymeric, injectable, intravitreal hydrogel devices for posterior segment applications and interventions, Artificial Cells, Nanomedicine, and Biotechnology, 46:sup2, 1074-1081, DOI: 10.1080/21691401.2018.1478845

2.1. Introduction

The structure of the eye is complex and as a result of this many disease states or abnormalities such as posterior segment inflammation and neovascularization-related diseases have been beyond the reach of proper medical intervention. The structure of the eye can be divided into the anterior segment, which consists of all the structures which lie anterior to the lens, and the posterior segment, which consists of all the structures that lie posteriorly to the lens (Choonara, et al., 2010). Causes of posterior segment inflammation can be classified as infectious and non-infectious and treatment may involve chronic therapy for long periods (Haghjou, et al., 2011). Posterior segment inflammation could potentially lead to visual loss as the disease progresses. Inflammation could lead to another possible complication, neovascularization. Ocular pathologies that present with neovascularization, which is characterized by the formation of new blood vessels in unwanted ocular regions, such as the wet form of age-related macular degeneration may also be implicated for loss of vision. Administration via indirect routes such as topical and systemic routes are not suitable as there would either be too little drug reaching the affected site, due to the presence of blood-ocular barriers, which prevents drug from passing through, or the side effects which would result in other morbidities or decreased patient compliance (Mohamed Khalil, et al., 2016). In an attempt to avoid these negatives coupled with the benefits of a direct route of administration, direct intravitreal (IVT) injections were developed (Mahlumba, et al., 2016). A comprehensive review by our department (Pillay, et al., 2016) outlines the various routes of administration such as systemic, topical, oral, periocular and intravitreal routes, and provides insight regarding some of the issues and challenges relating to each of these routes of administration.

Both biodegradable and non-biodegradable in-situ hydrogel forming injections are available. The hydrogels are formed by polymeric networks of a hydrophilic nature that have the ability to swell in water by maintaining the water within its structure whilst sustaining the integrity of the network (Kumar, et al., 2014). This swelling response is brought about due to the presence of crosslinking, whether chemical or physical as shown in Figure 2.1, between the constituent polymers (Klouda & Mikos, 2008)(Singh & Sung Lee, 2014). Physically crosslinked hydrogel structures are usually reversible hydrogels and are formed in the absence of an external chemical linking agent, by linkages that are formed using the chemistry already present on the polymer structures to form links by means of, for example, hydrogen bonds and ionic bonds. Whereas, chemically crosslinked hydrogels are formed employing an additional chemical substance to facilitate the linking of the components of the hydrogel. Furthermore, environment-sensitive polymeric hydrogels have been fabricated, such that they respond to stimuli such as high or low pH- utilizing poly(caprolactone-lactide)-poly(ethylene glycol)-poly-(caprolactone-lactide), temperature changes- utilizing N-isopropylacrylamide, poly(ethylene oxide)–b-poly(propylene oxide)–b-poly(ethylene oxide), poly(ethylene glycol), polysaccharides such as chitosan and dextran and proteins such as gelatin to name a few, and other molecules (Klouda & Mikos, 2008) (Singh & Sung Lee, 2014) (Klouda, 2015) (Ekenseair, et al., 2012) (Li, et al., 2018) (Ameeduzzafar, et al., 2018).

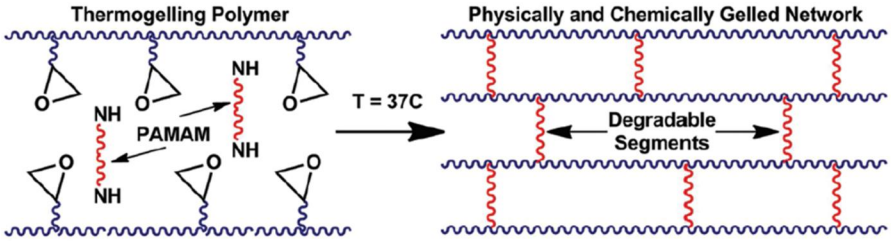

Figure 2.1: Schematic representation of the hydrogel epoxy cross-linking (Ekenseair, et al., 2012; Reproduced with permission from American Chemical Society © 2012).

2.2. Bioerodible injectable intravitreal hydrogel devices

Bioerodible injectable hydrogels for posterior segment diseases are highly attractive as intravitreal implants as they are broken down by the body over a period of time; the time taken would depend on the respective polymeric system used (Haghjou, et al., 2011). The advantage of a bioerodible intravitreal injectable hydrogel is that only one procedure i.e. the initial injection would be required for the implantation of the device, after which the device will be broken down and eliminated by the body, optimally without producing any possible inflammatory by-products. Non-removal of an exhausted non-biodegradable implant may act

as an irritation to the ocular tissue thus it is better if it is removed after the drug is exhausted (Lee, 2015). The absence of the need for removal surgery means that there would be a lesser risk of complications associated with major ocular surgery (Choonara, et al., 2010). Other advantageous properties of the various hydrogel systems can be found in Table 2.1 below. Bioerodible hydrogel implants can be made up of both synthetic and/or natural polymers as described in the following subsections.

2.2.1. Synthetic polymers employed for implantable ocular devices

Over the years, synthetic polymers are being used more than natural polymers due to availability of raw materials, better relative mechanical strength and durability. Synthetic polymers show more consistency between batches and most have properties that allows for easy modification to ensure optimum outcomes (Trimaille, et al., 2016).

It will be shown that poly (ethylene glycol)-based polymers are used extensively in the preparation of injectable intravitreal hydrogel devices. This is due to properties such as hydrophilicity, nontoxicity and tuneable degradability.

Poly(ethylene glycol)-based hydrogel devices

Multiarmed poly(ethylene glycol) hydrogels, commonly four-armed PEGs containing end group functionalization's, are gaining popularity as they allow for greater drug binding and improved drug release which is afforded due to the molecule having four binding regions as opposed to one binding region as found on commonly used single-armed PEGs (Hutanu, et al., 2014). They are also being formulated to produce tuneable and predictable degradation of the hydrogels by the use of linkers which possess the attribute of controllable degradation, thus the formulation can be optimized to release the respective drug molecule before the degradation of the hydrogel thus minimizing the risk of burst release (Henise, et al., 2015; Figure 2.2).

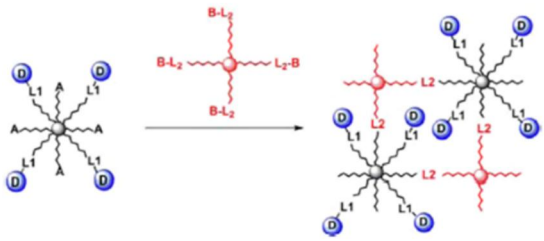

Figure 2.2: Formation of a hydrogel from an 8-arm PEG having four drugs, D, tethered via L1 linkers and four connecting groups, A, by reaction with four B connecting groups of a 4-arm

PEG containing cleavable cross-linkers L2 (Henise, et al., 2015; Reproduced with permission from American Chemical Society © 2015).

Multi-armed thiol and maleiamide end group functionalized PEG were used to create rapidly in-situ gelling hydrogels which were chemically cross-linked by the thiol-maleiamide reaction (Yu, et al., 2014). Gelation time of the transparent hydrogel was reported to be between 90-210 seconds depending on PEG-thiol concentration, a higher PEG-thiol concentration resulting in a quicker gelation time, at physiological conditions. The degree of swelling could be controlled, which is preferable in the ocular setting as excess swelling could be detrimental to the intraocular environment (Stoddart, 2017), by altering the pore size (Hayashi, et al., 2017). This formulation was shown to release drug, Avastin®, for up to 14 days, however, it still exhibited initial burst release which could be attributed to the drug from the surface of the hydrogel being released or possibly due to some of the drug not being fully trapped during the setting of the hydrogel or the initial absence of the diffusion barrier (Huang & Brazel, 2003). The respective duration of drug release of the different hydrogel delivery systems are found summarized in Table 2.2 below.

Another attractive advance to low swelling hydrogels involving the same multi-armed PEG-thiol and PEG-maleiamide was reported by Hayashi et al. (Hayashi, et al., 2017). Hayashi and co-workers (Hayashi, et al., 2017) formed groups of oligo-tetra-PEG by reacting the different PEGs with each other while adding an excess of the other, after which they were combined to form the final product (Figure 2.3). No drug was added as this implant was to serve as an artificial vitreous body. This oligo-tetra-PEG hydrogel which was formed with a low polymeric content, lasted over one year in vivo with a lack of any significant toxicity.

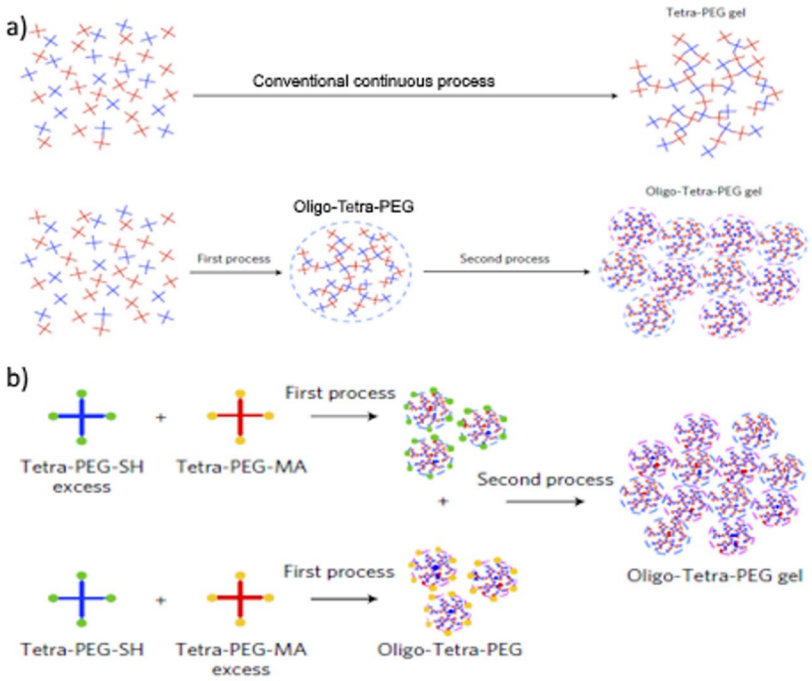

Figure 2.3: a) Gelation process of the conventional Tetra-PEG hydrogel and of the oligo-Tetra-PEG hydrogel and b) Synthesis of the oligo-Tetra-PEG hydrogel (Hayashi, et al., 2017; Reproduced with permission from Springer Nature © 2017).

Multi-arm PEG/silicate was also investigated as a biodegradable and biocompatible controlled release system due to the properties of silica which allow for degradation to be controlled via different mechanisms such as the water to alkoxide ratio and precursor concentration thus allowing greater control over the release of drugs (Lu, et al., 2014). The presence of silica would also affect the mechanical properties of the gel (Ilyin, et al., 2016). Gelation time ranged between 0.58 and 5.2 minutes and was directly affected by the water content within the sample, the greater the water content the longer the gelation time due to the decreased PEG/silicate concentration. Degradation tests were carried out for 13 days, after which the hydrogel with the highest water content was 73% degraded while the hydrogel with the least water content was fully degraded. Hydrophilic drug (dexamethasone sodium phosphate) release from the system showed that majority of the drug was released in the first 20 minutes while the hydrophobic drug (dexamethasone) was released at a lower concentration, which could be made even lower by increasing the water content of the PEG/silicate hydrogel.

A recent pilot study testing PolyActive™- a polyethylene glycol–polybutylphthalate (PEG-PBT) hydrogel device was carried out by Adamson et al. for IVT applications in the treatment of age-related macular degeneration (Adamson, et al., 2016). This copolymer is amphiphilic due to the hydrophilic PEG and hydrophobic PBT. This biodegradable copolymer was used due to its modifiability which controls the drug release rate and degradation. It was loaded with dual domain antibody anti-vascular endothelial growth factor (anti-VEGF) molecules, which were

released over a period exceeding 12 months in vitro and 6 months in vivo by diffusion after an initial burst release. This formulation resulted in ocular inflammation and displayed migration of particles into the anterior chamber of the eye in a primate model, both which are unwanted in posterior segment treatments.

Poly(lactic acid-co-glycolic acid)-based hydrogel devices

A novel multiblock copolymer hydrogel consisting of methoxy-poly (ethylene glycol)-block-poly (lactic-co-glycolic acid) (mPEG-PLGA) was synthesized by Hu et al. using 2,2-bis (2-oxazoline) as a cross-linking agent to prolong the release of anti-VEGF agent bevacizumab, which has an half-life of only approximately 4 days in vivo (Hu, et al., 2014). This biodegradable copolymer system was investigated for biomedical applications in bone, and it displayed promising properties such as lack of toxicity and almost linear drug release (Peng, et al., 2016). The formulation gelled from 10% weight at physiological temperature and exhibited sol-gel-sol behaviour above or at 45°C. Above the critical micelle concentration, this copolymer system forms micelles which encapsulates the drug thus improving the release of the drug and extending its release to 30 days, along with the absence of any significant burst release.

Thermoresponsive poly(lactic acid-co-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-co-glycolic acid) (PLGA–PEG–PLGA) hydrogels were synthesized as an IVT injectable device by Xie et al. (Xie, et al., 2015) and Zhang et al. (Zhang, et al., 2015) employing ring opening polymerisation. PLGA is useful in the ocular setting as it is non-toxic, non-irritant (Pandit, et al., 2017) and it did not elicit an inflammatory response in the above formulation (Li, et al., 2012). Zhang et al. (Zhang, et al., 2015) used two differing molecular weight PEGs and mixed the two copolymers in differing ratios to adjust the sol-gel transition temperature. The formulation released drug for over 10 days and an interesting find was that the hydrophobic dexamethasone was more soluble in the polymer solution compared to water. This could be attributed to the fact that PLGA–PEG–PLGA forms micelles and the dexamethasone was integrated into the micelle core and it was thus solubilized (Yu, et al., 2007). The hydrophobic dexamethasone was loaded into the hydrogel and injected into the vitreous of a rabbit model (Figure 2.4). Xie et al. (Xie, et al., 2015) used hydrophilic Avastin® which was released over a period of 14 days after gelation,which was reported to occur immediately after injection into the vitreous. Both group's formulations showed initial burst release and found that the greater the hydrophobic content of the formulation, the lower the sol-gel transition temperature.

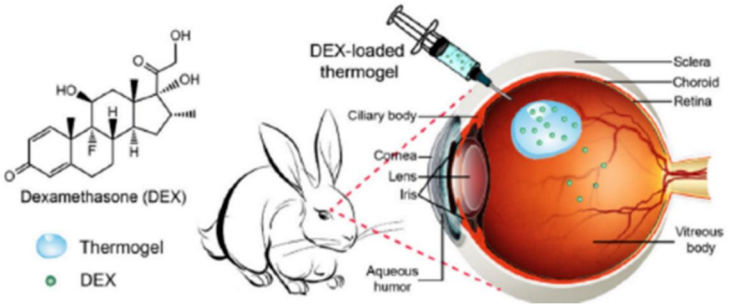

Figure 2.4: schematic diagram showing the molecular structure of DEX and intravitreal delivery of DEX using the mixture thermogel (Zhang, et al., 2015; Reproduced with permission from Elsevier B.V. Ltd. © 2015).

Silica based hydrogel devices

Biopharmaceutical company, DelSiTech, has developed an innovative modifiable and biodegradable silica-based hydrogel system to deliver a wide spectrum of drugs intraocularly (DelSiTech, 2016). The system consists of drug-containing silica microspheres, wherein the water solubility can be controlled, dispersed in a silica hydrogel polymer which is formed, as shown below, by the sol-gel method. The pre-gel silica solution environment can be modified to allow for an optimum environment to be created for the drug, thus allowing a wide spectrum of drugs to be used (Jokinen, et al., 2008). Drug release from the silica microspheres is regulated by erosion of the silica network and the initial burst release could thus be eliminated controlled. The hydrogel is formed in the syringe and then injected into the vitreous, unlike most other IVT hydrogels, which form in situ. A delivery system based on this can be made to release drug extending for a period over 12 months.

2.2.2. Natural polymers as implantable ocular devices

Natural polymers are used in hydrogel formulations due to the fact that they are natural, meaning that they may be similar to certain components that are found within the human body. Natural polymers are derived from nature and include polymers derived from plants, animals and microbes. Polymers derived from plants include polysaccharides such as cellulose and cyclodextrins, proteins and polyesters. Polymers derived from animals include polysaccharides such as chitosan, hyaluronan and chondroitin, proteins such as gelatin, collagen and silk, and resin in the form of shellac. Polymers derived from microbial sources include commonly used polysaccharides such as alginate and dextran, polyesters such as polyhydroxyalkanoates, polyamides such as poly-gamma-glutamate and polyanhydrides such as polyphosphate. The similarity of some of these polymers to endogenous components within

the human body would result in minimal toxicity, if any, and greater biocompatibility which would render natural polymers suitable to be used in most situations as a drug delivery system (Vasquez & Tumolva, 2015) (Zhao, et al., 2013) (Yuan, et al., 2017).

Hyaluronic acid/ Dextran blend hydrogel

A vinylsulfone functionalized glycosaminoglycan, hyaluronic acid (HA-VS), was chemically crosslinked to a thiol functionalised polysaccharide, dextran (Dex-SH), without the use of a catalyst to form a biodegradable in situ forming hydrogel for the controlled and extended release of anti-VEGF agent, bevacizumab by Yu and co-workers (Yu, et al., 2015). HA is a suitable delivery system to be used in the ocular environment as it already is a constituent of the vitreous and other ocular structures, along with its useful viscoelastic properties and degradability (Bora, et al., 2016) (Widjaja, et al., 2014). Dextran on its own is non-toxic, biocompatible and displays bioadhesive behaviour *in vivo* (Fathi, et al., 2015). The resultant product of the aforementioned study yielded a non-toxic, transparent hydrogel that was formed within approximately 30 seconds. It was loaded with anti-VEGF drug bevacizumab, which was released over a period of at least 3 months after an initial burst release, and was still in the vitreous in significant concentrations after 6 months. Biocompatibility tests showed an absence of inflammation and other significant associated pathologies.

Silk fibroin based hydrogel

Silk fibroin, which is derived from the *Bombyx mori silkworm* species, demonstrates biocompatibility, superior mechanical strength, stability on exposure to varying temperatures and easy functionalisation ability (Ming, et al., 2016) (Yin, et al., 2017). Silk fibroin is made up of hydrophobic and hydrophilic regions, which contributes to its attractive mechanical properties (Vepari & Kaplan, 2007). Lovett and co-workers (Lovett, et al., 2015) developed a sustained release, bevacizumab-loaded silk fibroin hydrogel for ocular applications. By day 90 it was observed that the hydrogel was not fully degraded and the concentration of the drug in the vitreous humor was still detectable in significant quantities. In vivo tests revealed drug migration into the aqueous humor, but this migration was reportedly found in the control as well. The group also reported non-serious transient inflammatory responses initially and around day 30. As with many hydrogels, the initial burst release of drug was observed, however, the drug concentration after 3 months in vivo was equivalent to the amount of drug normally available at 1 month with the standard dose. Drug release and biodegradation can be controlled by altering the ratio of high/low molecular weight silk and chemically modifying the polymer (Floren, et al., 2016). Gelation time can also be shortened/ modified by altering

formulation parameters and inclusion of additional reagents during formulation (Floren, et al., 2016).

Chitosan/ Alginate composite hydrogel

Chitosan, a hydrophilic polysaccharide produced by deacylation of chitin which is sourced from processed shellfish and more recently from fungus, is a biocompatible polymer that is biodegradable by enzymatic degradation in the human body (Ahmadi, et al., 2015)(Ghormade, et al., 2017). Alginate is another biocompatible hydrophilic polysaccharide material that is sourced from certain seaweeds and bacteria that is used to form hydrogels in biomedical applications (Lee & Mooney, 2012). Xu et al. formulated a transparent chitosan/alginate hydrogel based on mixing different amounts of glycol chitosan and oxidized alginate to encapsulate Avastin® (Xu, et al., 2013). Gelation time of the system varied between 10 seconds and 5 minutes depending on the glycol chitosan/ oxidized alginate ratio. Degradation of the gel and release of the drug is controllable by the oxidized alginate content, greater oxidized alginate concentration resulted in slower degradation but also resulted in a quicker rate of release due to a less densely packed structure. Initial drug release is controlled by diffusion of the drug while the latter release is controlled by degradation of the hydrogel. Drug release data showed an initial burst release of 30% of the drug and almost all of the drug was released by day 3. This links up to the disintegration of the hydrogel network which was also practically fully disintegrated by day 3, while degradation was mostly completed by day 18. This relatively quick degradation is possibly due to the presence of lysozymes which degraded the chitosan (Xu, et al., 2013).

2.3. Non-bioerodible injectable hydrogel devices

Non-bioerodible injectable IVT implants are one step in the right direction as major surgery will not be required to insert the implant. However, the implant will need to be removed after some time as it may cause unwanted effects within the ocular environment (Lee, 2015). Thus one minor surgery, to implant the device, plus an additional surgery to remove the non-biodegradable device will be required, which may reduce patient acceptance and compliance. Poly(N-isopropylacrylamide) (PNIPAAm)-based thermogels have been highly researched as drug delivery systems due to their attractive thermoreversible hydrogel properties (Klouda & Mikos, 2008)(Asghari, et al., 2017). They have been investigated for use in bone/cartilage and cell scaffolds (Ohya, et al., 2004)(Ren, et al., 2015). The application of Poly(N-isopropylacrylamide)-based hydrogels for intravitreal application has been further explored by Drapala and co-workers after initial work also conducted by them in 2011 (Drapala, et al., 2014)(Drapala, et al., 2011). This injectable intravitreous thermoresponsive hydrogel drug

delivery system was obtained by adding poly (ethylene glycol)-diacrylate (PEG-DA) onto the poly(N-isopropylacrylamide) chain (NIPAAm/PEG-DA). The addition of (PEG-DA) resulted in the hydrogel having a lower critical solution temperature greater than 32°C; which is the lower critical solution temperature of PNIPAAm without the PEG-DA chain attached (Gandhi, et al., 2015). Lower critical solution temperature is the temperature, below which the polymer is soluble within the medium and above which the polymer is insoluble within the medium (Kang-Mieler, et al., 2014). This insolubility is what is required to induce polymer swelling and thus the formation of the hydrogel is brought about. Anti-vascular endothelial growth factor agents (ranibizumab or aflibercept) were loaded into biodegradable PLGA microspheres which were added to the drug delivery system (Kang-Mieler, et al., 2014). Drug release studies showed the formulation to release drug for 196 days (Osswald & Kang-Mieler, 2016). However, as this system is, it lacks complete biodegradability. This issue has been addressed and it has been shown that the addition of poly(L-lactic acid)-polyethylene glycol diacrylate (PLLA-PEG-DA) and a chain transfer agent, such as glutathione, would render the formulation fully biodegradable by decreasing the length of the PNIPAAm chain, which studies have shown does make it fully biodegradable (Drapala, et al., 2014)(Osswald, et al., 2017).

Table 2.1: Polymer hydrogel sources and properties.

Material	Source	Properties
PEG thiol/maleiamide (Yu, et al., 2014)	Synthetic	Transparent Tuneable swell ratio and mechanical properties
Oligo tetra-PEG (Hayashi, et al., 2017)	Synthetic	Low swelling Low polymeric content
Multi-arm PEG/silicate (Lu, et al., 2014)	Synthetic	Tuneable drug release Better suited for hydrophobic drugs
PEG-PBT (Adamson, et al., 2016)	Synthetic	Amphiphilic Suitable for complex drugs such as antibodies
mPEG-PLGA (Hu, et al., 2014)	Synthetic	Thermoresponsive No significant burst release Formation of micelles- encapsulates drug
PLGA–PEG–PLGA (Xie, et al., 2015)(Zhang, et al., 2015)	Synthetic	Thermoresponsive Amphiphilic Formation of micelles- encapsulates drug

Silica (DelSiTech, 2016)	Synthetic	Non-mesoporous Drug release only by matrix erosion Suitable for any type of drug
Hyaluronic acid/ Dextran (Yu, et al., 2015))	Natural	Bioadhesive Transparent
Silk fibroin (Lovett, et al., 2015)	Natural	Desirable mechanical strength Controllable degradation and drug release
Chitosan/ Alginate (Xu, et al., 2013)	Natural	Hydrophilic Transparent Controllable degradation and drug release
Poly(N-isopropylacrylamide) (Drapala, et al., 2014) (Drapala, et al., 2011)	Synthetic	Thermoresponsive Can be made fully biodegradable Hydrophilic and hydrophobic (Pelton, 2010)

2.4. Therapeutics of injectable IVT hydrogel implants

Therapeutics employed in the reviewed hydrogel devices include an anti-inflammatory corticosteroid, dexamethasone and monoclonal antibodies ranibizumab and bevacizumab and antibody fragment aflibercept (Table 2.3). Dexamethasone has been used in IVT for the treatment of uveitis (Lee, 2015) and macular oedema (Unsal, et al., 2017). Despite the success of the use of steroidal compounds for the aforementioned conditions, the use of corticosteroids in the ocular setting, is marred by the fact that it, as a steroidal compound, might produce unwanted side effects as it is known to induce side effects in many systems. Neovascularization as found in age-related macular degeneration takes place on the expression of vascular endothelial growth factor. Age-related macular degeneration is found in a dry and a wet form with the wet form being more threatening to the patient's vision, thus more attention is afforded to the treatment of the wet form of age-related macular degeneration. Anti-VEGF agents inhibits the effects of vascular endothelial growth factor thus preventing the growth of excess blood vessels that are found in age-related macular degeneration. All of these drugs are available on the market for anti-VEGF treatment, bevacizumab is produced as Avastin®, ranibizumab as Lucentis® and aflibercept as Eylea®.

Table 2.2: Duration of drug release from various hydrogel compositions.

Hydrogel composition	Duration of drug release	Therapeutic agent
PEG thiol/maleiamide (Yu, et al., 2014)	14 days	Avastin® (Bevacizumab)
Oligo tetra-PEG (Hayashi, et al., 2017)	N/A	N/A
Multi-arm PEG/silicate (Lu, et al., 2014)	>13 days	Dexamethasone
PEG-PBT (Adamson, et al., 2016)	>12 months in vitro 6 months in vivo	VEGF dual dAB
mPEG-PLGA (Hu, et al., 2014)	30 days	Bevacizumab
PLGA–PEG–PLGA (Zhang, et al., 2015)	10 days	Dexamethasone
PLGA–PEG–PLGA (Xie, et al., 2015)	14 days	Avastin® (Bevacizumab)
Silica (DelSiTech, 2016)	>1 month to >1 yr	Any drug
Hyaluronic acid/ Dextran (Yu, et al., 2015)	>3 months	Bevacizumab
Silk fibroin (Lovett, et al., 2015)	>3 months	Bevacizumab
Chitosan/ Alginate (Xu, et al., 2013)	3 days	Avastin® (Bevacizumab)
Poly(N-isopropylacrylamide) (Drapala, et al., 2014) **(Drapala, et al., 2011)**	196 days	Ranibizumab/ aflibercept

Table 2.3: Intravitreal therapies, categorized and indications

Drug	Class/ Category	Ophthalmic Indications
Avastin®/ Bevacizumab	Humanized monoclonal antibody	Wet age-related macular degeneration
Dexamethasone	Steroidal anti-inflammatory	Inflammatory conditions such as uveitis and macular oedema
VEGF dual dAB	Anti-VEGF agent	Wet age-related macular degeneration
Ranibizumab	Humanized monoclonal antibody	Wet age-related macular degeneration Macular oedema
Aflibercept	Anti-VEGF agent	Wet age-related macular degeneration Macular oedema

2.5. Concluding remarks and Future considerations

To conclude, there have been many advances relating to the improvement of delivery systems for delivery to the posterior segment of the eye in the form of injectable IVT hydrogel devices, biodegradable and non-biodegradable. However, the unique nature of the human body, and especially of the eye, always produces trials for every ocular delivery system. A possible way forward would be the development of a delivery system that, apart from optimal features such as biodegradability, in situ gelling and absence of burst release, will also release drug only in response to a specific stimulus at the target site.

With the progression of science, ocular research and the information available relating to polymers and their respective attributes for biomedical applications, the design of a ground-breaking drug delivery system for the effective treatment of posterior segment diseases is nearing. A smart stimulus-responsive biodegradable intraocular implant, I^3, was developed by du Toit and co-workers for delivery of medicament to the posterior segment of the eye in response to inflammation as depicted in Figure 2.5 (du Toit, et al., 2014; du Toit, 2013). Using a similar bio-responsive behaviour as a basis for future delivery systems would be beneficial to patient treatments and would eliminate possible pharmaceutical drawbacks such as burst release of drugs from hydrogels.

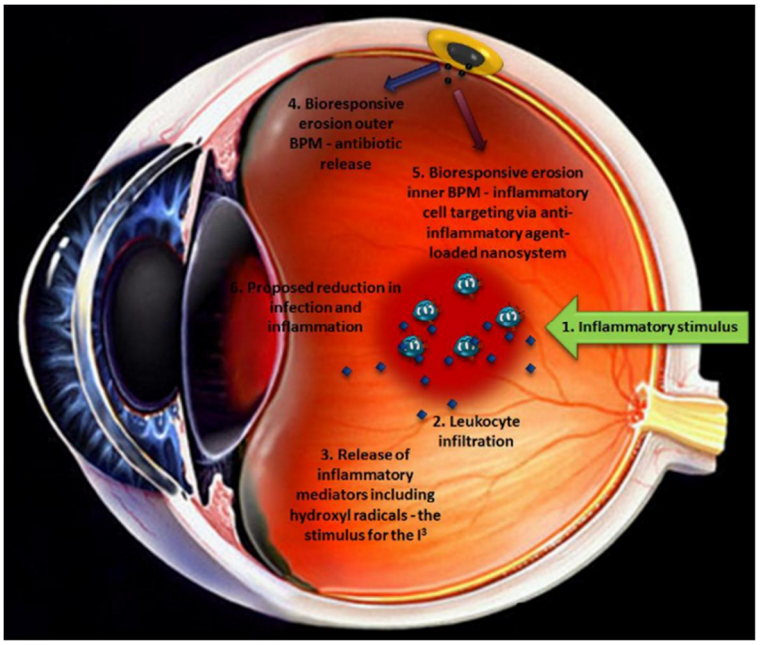

Figure 2.5: Schematic explicating the overall modus operandi of the I^3 in the inflamed eye (du Toit, 2013)

2.6. References

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CHAPTER 3

Preformulation studies for the synthesis of a thermoresponsive methoxy polyethylene glycol-polycaprolactone hydrogel as a vehicle for the novel delivery system

3.1. Introduction

In recent years, injectable thermogelling polymers for medical applications have gained much popularity. Pluronic F-127 was initially considered for use in this study. It is composed of tri-block copolymers of poly(ethylene oxide) poly(propylene oxide)-poly(ethylene oxide) (PEO-PPO-PEO) and it exhibits thermo-reversible gelling. Although it has been used for ocular formulations, evidence exists showing the potential toxicity of Pluronic-F127 to the ocular tissues (Su *et al.*, 2006). This, in combination with the high required gelling concentration (20%) of Pluronic-F127, ruled out Pluronic-F127 from the study.

Methoxy polyethylene glycol-polycaprolactone (mPEG-PCL) was identified as a viable drug delivery vehicle for the delivery of the BioRes system as it has been used previously in ocular studies and it has shown to be non-toxic to the ocular anatomy (Shi *et al.*, 2015; Luo *et al.*, 2016). It is widely used due to its properties of biodegradability, in situ gelling capability, allowing easier administration of drugs in the form of a solution, and lack of general toxicity (Gong *et al.*, 2009; Khodaverdi *et al.*, 2012). mPEG-PCL has a slow degradation which can be prolonged to over a year and has been found to be compatible for use with a wide variety of drug molecules (Danafar, 2016). This combination of hydrophobic PCL and hydrophilic mPEG results in an amphiphilic co-polymer which, on being introduced into an aqueous environment, results in micellar network formations that are subjected to organization in an ordered manner, under the influence of temperature, to form a hydrogel (Hyun *et al.*, 2014).

To prepare these thermo-responsive hydrogels, 2 different polymer chain lengths of methoxy poly(ethylene glycol) were used (750 and 4000 Mw) and the effect of these different chain lengths on the product would be documented and the more suitable of the two formulations (for purposes of ocular drug delivery) would potentially be used in the preparation of the final formulation of the vehicle for delivery of the BioRes system as an intravitreal drug delivery system.

3.2. Materials and methods

3.2.1. Materials

ε-caprolactone (ε-CL) methoxy poly(ethylene glycol) (MPEG, Mw = 750 and 4000,), stannous octoate ($Sn(Oct)_2$) and 1M hydrochloric acid in diethyl ether (HCl in DEE) were procured from Sigma-Aldrich® Inc. (St. Louis, MO, USA) and were used without any further purification. Acetone, dichloromethane and n-hexane was purchased from Merck Chemicals (Pty) Ltd (Gauteng, South Africa). All the materials used in this article were analytic reagent (AR) grade and used as received.

3.2.2. Methods

Preparation of the di-block mPEG-PCL copolymer

Two methods for the synthesis of the di-block mPEG-PCL copolymer were used and both involved ring opening polymerization. Ring opening polymerization is a polymerization technique that involves the end groups of a polymer chain reacting with cyclical (ring) monomeric units to create an extended chain polymer consisting of two or more polymeric "blocks". The first method, shown below in Figure 1, made use of metallic stannous octoate ($Sn(Oct)_2$) as a catalyst for the ring opening polymerization reaction as done in another study (Danafar, 2018). The second method which is also known as a "non-metallic catalysed" reaction, as shown in Figure 2, made use of a 1M hydrochloric acid in diethyl ether (HCl in DEE) as a catalyst for the reaction as established in a previous study (Hyun *et al.*, 2014). The use of HCl instead of stannous octoate as a catalyst does away with the need for the reaction to be carried out at high temperatures using solvents which have high boiling points such as toluene; which is difficult to remove from the product and may bring about the possibility for toxicity (Hyun *et al.*, 2014).

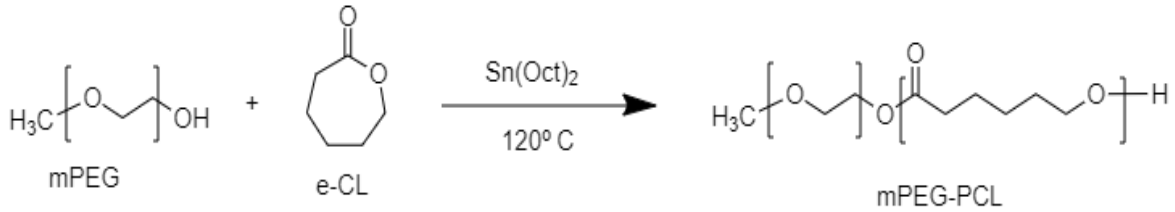

Figure 3.1: Reaction showing the formation of mPEG-PCL block copolymer using organometallic stannous octoate as a catalyst

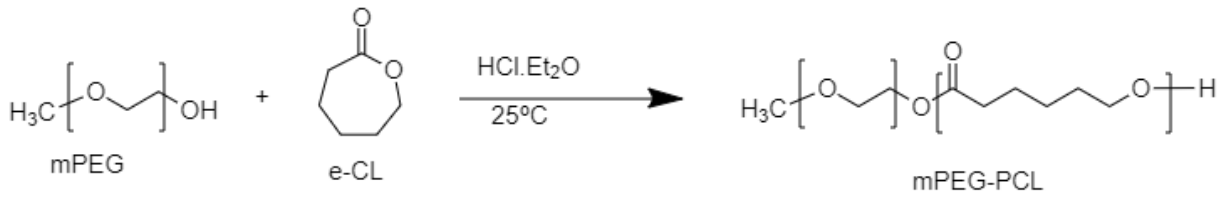

Figure 3.2: Reaction depicting the formation of mPEG-PCL block copolymer using 1M hydrochloric acid in ether as a catalyst

In method 1, a 2:1 ratio of ε-CL: mPEG was used. 4g of ε-caprolactone, 2g of mPEG, and 0.01 mmol of Sn(Oct)2 were heated to 120°C to initiate polymerization in an inert environment under a constant flow of nitrogen gas. After 12 h, the resulting polymer was cooled to room temperature and dissolved in chloroform, which was precipitated in cold diethyl ether. The copolymer was then dried under vacuum at room temperature for 24 h.

In method 2, as per Hyun et al. (Hyun *et al.*, 2014), 1.5g of mPEG and 30mL of toluene was added into a flask in order for azeotropic distillation to be effected to achieve the end of water removal. Toluene was then removed by distillation. Dichloromethane (91mL) was added after which 4.8g of ε-caprolactone was added. To initiate polymerization, 4mL of 1M HCl in DEE was added at room temperature. The reaction was left to stir on a magnetic stirrer bar equipped apparatus for 24 hours, after which it the mixture was precipitated into n-hexane to elucidate the polymer. The polymer was separated by decantation, redissolved in dichloromethane and filtered. The solvent was removed by rotary evaporation.

3.2.3. Characterization of the mPEG-PCL diblock copolymer

Characterization tests were to be carried out on the mPEG-PCL copolymers to determine the suitability of the copolymer for its intended use as an intravitreal drug delivery system. Tests that were carried out included Fourier Transform Infrared Spectroscopy (FTIR), differential scanning calorimetry (DSC) and gelation time/temperature tests. Based on the results of these characterizations, the appropriateness of the mPEG-PCL hydrogel for use as a vehicle would be decided.

Determination of Structural Transitions of the block copolymer by Fourier Transform Infrared Spectroscopy

FTIR spectra were recorded on a Perkin Elmer Spectrum 2000 FTIR spectrometer with a MIRTGS detector (PerkinElmer Spectrum 100, Llantrisant, Wales, UK), using an ATR-FTIR cell and a diamond crystal internal reflection element. Samples were analysed at a wavenumber range of 650–4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans per spectrum. FTIR was done to compare the surface structural composition of the final product relative to

the initial monomer and polymer units thereby enabling the confirmation of the successful formation of product.

Thermal transitions of the block copolymer by Differential Scanning Calorimetry

Comparative differential scanning calorimetry (DSC) analyses was performed on the two different chain length copolymer powders using a Mettler Toledo, DSC1, STARe System (Schwerzenback, Switzerland) at a heating rate of 10 °C/min from 0°C to 200 °C under a constant flow of N_2 gas. Accurately weighed samples were placed into a covered aluminium sample holder containing a central pin hole. Indium metal (99.99%) was used to calibrate the DSC modulus in relation to temperature and enthalpy. An empty sample holder was used as a reference. DSC thermograms were then be compared for differences in thermal transitions.

Gelation time and temperature

Gelation time and temperature was determined by exposing the dissolved powder in solution form to various temperatures and it was ascertained at what temperature it formed a gel and the time taken for the gel to form at that temperature was recorded (Yu, *et al*., 2014).

3.3. Results and Discussion

The reaction using the high molecular weight/ longer chain length mPEG resulted in a white powder that required a higher temperature to be dissolved an aqueous media relative to the lower molecular weight/ shorter chain length mPEG which dissolved with more ease at a lower temperature. Thus, the following link between mPEG chain length in the block copolymer and solubility can be deduced: The shorter the chain length (despite being hydrophilic), the more ease with which the copolymer would dissolve, at lower temperatures (Kim *et al.*, 2006). The product of the reaction wherein the shorter chain length mPEG was used yielded a highly viscous paste-like product which was difficult to work with.

Method 1 and method 2 yielded the same results but the ease of the reaction using HCl as a catalyst (method 2) rendered the synthesis of the product much easier as it does not require an inert setting and is thus a less sensitive reaction to work with. As stated earlier, it may also improve the toxicity profile of the copolymer due to the absence of a metallic agent in the form of $Sn(Oct)_2$.

Determination of Structural Transitions of the block copolymer by Fourier Transform Infrared Spectroscopy

FTIR spectroscopy of the individual components of the copolymer versus the copolymer showed the successful formation of the block copolymer. Characteristic peaks from the pristine compounds at ~3400-3600cm^{-1}; representing the OH groups in mPEG, 1727cm^{-1} and

2939cm^{-1} representing the carbonyl group (C=O) and CH group from polycaprolactone, were carried over into the spectrum of the final product, along with the C-O-C peak at 1163cm^{-1} which is characteristic of mPEG as well as at the linking point between the copolymeric units as shown in Figure 3 below (Brianezi *et al.*, 2018; Hsieh *et al.*, 2018; Rostamizadeh *et al.*, 2018).

Thermal transitions of the block copolymer by Differential Scanning Calorimetry

The thermograms of the mPEG–PCL copolymers displayed endothermic peaks at 48°C and 44°C for the copolymers formed using mPEG-4000 and mPEG-750 respectively. These endothermic peaks are indicative of the melting points of these copolymers in the micellar configuration, which is slightly lower than observed in the non-micellar configuration, as observed in another study (Manjili *et al.*, 2018). The exothermic peaks at 260°C (mPEG-750-PCL) and 270°C (mPEG-4000-PCL) in combination with the initial shoulder peak are indicative of the temperature of crystallization which is brought about the polymers forming ordered arrangements. It was noted that the long chain length, mPEG-4000-PCL, had a higher temperature of crystallization compared to the shorter chain length, mPEG-750-PCL. Thus, it follows that greater chain lengths of mPEG-PCL deblock copolymers possess higher melting and crystallization temperatures. DSC thermograms can be found below in Figure 4 for comparison.

Gelation time and temperature

Gelation studies on the various concentrations of used to formulate the gel showed that the minimum gelling concentration for mPEG-4000-PCL was 3% while the mPEG-750-PCL was left out due to it not having optimal workability due to its consistency which have posed a problem in the next phase of the BioRes gel system formulation. The test tube inversion method was not needed to determine gelation as the gel that formed underwent a very noticeable difference upon gelation, changing from a translucent solution to an opaque, white gel. The solutions formed gels within 15 to 60 minutes at 15°C and the higher concentrations (6%) displayed gelation within 7 minutes.

Tested gelation concentrations were kept below 7% as the use of high polymer concentrations may pose as a nuisance to the ocular structures. Gelation using the synthesized copolymers failed on certain occasions due to the unforeseen occasional lack of solubility. This was likely due to certain sensitivities in the formulation techniques that were not approached correctly.

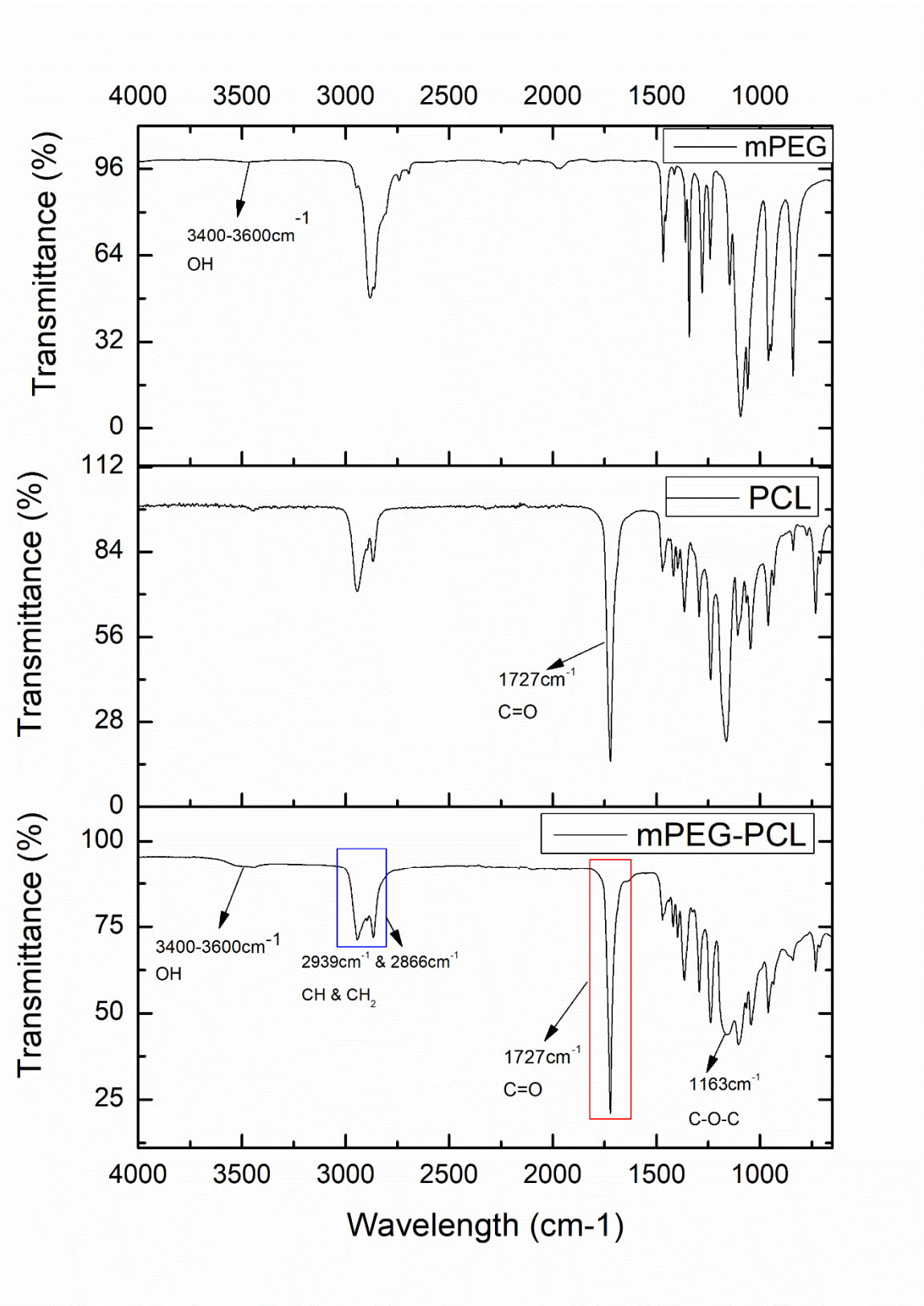

Figure 3.3: FTIR spectra showing the pristine compounds and final product (mPEG-PCL) with characteristic peaks from each compositional unit showing clearly the presence of both C=O as seen on PCL and C-H/ C-H_2 as shown on mPEG.

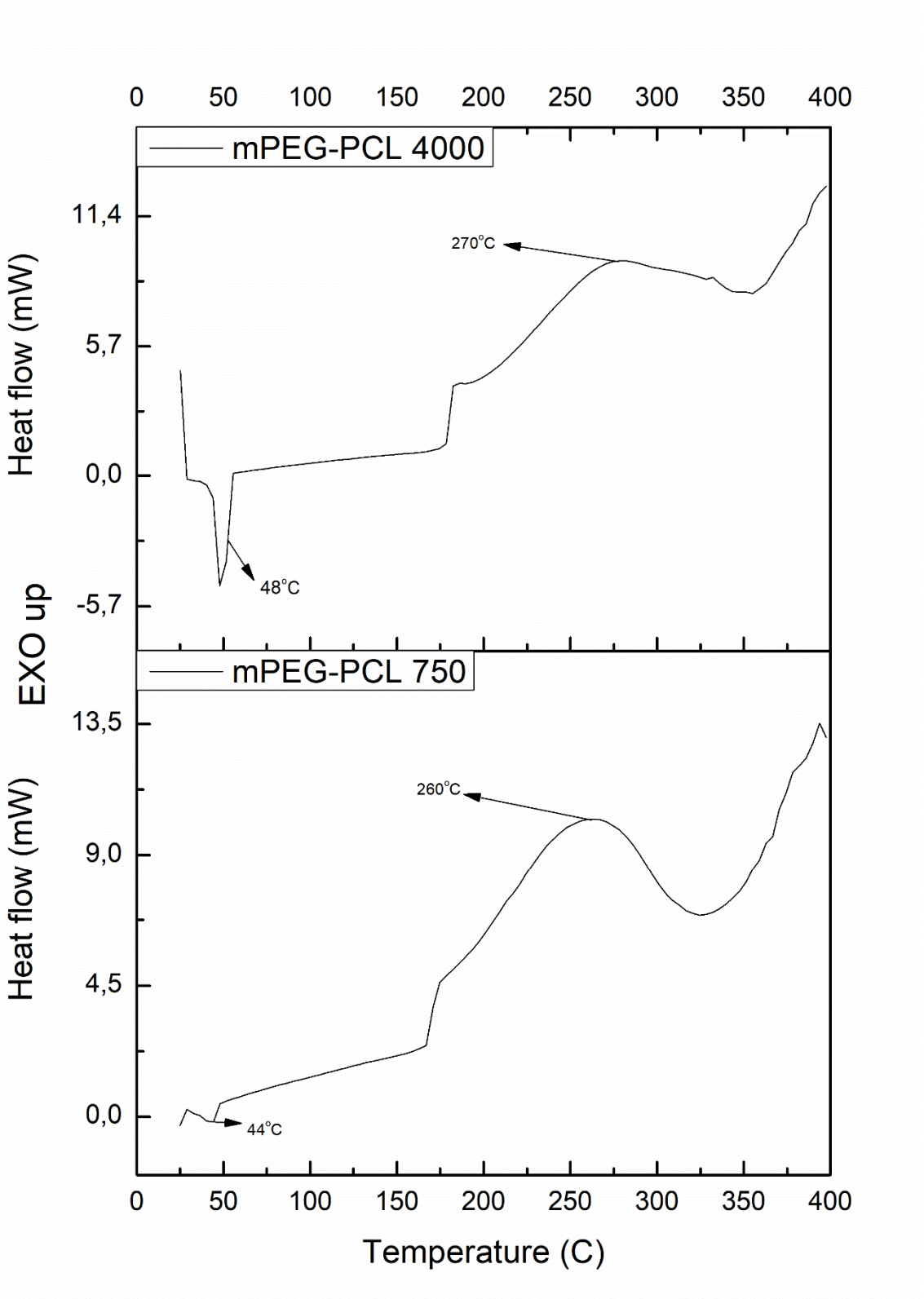

Figure 3.4: DSC thermograms of the mPEG-PCL di-block copolymers showing the slightly lower melting point of the smaller chain length mPEG highlighting the effect of chain length on melting point

3.4. Concluding remarks

It was thus concluded, due to the sensitivity of certain formulation parameters, that the synthesis of the mPEG-PCL di-block copolymer hydrogel possessed a certain degree of irreproducibility, and in combination with the relatively hefty price tag of procuring ready-made copolymer in a developing country as well as the relatively long formulation time required, that it would be in the better interests of this project that a more easily producible hydrogel, with greater reproducibility be used as the vehicle for the BioRes drug delivery system.

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Chapter 4

Synthesis, *In Vitro* Characterization, and *In Vivo* Analysis of a Biostimuli-degradable, Spacer-based, Polymer-drug Conjugate as an Injectable Intraocular Hydrogel System

4.1. Introduction

Posterior segment uveitis, along with other inflammatory ocular posterior segment disorders; which may be attributed to various aetiologies broadly divided into infectious and non-infectious such as autoimmune and trauma induced (Haghjou, Soheilian and Abdekhodaie, 2011); are implicated as being one of the major treatable causes of blindness (Tempest-Roe *et al.*, 2013). Greatly owing to the complexity of the ocular structural composition, there has been extensive research conducted in recent years with the aim of improving drug delivery to the various structures within the ocular setting, both in the anterior and posterior chambers. Again, due to the complex structure of the eye, the most efficient and cost-effective treatment of posterior segment disorders is currently only attainable by means of placing the treatment directly in the posterior segment or in surrounding structures in its immediate environment. Much research has been and is being conducted using polymers in intravitreal drug delivery systems (DDS) due to desirable properties such as easy structural modifiability, relatively good toxicity profiles, alterability of degradation profiles, biodegradability and possibility of creating or utilizing inherent stimuli-responsive behaviours to name a few of these properties (Anwary *et al.*, 2018; Nayak and Misra, 2018). Categorization of polymer-based intravitreal drug delivery systems can be done based on the biodegradability, or lack of it. Non-biodegradable systems are not preferred for intravitreal employment as they may prove to irritable or harmful to the surrounding ocular tissues (Lee, 2015). While research focusing on stimuli-responsive hydrogels for intravitreal employment has been explored to a great extent with many positive outcomes, non-hydrogel stimuli-responsive intravitreal drug delivery systems on the other hand have been studied to a much lesser degree.

Thus, this chapter was aimed at the design and formulation of the BioRes system which, in response to an effect that in almost all cases accompanies the biological inflammatory process viz. a drop in pH, releases drug from the conjugate via cleavage of the Schiff base spacer bond. The hydrogel vehicle of delivering the BioRes into the vitreous could also be adjusted (by altering the polymeric composition of the hydrogel vehicle) to further impart greater release control of the medicament from the BioRes. The BioRes consists of a polymer (methoxy polyethylene glycol- mPEG) with a functionalized end group and drug.

A hydrazone linker was isolated as a prime spacer to be used in the formulation owing to its stability at neutral pH (pH~7.2) and easily cleavable C=N bond at biological inflammatory pH (pH~5.4) (Kale and Torchilin, 2007; Sonawane, Kalhapure and Govender, 2017). The formation and breakdown of the hydrazone-based BioRes is as depicted in Figure 4.1, where at neutral pH the bond is stable and at inflammatory pH the backward reaction occurs. The reactions would conjugate the drug to the polymer by means of an imine bond formed between a carbon from the drug and the terminal amine of the polymer (Saito, Hoffman and Ogawa, 2007). Hydrolyzation of the bond in inflammatory conditions results in the release of the drug, which in this project was an anti-inflammatory agent. However, the possibility of significant burst release within the first 24 hours was still present, ascribable to the fact that spacer degradation is not rate limited by any sort of negative feedback system or matrix structure etc. which would control linker cleavage and therefore drug release. Thus, the linker-based system was carried in a hyaluronic acid (HA) hydrogel which was intended to act both as a vehicle and as a release control mechanism. Hyaluronic acid was selected as the vehicle as it possesses qualities of biocompatibility, biodegradability and mucoadhesive properties etc. which are essential for an ocular drug delivery system (Trombino *et al.*, 2019) in addition to its established use in drug delivery for a variety of applications (Jin, Ubonvan and Kim, 2010; Jiao and Zhai, 2016). HA is also one of the naturally occurring components of the vitreous humor (Lappas and Lappas, 2016). The integral targets of this drug delivery system were to achieve minimal drug release at normal ocular physiological conditions and to relatively increase the extent of drug release during inflammatory ocular conditions without producing toxic effects.

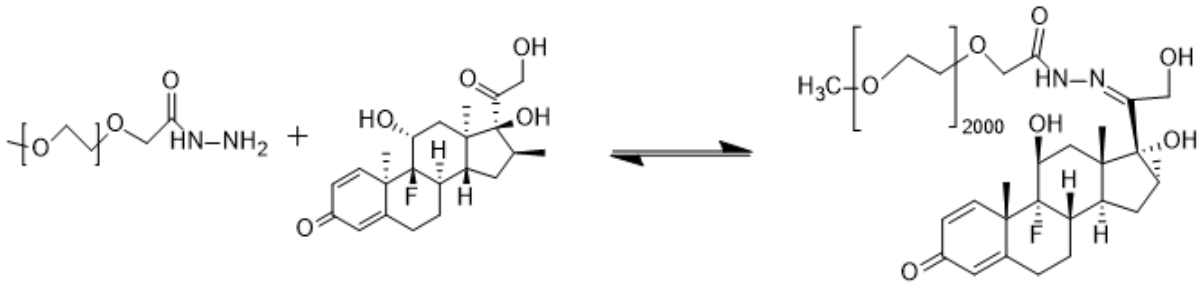

Figure 4.1: The formation and reverse reaction of the hydrazone-based BioRes system

4.2. Materials and Methods

4.2.1. Materials

For the Polymer-linker-drug system: Methoxy polyethylene glycol-Hydrazide (mPEG-Hz) (M_w ~2000 Da) was purchased from Biochempeg Scientific Inc. (Watertown, MA, USA). Dexamethasone (>98% HPLC) was procured from Sigma-Aldrich® Inc. (St. Louis, MO, USA). Glacial acetic acid was obtained from Associated Chemical Enterprises (Johannesburg, South

Africa). Dimethyl sulfoxide (DMSO) and methanol (MetOH) were obtained from Merck Chemicals (Pty) Ltd (Gauteng, South Africa). For the hydrogel vehicle: Hyaluronic acid was purchased from LEAPChem (Zhejiang, China).

4.2.2. Methods

Synthesis of the BioRes gel system

The system was designed as a linker-based bioresponsive polymeric drug release system utilising a pH/inflammation responsive hydrazone spacer bound to a biodegradable methoxy polyethylene glycol polymer on one end and an anti-inflammatory steroid (dexamethasone) on the other end, suspended within a hyaluronic acid hydrogel vehicle.

Synthesis of linker-based polymer drug conjugate

mPEG-Hz (350 mg) was dissolved 3 ml solution of DMSO/ MetOH (1:1 ratio). Dexamethasone (150 mg) was added to and dissolved in the solution followed by the addition of 10 µL of glacial acetic acid as a catalyst. The solution was left to stir for 16 hours in a 60 °C water bath equipped with a thermometer and magnetic stirrer. The solution was dialysed in deionized water using a 1000 molecular weight cut-off dialysis tubing for 6 hours to rid the product of excess reagent and removal of any catalyst present. The precipitate was dried was lyophilization. This yielded the spacer-based polymer drug conjugate to be added to the hyaluronic acid hydrogel vehicle.

Preparation of hydrogel vehicle

A 0.5%w/v HA hydrogel was prepared by adding 500 mg of HA to 100 mL of deionized water. The HA powder was then dissolved in the water using a magnetic stirrer apparatus at 25°C.

Physicochemical characterization of the BioRes

Evaluation of the chemical properties of the BioRes was accomplished using the following:

Confirmation of essential bonds in BioRes synthesis employing Nuclear Magnetic Resonance spectroscopy

(NMR) spectroscopy was carried out after dissolving the conjugated and non-conjugated BioRes in a suitable solvent, in addition to pristine drug in deuterated methanol at 25°C. ^{1}H

and ^{13}C NMR spectroscopy was done using a Bruker AVANCE II 500 MHz (Bruker Avance Biospin, Germany) at the School of chemistry at the University of the Witwatersrand, Johannesburg. NMR was carried out to ascertain the degree of conjugation of the polymer to the drug as well as to determine the correct procession of the reaction by observing peaks representing structures relating to essential chemical bonds.

Determination of Structural Transitions by Fourier Transform Infrared Spectroscopy

FTIR spectra were recorded on a Perkin Elmer Spectrum 2000 FTIR spectrometer with a MIRTGS detector (PerkinElmer Spectrum 100, Llantrisant, Wales, UK), using an ATR-FTIR cell and a diamond crystal internal reflection element. Samples were analysed at a wavenumber range of 650–4000 cm^{-1} with a resolution of 4 cm^{-1} and 64 scans per spectrum. FTIR was done to compare the structural composition of the final product relative to the reactants thereby enabling the reaction mechanism to be elucidated as well as confirmation of the successful formation of product.

Thermal transitions of the BioRes by Differential Scanning Calorimetry

Comparative (DSC) analyses was performed on lyophilized samples of the BioRes, pristine polymer and the drug of choice using a Mettler Toledo, DSC1, STARe System (Schwerzenback, Switzerland) at a heating rate of 10 °C/min from 0°C to 200 °C under a constant flow of N2 gas. Accurately weighed samples were placed into a covered aluminium sample holder containing a central pin hole. Indium metal (99.99%) was used to calibrate the DSC modulus in relation to temperature and enthalpy. An empty sample holder was used as a reference. DSC thermograms were then be compared for transitions.

Phase determination via X-ray powder diffraction (XRD)

AXRD was carried out on lyophilized samples to elucidate the crystalline properties of the drug-loaded and nondrug-loaded BioresG. XRD was carried out using the Miniflex 600 (Rigaku, Japan). Samples were scanned at 0°-100°/minute in a 2Ɵ range of 0°-60°.

Inflammation-responsive enhanced *in vitro* drug release capability of the BioRes system

In order to determine the enhanced release capability of the BioRes, the system was exposed to normal and inflammatory pH in a closed environment, which imitates the ocular setting.

Accurately weighed BioRes was added to the vehicle, which was then placed into a 1000 molecular weight cut-off dialysis tubing (Sigma-Aldrich® Inc., St. Louis, MO, USA) and placed into a 4 mL preparation of simulated vitreous humor (SVH); prepared using phosphate-buffered saline with 0.03% v/v hyaluronic acid at 37°C (Du Toit *et al.*, 2014); alternating between physiological pH and inflammatory pH every 48 hours.

Samples were placed in closed vials and placed in an oscillating laboratory incubator (Labcon® FSIE-SPO 8-35, California, USA), set to 20 rpm. Withdrawal of 0.2mL samples, and subsequent replacement by 0.2mL of SVH, were undertaken at 0, 4, 8, 24 hours and every 24 hours thereafter until 240 hours. Samples were appropriately diluted and subjected to Ultraviolet (UV-vis) Spectrophotometric analysis (Implen NanoPhotometer® NP80, Implen GmbH, Munich, Germany) at the λ_{max} for dexamethasone (240 nm) in SVH. The influence of the polymer on the absorbance readings was taken into consideration. Analyses were conducted in triplicate.

In vivo analysis to determine toxicity and inflammation-responsive behaviour of the BioRes in the rabbit eye

Ethics clearance for the *in vivo* study was obtained from the Animal ethics committee of the University of the Witwatersrand, ethics clearance number 2018/08 4/35/C (Appendix C).

In vivo design and surgical technique employed

In order to ensure statistical significance, the sample number for each group and time point was a minimum of three and a maximum of five animals. New Zealand albino rabbits were used for the study as the rabbit eye has a more workable nature in that the vitreous and aqueous humor volumes are greater and ocular structures are more easily accessible relative to that of rats/mice and is thus more commonly used in posterior segment ocular studies. 33 New Zealand albino rabbits were used. 10 rabbits each were in 3 groups: Positive control (Group 1) in which inflammation was induced without BioRes, BioRes with inflammation (Group 3) and BioRes without inflammation respectively (Group 4). Three (3) rabbits were used as the negative control (Group 2) in which neither intervention nor inflammation was induced/ injected. Rabbits were housed at room temperature with food and water accessible at their leisure. At day 0 (zero), 10 rabbits, 5 from group 3 and 5 from group 4 had the BioRes system injected into their posterior segment by the following procedure:

General anaesthesia was induced with intramuscular (IM) combination of ketamine (40mg/kg) and xylazine (10mg/kg). After washing the eyes with several drops of 5% povidone iodide and instillation of tetracaine HCl 0.5%, a 26-gauge needle containing the BioRes was introduced into the vitreous cavity, 2.0 mm posterior to the superotemporal limbus, and the needle tip was

directed into the midvitreous under direct visualization with external illumination of indirect ophthalmoscopy. One hundred microlitres (100µL) of BioRes was injected into the vitreous cavity. The needle was left *in situ* after injection of the BioRes system for 10 seconds to prevent reflux. Once the needle was withdrawn, direct pressure was placed over the injection site with a sterile cotton bud. Direct ophthalmoscopy was performed post injection to exclude a central retinal artery occlusion. Chloramphenicol ointment 1% w/w was applied post injection.

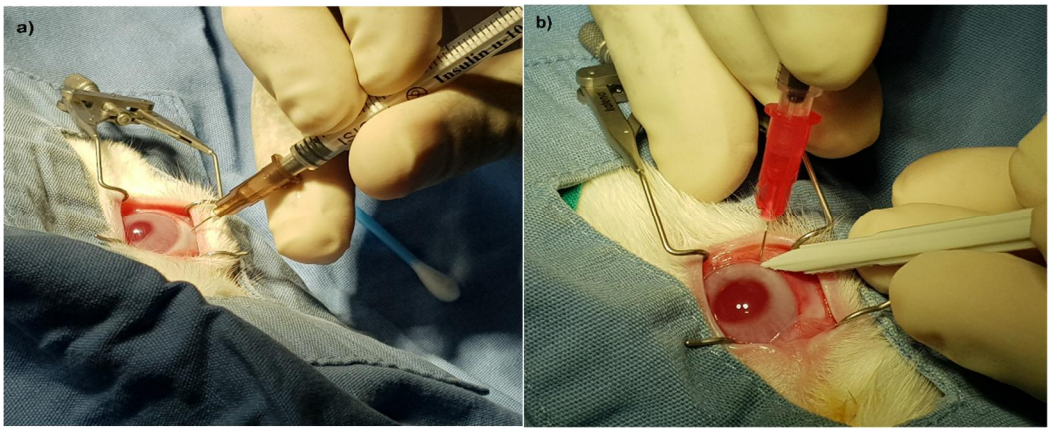

Figure 4.2: Photographic image during the process of an intravitreal injection of a) BioRes and b) lipopolysaccharide in rabbits from various groups as detailed below

On day 3, inflammation was induced in 10 rabbits, 5 from group 1 and 5 from group 3 by means of an intravitreal injection (100 µL) of Salmonella typhimurium lipopolysaccharide (LPS) (100–200 ng/ 10 µL of phosphate-buffered saline). The diluted LPS was injected into one eye intravitreally at the pars plana 2.0 mm posterior to the limbus using a needle, taking care to avoid injury to the lens, following general anaesthesia as described (Du Toit *et al.*, 2014).

On day 4, 5 rabbits from groups 1, 3 and 4 were euthanized by an overdose of IV sodium pentobarbital (> 50 mg/kg, ~2 mL) with consequent enucleation (Removal of eyeball leaving eye muscles and remaining orbital contents intact). Vitreous and aqueous humors were aspirated in volumes as allowed by physiological factors prior to enucleation using 18-gauge and 26-gauge needles. Aspirated humors for analysis were maintained at -80°C for analysis and enucleated ocular tissue from both eyes were immersed in 10% normal buffered formalin. Euthanasia on day 4 was carried out to determine the amount of drug released within the first 24 hours of administration, which could be related to the initial burst release.

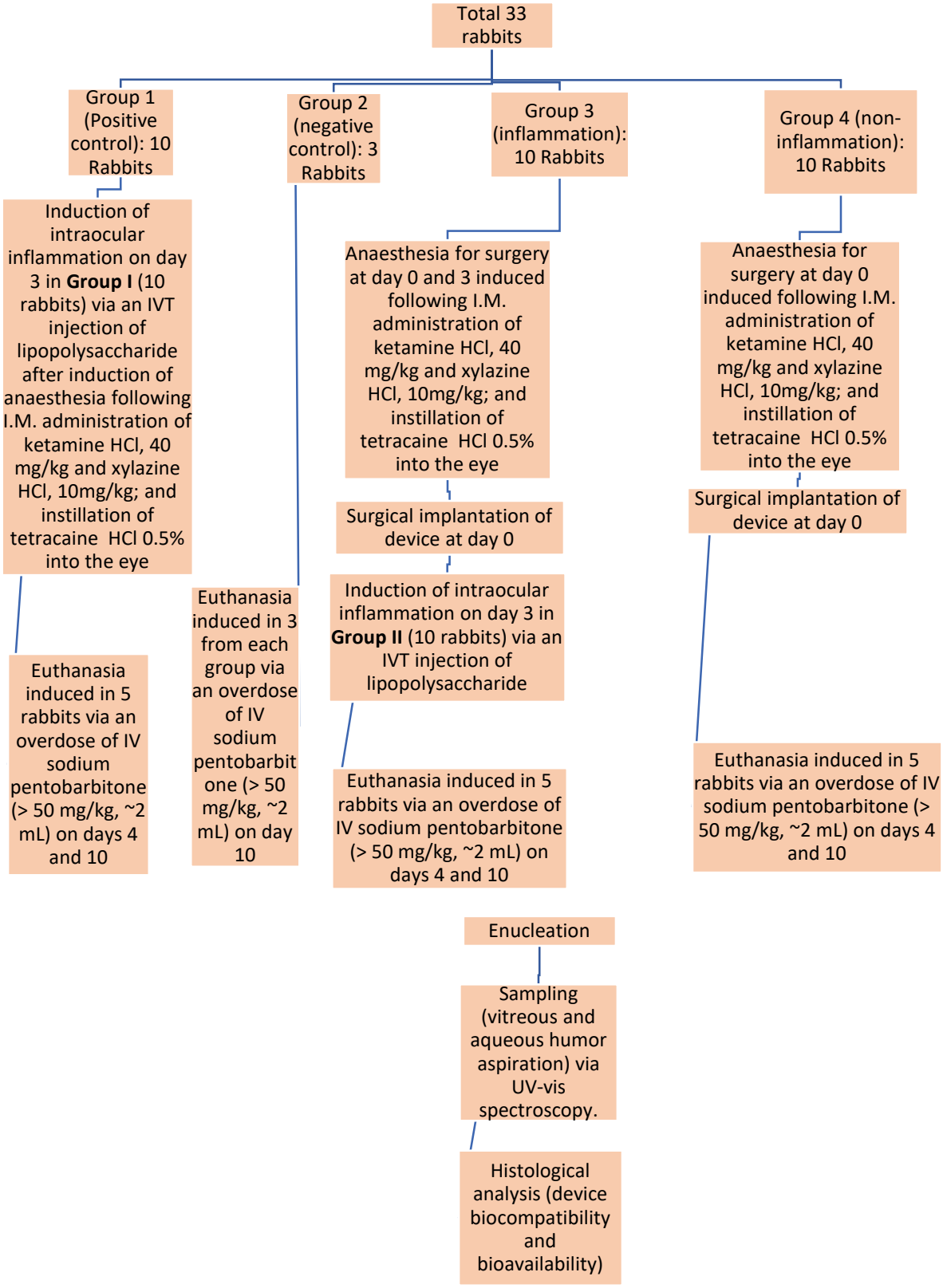

Figure 4.3: Flow chart for the design of the *in vivo* study that was conducted in a New Zealand white albino rabbit animal model

Histomorphological Analysis for Assessment of the Degree of Ocular Inflammation Following intravitreal injection of BioRes in the Normal and Inflamed Rabbit Eye

Enucleated eyeballs from the following groups were sent for analysis to determine degree of cellular infiltration:

- Positive control
- Negative control
- BioRes without inflammation
- BioRes with inflammation

The enucleated eyeballs (fixed in 10%v/v formalin) were sent to IDEXX laboratories (Pretoria, South Africa). The organs were cut according to standard IDEXX operating procedure (IdexxSA-AP-SOP-26) and processed according to routine histological tissue processing in an automated tissue processor with standard operating procedures IdexxSA-AP-SOP-27. Following tissue processing, sections were cut of 5-6µm (IdexxSA-AP-SOP-30) and the slides produced stained in an automated Haematoxylin and Eosin tissue stainer (IdexxSA AP-SOP-205) before histological evaluation.

Cellular infiltration (degree of infiltration by lymphocytes, plasma cells, and mononuclear and polymorphonuclear leukocytes) was graded from 0 to 3+ in accordance with IDEXX laboratory protocols, as done previously in a similar investigation (Du Toit *et al.*, 2014).

4.3. Results and Discussion

The BioRes product formed a stable suspension once it was added to the required quantity of hydrogel, hyaluronic acid, which was injectable through a 26 gauge needle. The suspension showed no caking and was easily redispersed after settling. Settling occurred at a slow rate and the formulation completely settled after standing for a period greater than 24 hours Physicochemical tests such as NMR, FTIR and XRD confirmed the formation of the BioRes system as well as the stability of the system subject to addition of vehicle of an aqueous nature and temperature changes. *In vitro* and *in vivo* studies tested the efficacy and toxicity of the spacer-based drug release system.

Confirmation of essential bonds in BioRes synthesis employing Nuclear Magnetic Resonance spectroscopy

1H NMR was carried out on the pristine components, mPEG-Hz as well as the product, BioRes, to confirm the formation of the product by establishing the disappearance and shifting of vital peaks. The disappearance of the broad N-H_2 peak in the spectrum of the product

(BioRes), found at 1.9-2.4ppm in the polymer-linker conjugate (mPEG-Hz); shown below in Figure 4.4; confirms that the N-H_2 bond was broken to form the core C=N bond of the hydrazone structure, which is responsible for the stimuli-responsive behaviour of the product. This is further confirmed by the downfield shift (deshielding) of the N-H peak in Figure 4.4b from 6.23ppm to 6.76ppm which is due to the absence of the two protons in the product (BioRes) that are found on the N-H_2 in the reactant (mPEG-Hz). To confirm the presence of dexamethasone in the product, 19F NMR was carried out on dexamethasone and BioRes; the spectra which are found below in Figure 4.4c. The presence of the peak in BioRes shows successful product formation while the large downfield shift from ~-170ppm to ~70ppm is proposed to be brought about by steric deshielding which is attributed in this scenario to the many alkyl groups of the 2000Mw mPEG polymer (Dolbier, 2016).

Determination of Structural Transitions by Fourier Transform Infrared Spectroscopy

The FTIR spectra for each of the pristine components, mPEG-Hz, Dexamethasone and HA, as well as the product, BioRes, can be found below (Figures 4.5- 4.7) for comparison. The end product (Figure 4.6) showed characteristic bands of both the polymer and drug, such as the sharp peak at 1340 cm^{-1} indicating the presence of non-aromatic C-H bonds as found in the polymer, as well as the broad peak in the 3200-3600 cm^{-1} region (albeit greatly reduced in intensity due to the relatively large size of the polymer) indicating the presence of the O-H bonds found in dexamethasone as well as the N-H group from the polymer (Seker, Arslan and Chen, 2019). The significant reduction in the intensity of the C=O peak found at 1704 cm^{-1} is indicative of the removal of oxygen from the carbonyl group of dexamethasone at the more reactive C=O to make way for the formation of the imine linker bond (C=N) to be formed. Other peaks specific to polyethylene glycols and dexamethasone have been elucidated in previous works (Shoaeifar, Abbasian and Entezami, 2007; Shameli *et al.*, 2012; Loganathan and Sankaran, 2017; Seker, Arslan and Chen, 2019).

The introduction of the BioRes powder into the HA, as the system would be in its final form pre-administration, elicited no changes in the chemical compositions/ bonds or either of the components of the BioRes-gel combination system. This proves optimal, as it shows that BioRes is stable once it has been formed and could safely be used in a hydrogel vehicle without having any of its vital properties altered. The layered graphs can be found below in Figure 4.7 for comparison and it can be seen that no new bond formations/peaks are seen in the spectrum of the BioRes gel system (black) relative to pristine HA (red) or BioRes (blue).

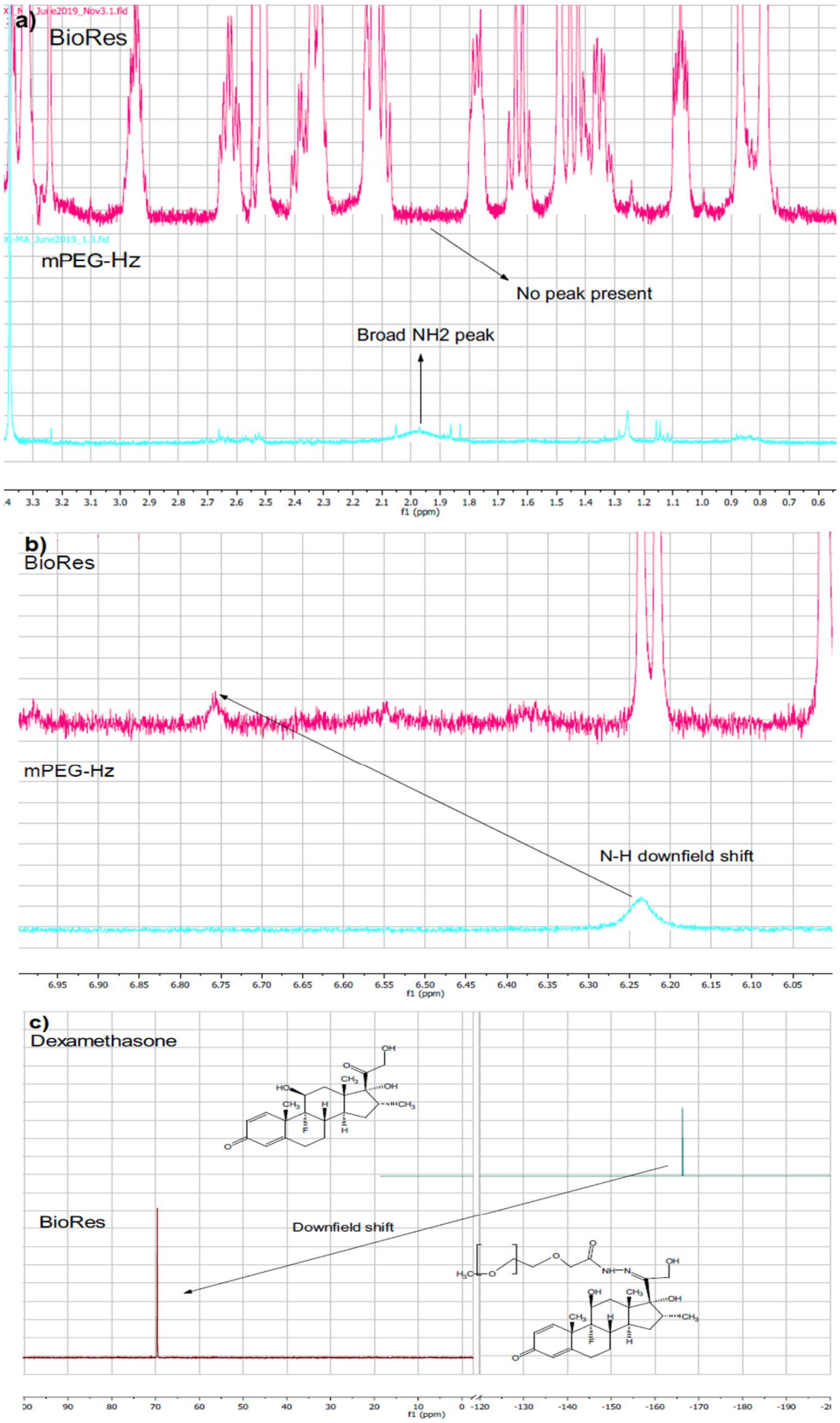

Figure 4.4: NMR Spectra showing a) the disappearance of the N-H_2 peak b) the downfield shift of N-H due to deshielding and c) the presence of and the shift of the fluorine peak in 19F NMR; all indicating towards the formation of the hydrazone bond

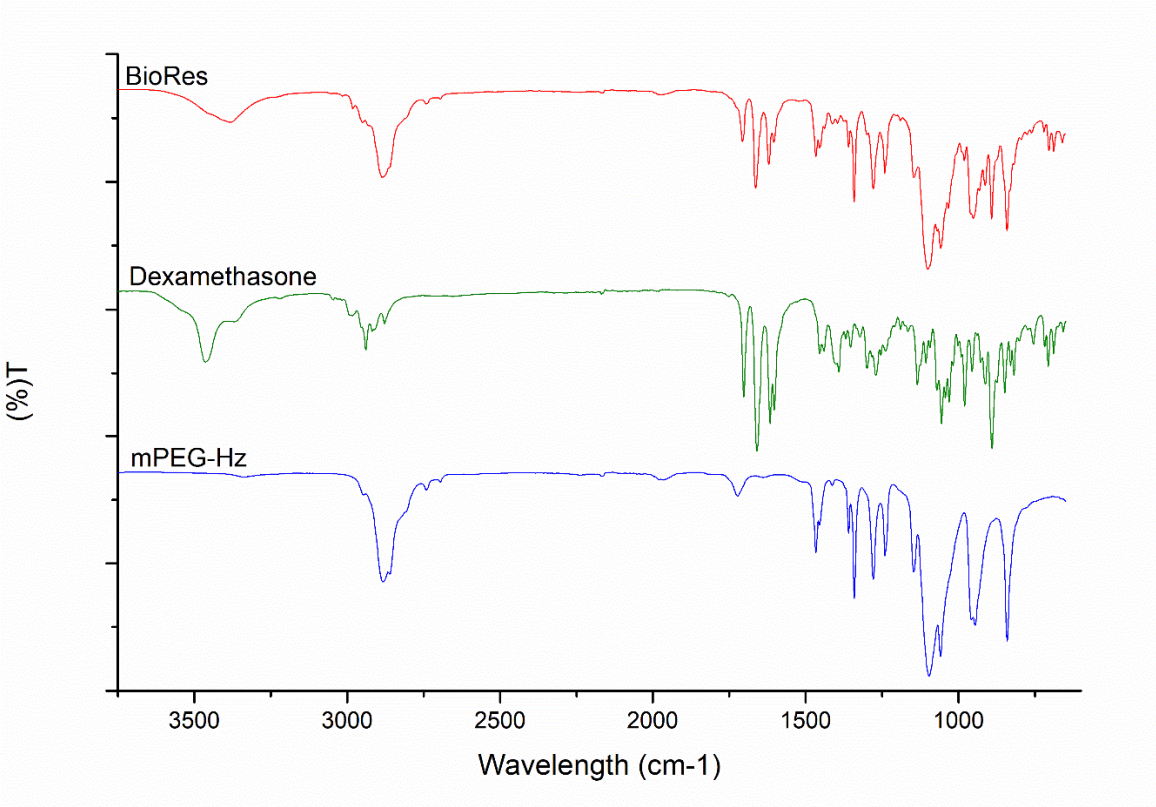

Figure 4.5: FTIR spectra of pristine reactants and product (BioRes). Characteristic peaks of the product are shown in Figure 4.6

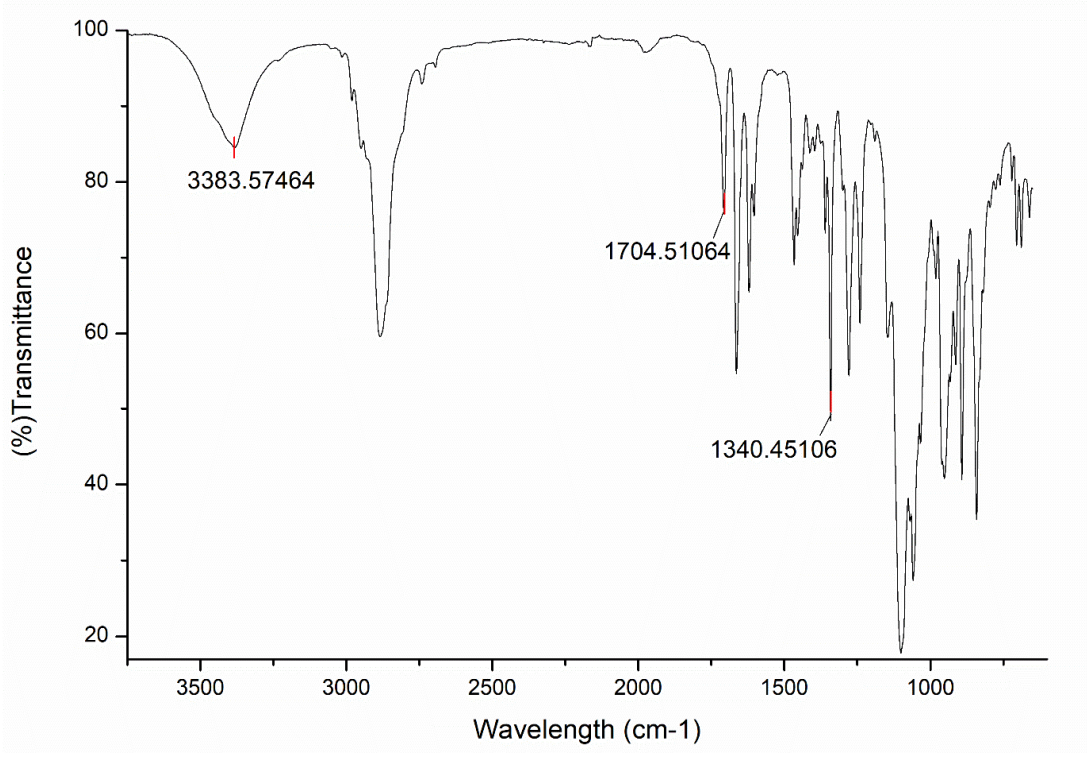

Figure 4.6: Expanded FTIR spectra of BioRes showing characteristic peaks C=O (1704 cm^{-1}), -OH (3200-3600 cm^{-1}) and non-aromatic C-H (1340 cm^{-1}).

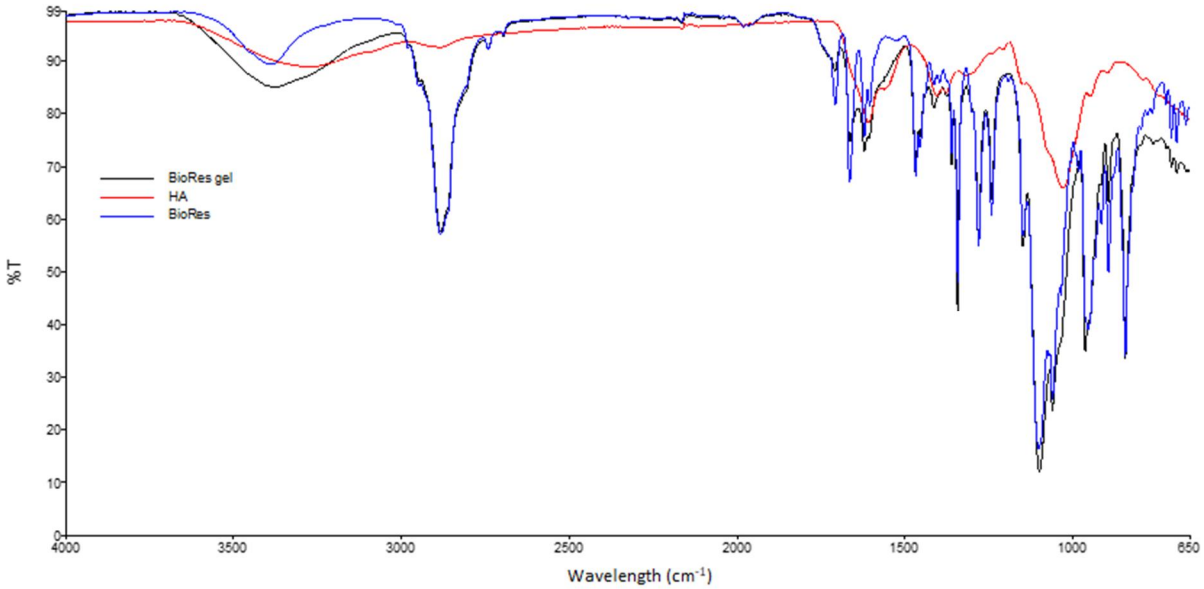

Figure 4.7: FTIR spectra highlighting the effect of addition of the BioRes system into the hyaluronic acid (HA) hydrogel. BioRes gel system (black), HA (red) and BioRes (blue).

Thermal transitions of the BioRes by Differential Scanning Calorimetry (DSC)

DSC was carried out on mPEG-Hz, Dexamethasone and BioRes as depicted in Figure 4.8 below. The Thermogram of mPEG-Hz and dexamethasone show endothermic peaks at 54ºC and 272ºC representing melting temperature of the two reactants. The peak at 55.16ºC in the thermogram of BioRes is representative of the melting temperature which indicates that once the drug has been bound to the system, the melting point of the system is closer to that of the polymer than that of the drug (Chu *et al.*, 2016). This suits the BioRes' drug delivery system as the temperature of the human eye is well below the melting temperature of the system thus the BioRes would remain intact at intraocular temperatures.

Phase determination via X-ray powder diffraction (XRD)

XRD analysis showed that both mPEG-Hz and dexamethasone have a high crystallinity, although mPEG-Hz shows a few small amorphous regions, as shown in Figure 4.9. This high relative crystallinity carries through into the spectrum of the BioRes product which is also represented in the DSC spectrum as a slight increase in the melting point of BioRes with the addition of crystalline dexamethasone (Chu *et al.*, 2016). There are no indications of significant amorphous properties present. With regards to mPEG-Hz, the effect of high crystallinity is a lower rate of degradation (Jenkins and Harrison, 2008), which is optimal in this formulation as the polymer would have to remain intact long enough for the drug to be released via the pH-responsive mechanism rather than by degradation of the polymer carrier.

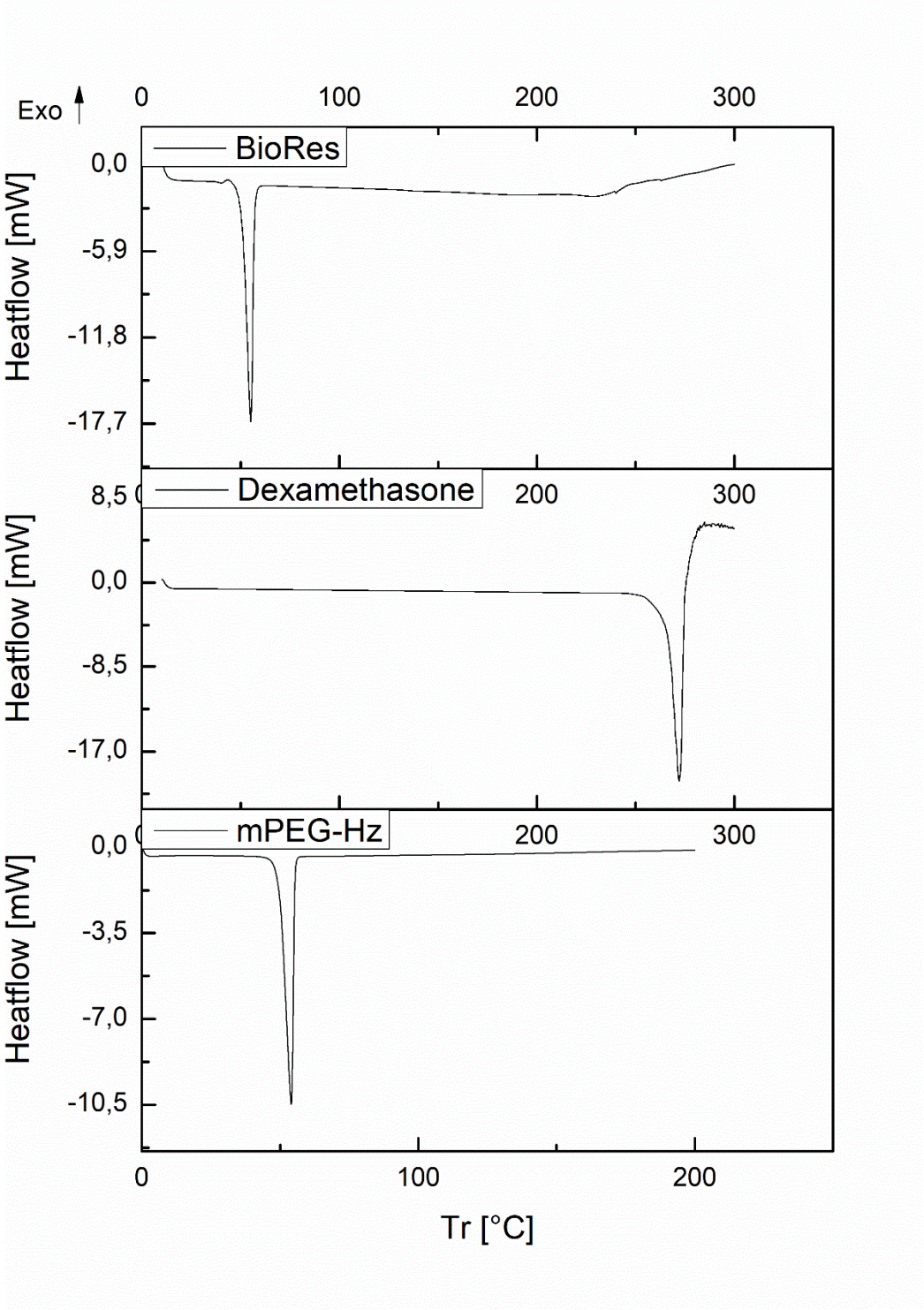

Figure 4.8: Depicting a comparison of the DSC thermograms of components, mPEG-Hz and dexamethasone, and product (BioRes) in which the melting point of the BioRes is closer to that of the polymer.

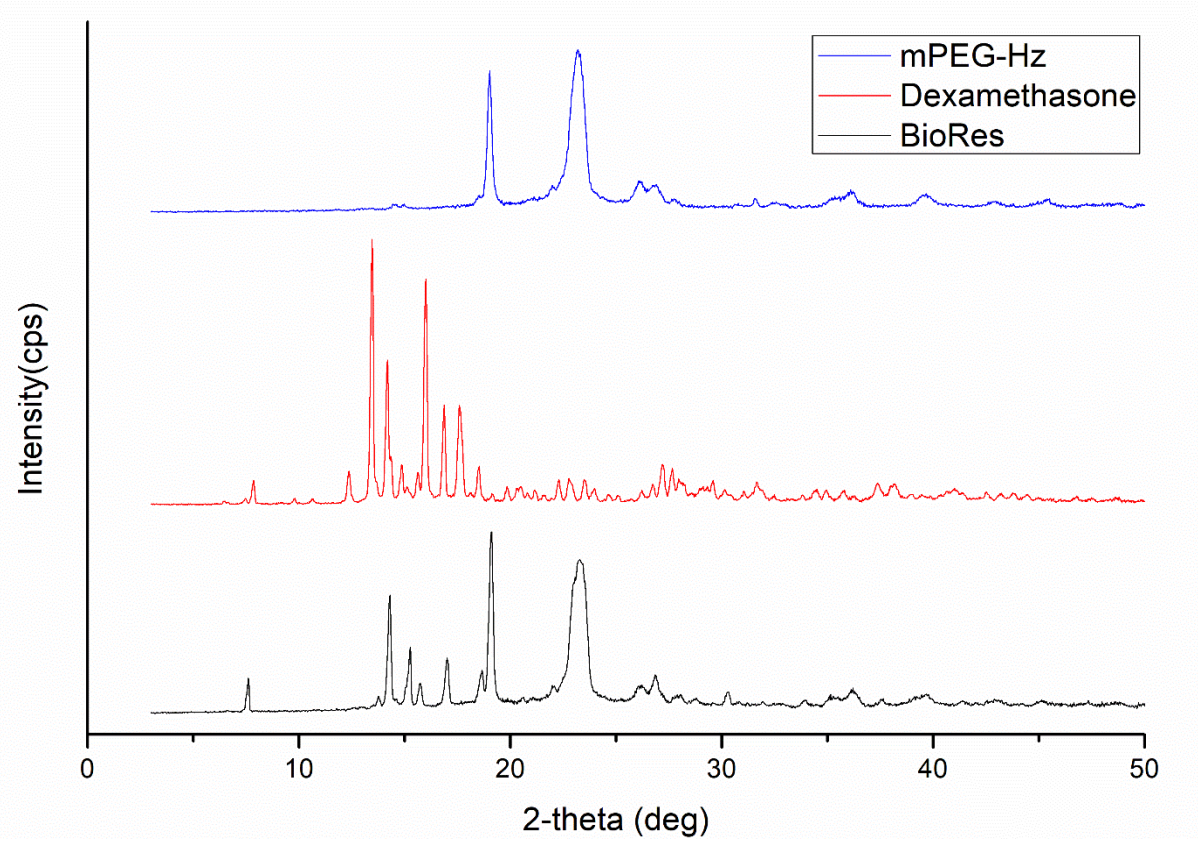

Figure 4.9: XRD Spectra of mPEG-Hz, dexamethasone and BioRes

4.3.1. Inflammation-responsive enhanced *In vitro* drug release capability of the BioRes system

The BioRes system exhibited an initial burst release of dexamethasone as shown in Figure 4.10 during the first 24 hours, followed by a steady increase through the first 2 day period of inflammatory pH conditions (48 through 96 hours) after which the release is significantly slowed down during 96 -144 (4% increase) hours as an effect of bringing the pH back to normal physiological pH; as can be seen by the plateau between those time points. The next drop in pH shows an 8% increase in drug release as expected for the stimulus-responsive system. The ensuing increase of pH back to non-inflammatory physiological pH shows a 2% decrease in drug release. The small drop in % drug released could be attributed to the lack of dexamethasone release in conjunction with the loss of drug during removing of samples for analysis and replacement by fresh SVH.

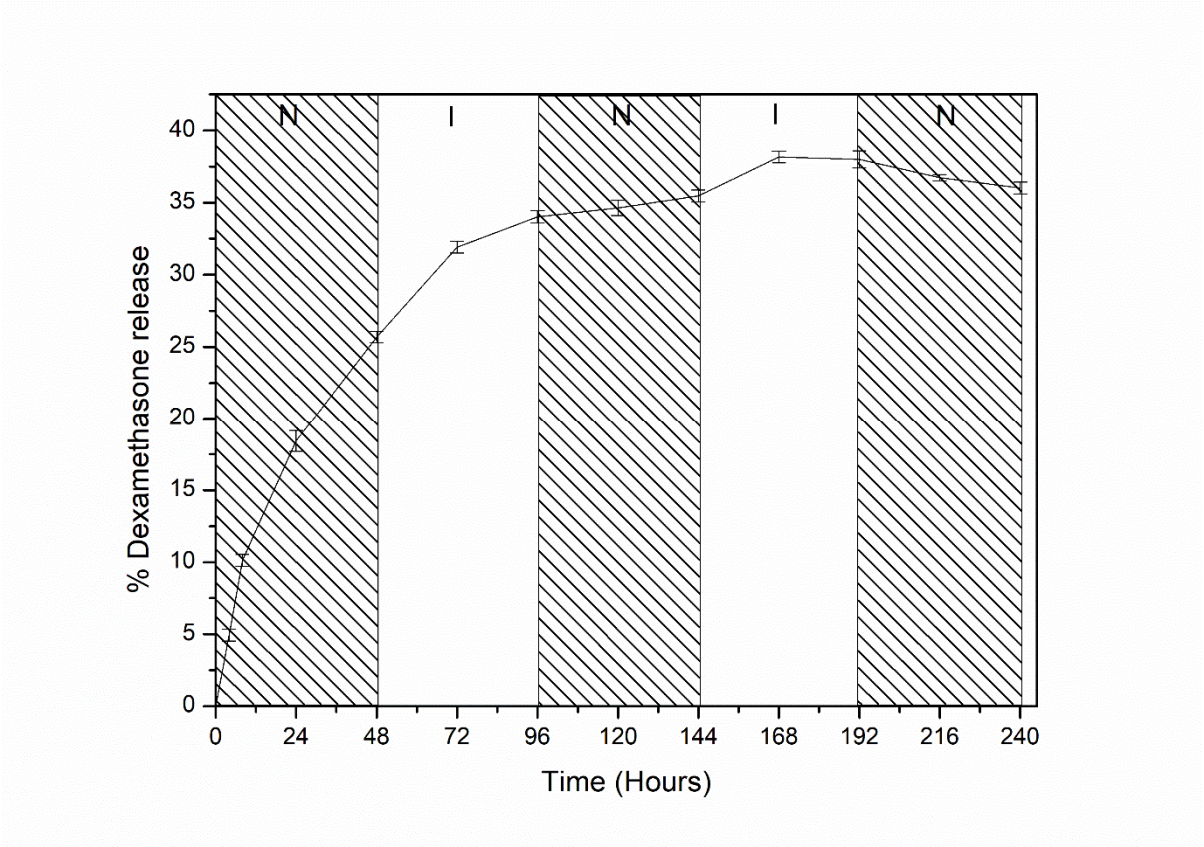

Figure 4.10: *In vitro* drug release at normal pH (N) and inflammatory pH (I)

4.3.2. *In vivo* analysis to determine toxicity and inflammation-responsive behaviour of the BioRes in the rabbit eye

In vivo evaluations revealed that the BioRes system had a significantly higher release in the inflammation group relative to the non-inflammation group as shown in Figure 4.11 below. Drug concentration in the non-inflammation group had experienced a decline at day 10. This could be accounted for by the fact that slight ocular inflammation is induced when any invasive procedure is carried out, such as the intravitreal BioRes injection in this scenario, which would result in the release of a certain amount of drug due to the drop in intravitreal pH. Thus, as inflammation disappears, building up to the latter days of the study, when there is an absence of associated inflammatory conditions such as a lowered pH (as evident in the proceeding histomorphological results), drug seizes to be released and thus the concentration declines.

Dexamethasone concentration in the non-inflamed versus inflamed aqueous humor displayed a similar pattern, albeit in insignificant amounts. It should be noted that a minimum effective concentration for intraocular dexamethasone has not yet been identified (Papangkorn et al., 2018). Drug concentrations in the aqueous humor of the inflamed eye (0.691µg/mL) was found

to be slightly over 10 times the drug concentration in the aqueous humor of the non-inflamed eye (0.0634µg/mL). This further supports the stimuli-responsive drug release behaviour of the BioRes system *in vivo.*

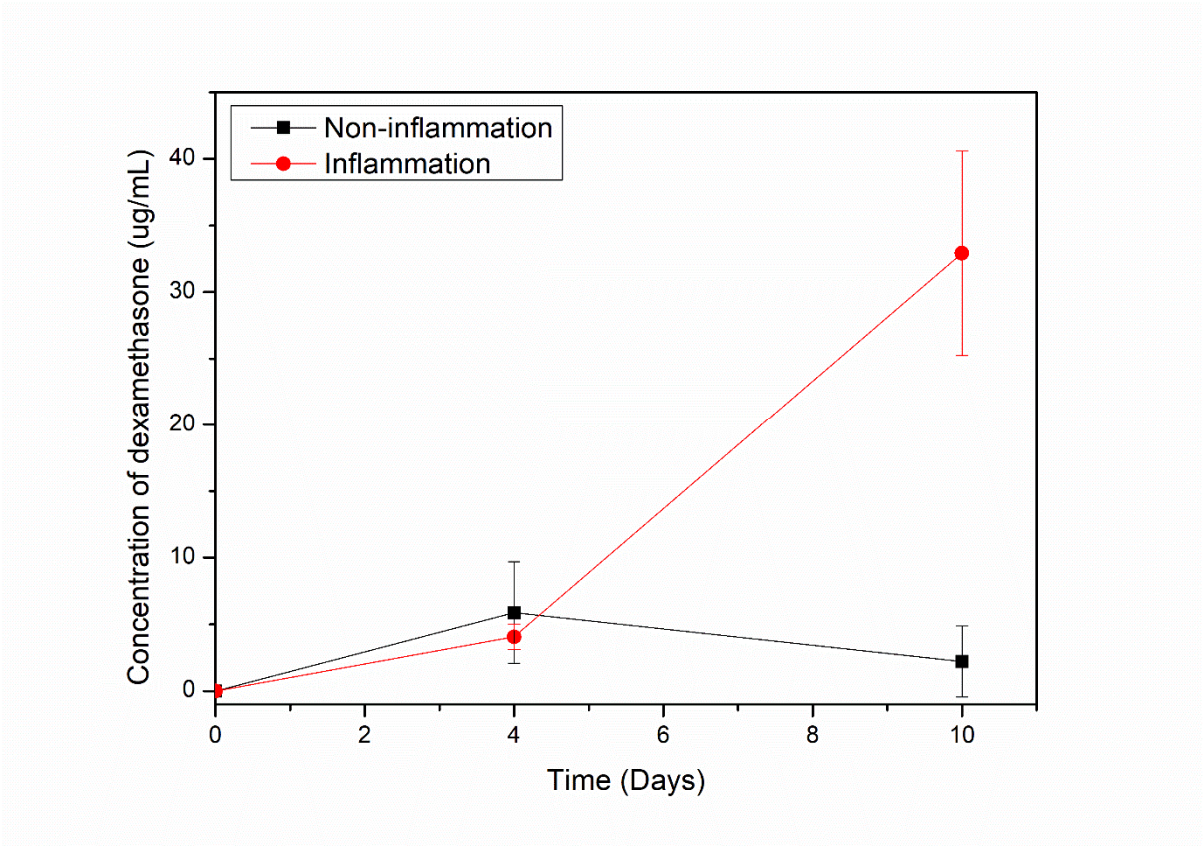

Figure 4.11: *In vivo* drug release of the BioRes system in the non-inflamed and inflamed New Zealand White Albino rabbit model (P<0.001)

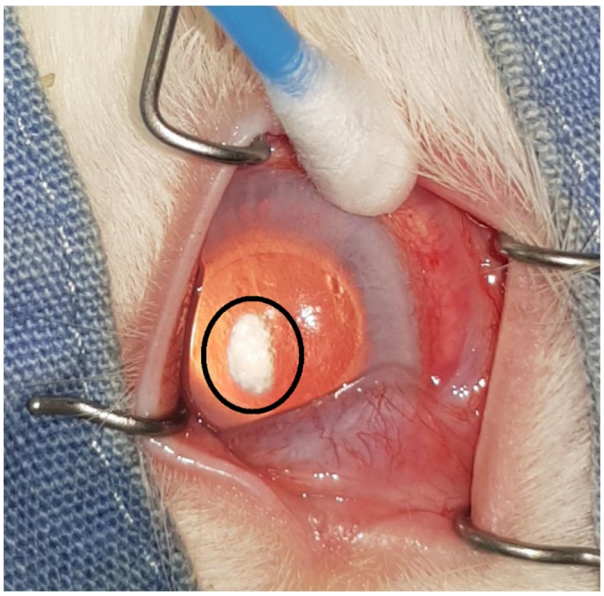

Figure 4.12: Photographic image of the BioRes system (circled) formed *in situ* within a few minutes after the intravitreal injection. It appears as an opaque, oval shaped mass adhered to the vitreous wall.

Histomorphological Analysis for Assessment of the Degree of Ocular Inflammation Following intravitreal injection of BioRes in the Normal and Inflamed Rabbit Eye

Histomorphological evaluation as well as relative degree of inflammation of ocular structures was undertaken on to determine toxicity of BioRes in the ocular setting. Table 4.1 in conjunction with Figure 4.13 (a-e) shows the levels of inflammation present in the anterior and posterior chambers as well as the vitreous body of the rabbit eye. Histological assessment revealed that levels of inflammation the BioRes group (0 grading) were comparable to the negative group (0 grading) in which there was no signs of inflammation present and thus significantly lower than the respective positive control group at days 4 (1 grading) and 10 (2, 3 grading). This thus shows the lack of toxicity of the BioRes formulation to the ocular environment in the rabbit eye. Another study that also utilized a bioresponsive system was carried out (Du Toit *et al.*, 2014), and showed similar results, in that the drug-free system showed a significantly greater presence of inflammatory infiltrate in comparison to the drug-loaded device, which did not elicit an inflammatory immune response, rather it elicited a definite decrease in inflammation.

Table 4.1 Levels of anterior/ posterior chamber and vitreous body inflammation in the controls and treatment eyes at days 4 and 10

Group	Anterior/ Posterior Chamber Inflammation	Vitreous Body Inflammation	Additional Observations	Histological Image (Figure 4.13)
Negative Control	0	0	No indicators of inflammation present	(a)
4 Day BioRes	0	0	No clear indications of inflammation in any of the structures of the globe.	(b)
4 Day Positive Control (LPS injection)	1	1	The posterior chamber of the eye contains heterophils associated with fibrin deposition. The retinal layers show fibrinopurulent exudate on the anterior surface suggestive of inflammation also involving the vitreous chamber. The optic nerve contains mild heterophilic presence in the perivascular spaces, in few areas showing single heterophils extending into the peripheral nerve tissue itself.	(c)
10 Day BioRes	0	0	No lesions are visible in the structures of the globe and there is no inflammatory exudate present in the chambers of eye.	(d)
10 Day Positive Control (LPS injection)	2	3	Severe accumulation of heterophils in the vitreous body admixed with fibrillar exudate extending into the inner nerve fibre layer of the retina	(e)(f)

Inflammation: 0= no detectable; 1= mild; 2= moderate; 3=severe, LPS= lipopolysaccharide

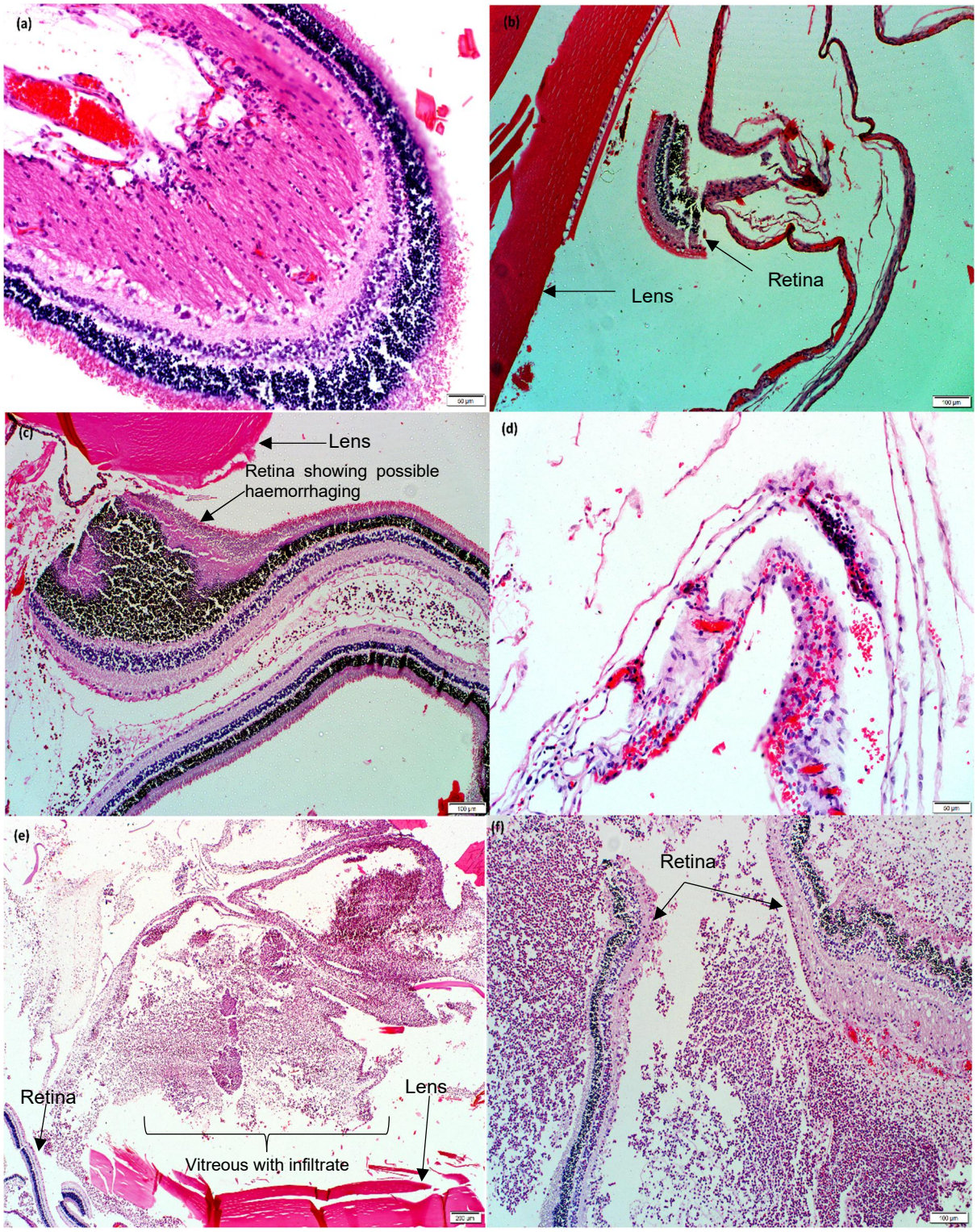

Figure 4.13: Histological slides at days 4 (a-c) and 10 (d-f) for (a) the negative control eye showing the normal retina at 20x magnification (b) BioRes injected eye showing the choroid, retina and lens at 10x magnification (c) LPS injected eye showing the retina and lens at 10x magnification (d) BioRes injected eye showing the sclera at 20x magnification and (e,f) LPS injected eye showing the vitreous, retina and lens (e) and lens (f) at 4x and 10x magnification.

4.4. Concluding remarks and future prospects

A biostimuli-degradable, spacer-based, polymer-drug conjugate was formulated and evaluated as an injectable intraocular hydrogel system. FTIR, ^{1}H NMR and DSC confirmed the successful formation of the pH-responsive spacer within the polymer-drug conjugate using mPEG-Hz and dexamethasone. *In vitro* studies conducted over a period of 10 days demonstrated stimuli-responsive release at inflammatory pH and In vivo studies carried out on New Zealand albino rabbits elucidated the further enhanced release when the DDS was within the actual ocular environment. Histomorphological analysis determined the biocompatibility of the BioRes which showed a lack of toxicity to the ocular structures. It can be concluded that the BioRes has potential for treatment of posterior segment inflammatory disorders.

It is worth mentioning that further release control could be attained by slightly altering the chemical composition of the of the DDS vehicle/ gel which would produce an enhanced pH and matrix-degradation dual-controlled DDS that would be capable of releasing a host of drugs over a more extended period of treatment for posterior segment inflammatory disorders.

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Chapter 5

Conclusions, Challenges and Recommendations

5.1. Conclusions

The advancement of drug delivery has paved the way for bettered diagnostics and treatments, including the invention of new treatments for posterior segment ocular diseases such as age-related macular degeneration, diabetic retinopathy and uveitis by a variety of novel technologies as mentioned previously in this dissertation. Research relating to intelligent stimuli-responsive pharmaceutical systems is being conducted in-depth and at a high-level owing to its many benefits concerning control of drug release. To date, much research has been conducted on the use of thermoresponsive drug delivery systems, however the same cannot be said for pH or inflammation-responsive systems. Research relating to pH-responsive systems, particularly in the posterior segment ocular department, is relatively scarce. The developed system would thus contribute to the furtherance of research in this field.

The BioRes system that was formulated in this study has proved to be an effective injectable and biodegradable intravitreal drug delivery system that provides stimuli-responsive drug release of anti-inflammatory drug, dexamethasone, to the posterior segment of the eye. A hydrazone linker, which is a type of Schiff base, was identified to be the appropriate spacer as it exhibited excellent responsiveness to pH variability and was thus successfully incorporated by means of a relatively well known Schiff base reaction wherein a carbonyl group and amine are reacted in the presence of an acid catalyst to form an imine bond. The physicochemical properties that were tested confirmed the successful achievement of the aforementioned bond as required as well as the chemical and physical stability of the formulation at physiological temperatures. The drug release of the BioRes formulation was tested *in vitro* over a period of 240 hours in alternating pH conditions (pH 5.2 and pH 7.4). The BioRes system was found to exhibit a certain degree of enhanced stimuli responsive drug release *in vitro* by manifesting an increase in dexamethasone release at low pH and a decrease in dexamethasone release at normal physiological pH as expected from a Schiff base compound.

This was then followed up and confirmed with the *in vivo* animal studies which were conducted in the model of the New Zealand White Albino rabbit eye. The *in vivo* study was carried out on 33 rabbits divided into 4 groups as detailed previously wherein vitreous and aqueous samples were collected at days 4 and 10. The *in vivo* study also revealed greatly enhanced stimuli

responsive release. Ten day vitreous concentrations of drug in the of the inflammatory group were found to be 32.97µg/mL (±7.6µg/mL), which was significantly greater than the group in which no inflammation was induced, wherein the concentration was found to be 2.21µg/mL (±2.6µg/mL). similar findings were observed in the anterior chamber of the eye. The concentration in the inflamed eye was also significantly greater when compared to the non-inflamed eye, albeit both concentrations were of insignificant amount; 0.691µg/mL and 0.00634µg/mL. There were no observed surgical complications which can be ascribed to the ease of the injection procedure which can be considered as the least invasive of any invasive ocular procedure and to the biodegradability of the system thus doing away with the need for removal surgery. As part of the *in vivo* study, the safety of the formulation was also tested by means of histomorphological analysis that was carried out on the ocular tissue of the rabbits. The histomorphological results showed the safety of the BioRes system in the ocular setting. To bring this research to a close, it can be said that that the BioRes has the potential to be further researched as an intravitreal, stimuli-responsive drug delivery system for posterior segment inflammatory disorders.

5.2. Challenges and shortcomings

The initial shortcoming that was encountered was that of the effective synthesis of the mPEG-PCL hydrogel. The hydrogel formulation synthesis parameters need to be strictly adhered to in order for the copolymer gel to be formulated with reproduceable results. However, this shortcoming was overcome by the use of a hyaluronic acid and its corresponding hydrogel, which was optimal for this formulation as results using this compound were reproduceable and more feasible for this study.

The BioRes powder showed a lack of clear solubility in aqueous media at the desired concentrations. The challenge that this potentially posed was the possibility of a decrease in the ease of injectability of the system when administering the formulation via the intravitreal route. The most desirable needle size to be used was a 29- or 30-gauge needle. Due to the formulation being in suspended rather than dissolved, a 26 needle was used, as is commonly used for daily ocular procedures. Thus, this challenge was overcome.

5.3. Recommendations

a) The BioRes could be further optimized to improve injectability to the end that a smaller needle could be used for administration of the BioRes.
b) The utilization of a thermo-responsive hydrogel in order to impart better injectability as well as a dual-responsive property to the delivery system.

c) It could be used for other disease conditions such as retinitis as induced by the cytomegalovirus which is seen in many HIV cases, subject to changing the drug to be used. This is possible due to the chemical nature of the polymer-linker system. A vast array of drugs could be attached to the system without the need for pre-reaction chemical changes to the structure of the drug.
d) It can be used in non-ocular diseases in other body systems as inflammation (and its accompanying drop of pH) is present in almost all body systems.

APPENDICES

Appendix A: Published Review Article

ARTIFICIAL CELLS, NANOMEDICINE, AND BIOTECHNOLOGY
2018, VOL. 46, NO. S2, S1074–S1081
https://doi.org/10.1080/21691401.2018.1478845

Polymeric, injectable, intravitreal hydrogel devices for posterior segment applications and interventions

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ABSTRACT

Posterior segment diseases are commonly implicated in the occurrence of curable as well as incurable diseases which could lead to blindness. An example of one such disease is age-related macular degeneration, which would require chronic treatment over an extended period and as such the development of an intravitreal drug delivery system that would optimize patient outcomes is essential. The major contributor of the difficulty in treating diseases in the posterior segment is the structure of the eye, particularly the blood–ocular barriers. This review will focus on the various biodegradable and non-biodegradable injectable intravitreal polymeric hydrogel devices, such as poly(ethylene glycol), poly(lactic-co-glycolic acid), silica, hyaluronic acid/dextran, silk, chitosan/alginate and poly(*N*-isopropylacrylamide)-based polymers, their compositions as well as the effects and results of these polymeric components on the device as a whole. Furthermore, it will briefly discuss therapeutic agents used in these devices, such as ranibizumab, dexamethasone, Avastin®/bevacizumab and aflibercept, and a potential way forward by employing intelligent polymeric systems.

ARTICLE HISTORY
Received 11 April 2018
Revised 12 May 2018
Accepted 15 May 2018

KEYWORDS
Ocular drug delivery; posterior segment; intravitreal devices; injectable hydrogels; injectable intravitreal devices; biodegradable hydrogels

Introduction

The structure of the eye is complex and as a result of this many disease states or abnormalities such as posterior segment inflammation and neovascularization-related diseases have been beyond the reach of proper medical intervention. The structure of the eye can be divided into the anterior segment, which consists of all the structures which lie anterior to the lens, and the posterior segment, which consists of all the structures that lie posteriorly to the lens [1]. Causes of posterior segment inflammation can be classified as infectious and non-infectious and treatment may involve chronic therapy for long periods [2]. Posterior segment inflammation could potentially lead to visual loss as the disease progresses. Inflammation could lead to another possible complication, neovascularization. Ocular pathologies that present with neovascularization, which is characterized by the formation of new blood vessels in unwanted ocular regions, such as the wet form of age-related macular degeneration may also be implicated for loss of vision. Administration via indirect routes such as topical and systemic routes are not suitable as there would either be too little drug reaching the affected site, due to the presence of blood–ocular barriers, which prevents drug from passing through, or the side effects which would result in other morbidities or decreased patient compliance [3]. In an attempt to avoid these negatives coupled with the benefits of a direct route of administration, direct intravitreal (IVT) injections were developed [4]. A comprehensive review by our department [5] outlines the various routes of administration such as systemic, topical, oral, periocular and intravitreal routes, and provides insight regarding some of the issues and challenges relating to each of these routes of administration.

Both biodegradable and non-biodegradable in-situ hydrogel forming injections are available. The hydrogels are formed by polymeric networks of a hydrophilic nature that have the ability to swell in water by maintaining the water within its structure whilst sustaining the integrity of the network [6]. This swelling response is brought about due to the presence of crosslinking, whether chemical or physical as shown in Figure 1, between the constituent polymers [7,8]. Physically crosslinked hydrogel structures are usually reversible hydrogels and are formed in the absence of an external chemical linking agent, by linkages that are formed using the chemistry already present on the polymer structures to form links by means of, for example, hydrogen bonds and ionic bonds. Whereas, chemically crosslinked hydrogels are formed employing an additional chemical substance to facilitate the linking of the components of the hydrogel. Furthermore, environment-sensitive polymeric hydrogels have been fabricated, such that they respond to stimuli such as high or low pH- utilizing poly(caprolactone-lactide)-poly(ethylene glycol)-poly(caprolactone-lactide), temperature changes-utilizing N-isopropylacrylamide, poly(ethylene oxide)–b-poly(propylene oxide)–b-poly(ethylene oxide), poly(ethylene glycol), polysaccharides such as chitosan and dextran and proteins such as gelatin to name a few, and other molecules [7–12].

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Appendix B: Research Paper to be Published

Synthesis, Characterization, *In vitro* and *In vivo* Analysis of a Biostimuli-degradable, Spacer-based, Polymer-drug Conjugate as an Injectable Intraocular Hydrogel System.

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Abstract

A biostimuli-degradable, spacer-based, polymer-drug conjugate was formulated and evaluated as an injectable intraocular hydrogel system for delivery of drug in the posterior segment of the eye in response to a pH drop generated during the inflammatory process. mPEG capped with a stimulus responsive hydrazide group was used as the polymeric component of the polymer-drug conjugate. Dexamethasone was used in the drug delivery system as it is currently used in the market for posterior segment inflammation. Physico-chemical and characterization tests were carried out using an array of equipment to ascertain the correct formation of the system as well as other properties of the DDS. In vitro and In vivo analyses were conducted to determine the release of drug in normal and inflammatory pH surroundings and the results were promising. The results of these analyses showed potential for future application of the BioRes as a stimuli-responsive DDS for posterior segment inflammatory disorders.

Appendix C: Animal Ethics Clearance Certificate

STRICTLY CONFIDENTIAL

ANIMAL ETHICS SCREENING COMMITTEE (AESC)

CLEARANCE CERTIFICATE NO. 2018/09/39/C

APPLICANT: Mr M Anwary

SCHOOL: Pharmacy
DEPARTMENT:
LOCATION:

PROJECT TITLE: Biostimuli-degradable, spacer-based, polymer-drug conjugate as an injectable system for ocular posterior segment inflammation intervention in New Zealand white rabbits

Number and Species

33X-1.5-2.5kg male New Zealand white rabbits

Approval was given for the use of animals for the project described above at an AESC meeting held on 2018/09/25. This approval remains valid until 2020/12/04.
Unreported changes to the application may invalidate the clearance given by the AESC
An annual progress report must be provided

The use of these animals is subject to AESC guidelines for the use and care of animals, is limited to the procedures described in the application form and is subject to any additional conditions listed below:

Signed: _____ (Chairperson, AESC) Date: 10/12/2018

I am satisfied that the persons listed in this application are competent to perform the procedures therein, in terms of Section 23 (1) (c) of the Veterinary and Para-Veterinary Professions Act (19 of 1982)

Signed: _____ (Registered Veterinarian) Date: 06 November 2018

cc: Supervisor: Prof V Pillay and Dr P Kumar
Director: CAS

Works 2000/lain0015/AESCCert.wps

Appendix D: Turn-it-in Report

Turnitin Originality Report

Processed on: 24-Mar-2020 9:52 AM SAST
ID: 1280995925
Word Count: 10249
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