A Long-acting Ocular Lubricant Eye Drop based on Dextran Hydrogel Nanoparticles

by

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Author's Declaration

This thesis consists of material all of which I authored or co-authored: see Statement of Contributions included in the thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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

Statement of Contributions

All experimental design, methodology development, and data analysis work presented herein is my own, with guidance from Professor Gu and Dr. Shengyan Lui.

Many of the data sets included in Chapters 3 and 4 of this thesis were obtained through experiments conducted in part or full by Sabrina Zuccaro, Amelia Caza, or Wenxuan (Ivy) Liu, each of whom were undergraduate co-op students under my direct guidance and supervised by Professor Gu. Each of these students also assisted with some data analysis work under my guidance.

The histopathological analysis featured in Section 4.5 was conducted by Dr. Denise Hileeto, Clinical Associate Professor at the University of Waterloo School of Optometry. The microscope images were provided to Dr. Hileeto, and the written description included herein is my own based on her analysis report and our verbal discussions.

Abstract

Dry eye disease is a highly prevalent condition that affects hundreds of millions of people worldwide. In moderate to severe cases, affected individuals experience considerable difficulty in carrying out their day-to-day activities due to discomfort and pain, and may even develop symptoms of depression. Ocular lubricants (sometimes referred to as artificial tears) are the most widely-used method of dry eye management. Despite being available over-the-counter and in myriad varieties and brands, current formulations all suffer from either short duration of action or high inconvenience due to vision obstruction, unwanted residue, and/or invasiveness. There is therefore great need for long-lasting, inconspicuous, and convenient ocular lubricant formulations to address the shortcomings of current market offerings.

This thesis describes the development of a novel ocular lubricant technology based on mucoadhesive dextran hydrogel nanoparticles (DH-NPs) that shows considerable promise in addressing these needs. The nanoparticles feature a dextran hydrogel core synthesized using a water-in-oil nanoemulsion method. The hydrogel core is designed to enable sustained release of ocular lubricant through two distinct mechanisms, namely degradation and diffusion. The nanoparticles are also coated with phenylboronic acid (PBA) to impart mucoadhesion and cause them to be retained on the ocular surface for approximately 24 hours. By continuously releasing lubricant during this time, DH-NPs are anticipated to provide long-lasting and more effective DED symptom relief than the ocular lubricants currently on the market.

In addition to the methods of DH-NP synthesis and characterization, various parameters capable of tuning key properties such as diameter, synthesis yield, PBA conjugation, and mucoadhesion strength are described herein. In vitro release experiments were also performed to

characterize the kinetics of ocular lubricant release from DH-NPs. Various parameters that affect

release rate and quantity were identified to enable tuning and optimization towards achieving the

ideal clinical ocular lubricant dose. An acute in vivo biocompatibility study was also performed

using a rabbit model, in which the novel formulation was well-tolerated. Overall, DH-NPs were

found to be a highly promising technology for DED treatment, and further development towards

clinical evaluation is recommended.

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Dedication

To mom, Sultana Zahan,

and dad, Joinal Abedin.

Whatever I achieve is mere a reflection of your awesomeness.

And to my younger brother, Saif-ul Abedin.

My biggest fan and most outspoken critic,

you help me grow every day.

Now that I'm done this thesis I'm going to practice NHL till I finally beat you!

Table of Contents

List of Figures

List of Tables

List of Abbreviations

NP: nanoparticle

DH-NP: dextran hydrogel nanoparticle

DED: dry eye disease

DexOx: oxidized dextran

PBA: phenylboronic acid

ADH: adipic acid dihydrazide

PVP: polyvinylpyrrolidone

1.0 Introduction

1.1 Overview

Dry eye disease (DED) is a highly prevalent condition that affects hundreds of millions of people worldwide, reaching prevalence rates of up to 75% in some populations[1,2]. DED is the most common cause of eye-related physician visits, and can cause patients to experience significant pain, poor general health, considerable impairment in conducting daily activities, and symptoms of depression[1,2]. The economic effects of the condition are also extensive, with a 2011 estimate placing the total burden of disease in the United States alone at approximately $3.2 billion annually in direct treatment costs and an additional $55.6 billion in lost productivity[3].

Due to the complex and varied physiological origins of dry eye disease, treatment regimens must be tailored to each patient's unique condition, and often involve the simultaneous use of a variety of methods. In virtually all cases however, ocular lubricant eye drops (also known as artificial tears) are a major component of treatment due to their high level of safety and the symptom relief and ocular protection they offer[4–8]. Unfortunately, the ocular lubricant eye drops currently on the market suffer from short duration of action, requiring patients with moderate to severe dry eye to administer them as frequently as every 1-2 hours or more[9]. In addition, literature published to date shows no definitive advantage in efficacy of any single formulation, despite the large variation in lubricating polymers, pH, osmolarity, viscosity, and other ingredients amongst commercially available eye drop formulations[6]. Patients who require ocular lubricants 3-6 times a day or more are also required to take preservative-free formulations, which are usually more expensive and inconvenient due to the need for many single-use vials.[7,10–13] Higher viscosity ocular lubricant formulations often provide a higher level of efficacy and have a longer duration of action,

but are often unusable during daily activities due to blurring of vision and unpleasant residue deposition on the perimeter of the eye.[4] As a result of all of these considerations, ocular lubricants on the market today suffer from a seemingly insurmountable trade-off between efficacy and convenience. The products that offer effective symptom relief are troublesome to use amidst the business of everyday life, while those products that are highly convenient offer very low efficacy.

Extension of ocular retention time is one of the main strategies identified by industry for improving both the efficacy and convenience of ocular lubricant formulations. Traditional low-viscosity ocular lubricants ("aqueous drops") are known to have an ocular retention time of approximately 20-30 minutes[14], after which they are completely eliminated from the ocular surface and no longer effective. Multiple methods of extending this duration of action (beyond viscosity increase) have been explored by manufacturers, including in-situ gelation, mucoadhesion, and nanocarriers. Clinical studies published to date on in-situ gelation systems (namely Alcon's Systane line of ocular lubricants) demonstrate conflicting results, and it is not possible to deduce any definitive advantage of the in-situ gelling formulation over other ocular lubricants.[6] Mucoadhesive and nanocarrier-containing formulations are a relatively new development, and can only be evaluated after further testing and patient exposure.

To address the shortcomings of ocular lubricant products currently on the market, we propose a novel ocular lubricant eye drop based upon mucoadhesive dextran hydrogel nanoparticles (DH-NPs). By maintaining the low-viscosity eye drop dosage form, this ocular lubricant will offer patients the advantages of easy, convenient administration, familiarity, and inconspicuousness. However, the formulation will also provide effective ocular lubrication for an entire day with a single administration through sustained release of ocular lubricant, a feature traditionally found exclusively in cumbersome and invasive ocular insert products. The

polysaccharide-based composition of the DH-NPs is also designed to reinforce the glycocalyx, potentially offering substantial enhancement in ocular lubrication and combatting the vicious cycle of DED propagation. It should also be noted that the proposed DH-NP ocular lubricant is designed to augment the aqueous portion of the tear film, while novel nanocarrier-based ocular lubricants proposed to date are designed for the lipid tear film layer only. In this manner, the proposed ocular lubricant formulation is expected to offer patients enhanced symptom relief and the convenience of one-time administration, while maintaining the ease-of-use of a low-viscosity eye drop. By providing long-term action and glycocalyx reinforcement, it is also anticipated that this formulation may offer DED patients substantially greater treatment efficacy than other ocular lubricant products currently on the market.

1.2 Research Objectives

The over-arching objective of this research project was to develop a next-generation ocular lubricant for dry eye disease treatment. To work towards this goal, we aimed to synthesize and characterize dextran hydrogel nanoparticles (DH-NPs), and evaluate their performance as vehicles for ocular lubricant delivery. Each of these goals included multiple specific objectives as follows:

1. Synthesize DH-NPs

    a. Demonstrate reliable and repeatable synthesis of DH-NPs. Achieve NP yields as high as possible.

    b. Develop methods for successfully purifying all DH-NP variants, including those with high PBA content.

    c. Develop understanding of process such that synthesis parameters can be deliberately adjusted to achieve desired DH-NP properties.

d. Develop method of producing DH-NPs in solid powder form to enable precise control of concentration in subsequent experiments.

2. Characterize DH-NPs

a. Develop a full suite of methods to characterize DH-NPs, including DexOx oxidation degree, NP diameter, NP yield, crosslinking degree, PBA conjugation, mucoadhesion strength (K_{SV}), and lubricant release rate.

3. Evaluate performance as lubricant delivery vehicle

a. Characterize rate and duration of lubricant release. Explore parameters that may allow tuning of the release kinetics.

b. Verify and optimize mucoadhesive property through in vitro and in vivo mucoadhesion tests.

c. Evaluate efficacy of lubrication through in vivo trials in animal model of DED

1.3 Thesis Outline

This thesis is organized into five sections. After this introduction, Chapter 2 contains a survey of relevant literature, including a detailed overview of dry eye disease and a summary of some novel ocular lubricants used for its treatment. Chapter 3 describes the synthesis and characterization of DH-NPs, including parameters that can be used to tune the final properties of the synthesis process and resulting nanoparticles. Chapter 4 describes the studies conducted on the biocompatibility of DH-NPs, as well as characterization and tuning of their lubricant release capabilities. Chapter 5 summarizes the thesis and provides recommendations for future work.

2.0 Literature Review

2.1 Dry Eye Disease

2.1.1 Definition

Dry eye disease (DED) is known to the medical community and general public by a variety of names, including keratoconjunctivitis sicca, dysfunctional tear syndrome, dry eye syndrome, and dry eye.[10] DED was defined by the authoritative report of the Dry Eye Workshop II as:

> A multifactorial disease of the ocular surface characterized by a loss of homeostasis of the tear film, and accompanied by ocular symptoms, in which tear film instability and hyperosmolarity, ocular surface inflammation and damage, and neurosensory abnormalities play etiological roles.[1]

As indicated by this definition, DED is a complex condition, with multiple factors and processes interacting to contribute to its pathophysiology, epidemiology, diagnosis, and management/therapy.

2.1.2 Symptoms

Symptoms of DED may vary significantly between patients. In the majority of cases, DED is accompanied by multiple of the following sensations in the eye: dryness, gritty or burning feeling, itching, pain, blurry vision, foreign body sensation, excessive tear production, redness, sensitivity to light, and stringy discharge.[10,11,15] Symptoms may often become aggravated towards the end of the day, in low-humidity environments (e.g. indoors due to air conditioning or heating), and due to reading or computer use (as a result of reduced blink rate).[11] In some cases however, individuals may experience no symptoms at all despite clear clinical signs of DED (this is usually

due to neurosensory malfunction, and calls for DED treatment to prevent further damage to the eye).[10,16]

2.1.3 Effect on Quality of Life

DED may have a significant effect on the quality of life of affected individuals, causing substantial impairment to daily functioning, visual acuity, and workplace performance.[10] Utility scores, metrics used to quantify the effect of a health state on quality of life, show that the impact of moderate to severe DED is similar to the impact on patients' lives of experiencing moderate to severe angina (chest pain due to cardiovascular disease) or undergoing dialysis for kidney failure.[17–19] This highlights the seriousness of DED and the importance of effective diagnosis, treatment, and further research to advance our understanding of the condition and reduce its impact on affected persons.

2.1.4 Epidemiology

It is estimated that DED affects hundreds of millions of people worldwide, with individual studies reporting prevalence rates between 5% and 75% in the populations studied.[1,2] The large variability in prevalence estimates reflects the general inconsistency in DED epidemiology data available to date, due in large part to lack of standardization in diagnostic criteria, as well as a shortage of studies for younger demographics (below age 40) and absence of studies in locations outside of Europe, Asia, and the United States.[1,2] Nevertheless, epidemiological studies have been successful in identifying certain risk factors for DED with a high degree of certainty. For example it is known that DED becomes more common with age, and that women become significantly more likely to develop DED than men as they age[1,10,20] (due in large part to reduced levels of androgen production and potential use of hormone replacement therapy[11,20]). Besides demographic risk

factors, certain medical conditions are also known to increase the chance of developing DED, such as meibomian gland dysfunction and Sjögren's syndrome.[1,10,20] The same is true for certain medical interventions (such as estrogen replacement therapy, and use of antidepressants or antihistamines), lifestyle (contact lens wear and computer use), and environmental conditions (such as low humidity and pollution).[1,10,20] Table 1 presents a detailed list of some risk factors identified in literature and the relative degree of certainty with which they are known to be associated with DED.

Table 1:Risk factors for development of DED

		Degree of Certainty		
		High	Moderate	Low
Type of Risk Factor	Demographic	- Age[1,10,20] - Female gender[1,10,20] - Race[1]		- Hispanic ethnicity[1]
	Medical condition	- MGD[1,10,20] - Connective tissue disease[1] - Sjögren syndrome[1,10,20] - Androgen deficiency[1,10,20] - Vitamin A deficiency[10,11,20]	- Diabetes[1,10] - Rosacea[1] - Viral infection[1] - Thyroid disease[1,20] - Psychiatric conditions[1] - Pterygium[1] - Allergic conjunctivitis[1,20]	- Menopause[1] - Pregnancy[1] - Acne[11] - Sarcoidosis[1] - Demodex infestation[1]
	Medical intervention	- Estrogen replacement therapy[1,20] - Topical drugs containing preservatives[1,10,20] - Antihistamines[1,10,20] - Antidepressants[1,20] - Anxiolytics[1] - Isotretinoin[1,10] - Hematopoietic stem cell transplantation[1,20]	- Refractive surgery[1,10,20] - Anti-cholinergic agents[1,10,20] - Diuretics[1] - β-blockers[1]	- Menopause[1] - Botulinum toxin injection[1] - Multivitamin use[1] - Oral contraceptives[1]
	Lifestyle	- Contact lens wear[1,10,20] - Computer use[1,20]	- Low fatty acid intake[1]	- Smoking[1] - Alcohol use[1]
	Environmental condition	- Pollution[1,20] - Low humidity[1,10,20] - Sick building syndrome[1]		

2.1.5 Classification and Etiology

Dry eye disease has traditionally been classified into two essential types, evaporative dry eye (EDE) and aqueous deficient dry eye (ADDE).[1,10,20] EDE was defined to encompass those cases of dry eye that resulted from excessive tear evaporation, while ADDE included cases where the root cause was insufficient volume of aqueous tear production.[21] However, evidence increasingly suggests that the mass majority of dry eye cases are in fact a result of the simultaneous action of both EDE and ADDE mechanisms. As a result, the panel of experts who contributed to the TFOS Dry Eye Workshop II recommend that DED be conceptualized as a spectrum between EDE and ADDE, with factors from both subtypes of dry eye contributing to a given individual's unique presentation.

Clinical evidence shows that in most cases of DED, evaporative dry eye mechanisms have a greater contribution to the patient's overall disease state than ADDE mechanisms.[1,20] EDE may be caused by several underlying factors, including meibomian gland dysfunction (MGD), low blink rate, lid aperture disorders, vitamin A deficiency, and others.[1,11,20] The most common of these is MGD (also known as posterior blepharitis), in which meibum secretions (the mixture of lipids released by the meibomian glands that forms the lipid layer of the tear film) are insufficient in quantity or inadequate in quality.[1] This impairs the effectiveness of the lipid layer in serving as a barrier to evaporation for the underlying aqueous layer, resulting in accelerated evaporation of the tear film and consequently EDE.[10,11]

The insufficient volume of aqueous tear production characteristic of ADDE also has several potential causes, each involving some form of deficiency in the main lacrimal gland.[1,11,20] Despite contribution from the accessory lacrimal glands, conjunctiva, and even corneal epithelium,[22] about

95% of aqueous tear secretion originates from the main lacrimal gland.[11] Several forms of main lacrimal gland malfunction therefore result in reduced aqueous tear secretion and ADDE, including Sjögren's syndrome, age-related lacrimal gland dysfunction, conjunctival scarring (e.g. due to trachoma or mucous membrane pemphigoid), and others.[10,11]

2.1.6 Pathophysiology

Regardless of the relative contributions of EDE and ADDE to a patient's disease state, tear film hyperosmolarity has been found to be a consistent and central driving factor for all cases of dry eye[1,10,20,21]. Healthy persons have an average tear osmolarity of approximately 300 mOsm/L.[23] This value becomes elevated in persons with dry eye, primarily due to evaporation of the aqueous component of the tear film[20,23–25] (some evidence indicates that the main lacrimal gland may also secrete an elevated concentration of electrolytes when its secretion rate becomes low[24,25]). Both EDE and ADDE mechanisms therefore cause increased tear evaporation in some capacity, albeit through different pathways.

Increased tear evaporation is the defining characteristic of all EDE mechanisms, and hyperosmolarity therefore results as a direct consequence of the characteristic deficiencies in meibum that accelerate the rate of aqueous tear film evaporation. In the case of ADDE mechanisms, the primary effect is a reduction in volume of the aqueous tear film, and the development of hyperosmolarity is indirect. One contributing factor is the increase in surface area to volume ratio of the tear film. Since the surface area of the interpalpebral space remains constant while the tear volume decreases, a greater portion of the aqueous tear film evaporates between blinks (since the volumetric evaporation rate remains the same, while the total tear volume is less), resulting in an overall state of hyperosmolarity.[20,23–25] Osmolarity increases further if aqueous tear

volume becomes sufficiently low to cause tear film breakup (i.e. complete evaporation of the tear film in an area of the interpalpebral space, exposing the underlying epithelial cells)[20,23,24], as the extremely low aqueous tear volumes that are produced result in spikes in local osmolarity as high as 1900 mOsm/L in the area of the tear film breakup.[23]

Hyperosmolarity of the tear film has a number of physiological effects, and acts as the backbone of a vicious cycle that propagates DED.[1,11,20] Firstly, hyperosmolarity causes direct ocular surface damage, including loss of corneal and conjunctival epithelial cells, loss of goblet cells, and damage to the glycocalyx (the polysaccharide coating found on the outermost layer of corneal and conjunctival epithelial cells that hydrates, lubricates, and protects the eye[22,26]).[1,20] Hyperosmolarity also triggers inflammation of the ocular surface, activating various signalling cascades to cause the release a number of inflammatory signal carriers and recruit inflammatory immune system cells.[1,20,26] This inflammation also causes extensive damage to the glycocalyx and death of epithelial and goblet cells, reinforcing the direct effects of hyperosmolarity.[1,20,26] This ocular surface damage makes the tear film more prone to evaporation (tear film instability), causing a further increase in hyperosmolarity and inflammation, and creating a self-perpetuating cycle that maintains and aggravates the dry eye state.[1,20,26].

2.1.7 Diagnosis

Dry eye is diagnosed through the combined findings of a variety of qualitative and quantitative tests. No single test has been found to be capable of accurate diagnosis when used alone,[10] but studies have revealed a general order of importance that can be assigned based on the relative usefulness and degree of difficulty in conducting each test. It is usually recommended for the assessment of a potential DED patient to begin with an evaluation of their symptoms, as they

have been found to be the most consistent indicator of dry eye, and provide valuable information regarding DED severity and the presence of other morbidities (i.e. differential diagnosis).[1,10,27] Several standardized questionnaires are available to assist in objective and quantitative symptom evaluation (particularly important for assessing disease progression and for clinical trials), including the Ocular Surface Disease Index (OSDI), Dry Eye Questionnaire-5 (DEQ-5), Impact of Dry Eye on Everyday Life (IDEEL), and others.[10,27] An examination of the clinical signs of DED should always accompany symptom evaluation, in order better elucidate the disease mechanisms leading to DED in the patient (and thereby optimize treatment) and rule out other diseases that mimic the symptoms of DED (such as neuropathic pain).[10,27] The most reliable and commonly used clinical signs include short tear break-up time, elevated or highly variable tear osmolarity, and ocular surface staining (using the dyes fluorescein, rose bengal, and lissamine green).[1,10,11,27] Amongst these tests, osmolarity has been shown to be the most reliable and consistent, although the best numerical cut-offs to identify DED remain under debate. The recent commercialization of a point-of-care nanolitre osmolarity measurement device (TearLab) is increasing the accessibility of the test, although performance is not yet sufficient for it to serve as the standalone DED diagnostic method.[20,27] Further specialized tests can be performed to elucidate the subtype of DED and relative contribution of the various etiological factors to a patient's condition, including meibography, lipid interferometry, and tear volume measurement (Schirmer's test).[27] Although beneficial, it should be noted that all of the above tests are not usually performed in the diagnosis of each DED patient, and the clinician uses his/her professional judgement to determine which investigations are beneficial in confirming DED and identifying the root causes of a patient's condition.[27] However, it is recommended that clinicians identify the presence of DED symptoms and at least once major clinical sign in order to diagnose DED in a patient.[27] Additionally, some

11

patients are known to report DED symptoms while clinical signs are not present, for which clinicians are recommended to provide preventative DED treatment and education.[27]

2.1.8 Treatment

Treatment for DED is highly customized, with clinicians selecting the most suitable treatments for their patients on a case-by-case basis, and through iterative adjustments. Such an approach is necessary due to the complex and highly variable etiology of DED, which has resisted the development of any single regimen or highly structured protocol that is appropriate for all patients. Efficacy of DED treatment therefore relies heavily upon the professional judgement and clinical skills of eye care practitioners, guided by the latest scientific evidence available in the literature. The current body of evidence continues to suggest that a staged approach is most effective in treating DED. The first stage of treatment includes methods that are low-risk, easily accessible, and likely to provide benefit for a wide range of underlying disease etiologies. Each subsequent stage contains treatments that may be effective in treating more severe cases of DED, but carry a greater risk of side effects and/or may be less accessible due to cost or logistical reasons. Treatments in latter stages also tend to be more specialized, targeting a particular disease process that contributes to DED for the patient in question. This highlights the importance of diagnostic tests in subtyping and identifying the root causes of DED for each patient, as many of the latter-stage treatments will only be effective if the specific disease processes causing DED in the patient are known.[4]

The first stage of DED therapy includes ocular lubricants, eyelid therapy, patient education, environmental controls, diet modification, and review of contact lens wear and medications. At this stage, ocular lubricants containing preservatives are usually suitable, and low-viscosity

formulations are most often the products of choice. Suitable eyelid therapies include warm compresses and lid hygiene techniques that the patient can administer at home. Environmental controls include avoidance of low-humidity environments, developing a habit of blinking regularly even during attentive tasks (such as computer use or reading), and avoiding polluted air (including cigarette smoke). Contact lenses and any topical or systemic medications used by the patient should also be reviewed by the clinician, to assess their possible role in contributing to DED, and adjustments and/or alternatives should be identified if contribution to DED is confirmed. The patient's diet should also be reviewed to identify potential modifications that may reduce their DED, including omega fatty acid supplementation.[4,10,11]

The second stage of DED treatment includes more advanced ocular lubricants and eyelid therapy, prescription medications, and tear conservation techniques. Increasing the dose of ocular lubricants up to once hourly is often beneficial, with preservative-free formulations required when the prescribed dose surpasses 3-6 times per day.[7,10–13] Higher viscosity formulations such as gel drops and ointments are also recommended, although they are often only suitable for use at bedtime due to blurring of vision upon instillation. Meibomian gland dysfunction treatments administered professionally may also provide benefit, including unblocking/expression of the meibomian glands by heat and/or mechanical force, and intense pulsed light therapy. Prescription medications that may provide benefit include antibiotics, corticosteroids (a short-term regimen), non-steroidal immunomodulatory drugs such as cyclosporine, and LFA-1 antagonists such as lifitegrast. Recommended tear conservation techniques include removable punctal plugs and moisture chamber goggles.[4,11]

The third stage of treatments recommended for DED therapy includes serum eye drops, oral secretagogues, and therapeutic contact lenses designed to enhance moisture retention on the eye.

The fourth stage of DED therapy includes systemic anti-inflammatory drugs, corticosteroids for longer duration, permanent punctal blocking, and other surgical procedures (including eyelid correction, salivary gland autotransplantation, and mucous/amniotic membrane transplantation).[4,11]

DED therapy is usually continued for the duration of a patient's lifetime, as most treatments currently available provide management for a particular disease process but do not resolve the underlying etiological cause.[4,10] However, it is common for patients' treatment regimens to be adjusted over time, and individuals with good response to treatment may be shifted to earlier stage, lower risk therapies as time progresses.

2.2 Ocular Lubricants

Ocular lubricants (also known as "artificial tears") are a key component of DED therapy for the majority of patients, and are thus often referred to as the mainstay of DED treatment.[28] A variety of dosage forms are currently available on the market, including low viscosity aqueous eye drops, intermediate viscosity gel drops, high viscosity gels and ointments, and ocular inserts. Ocular lubricants are prescribed as the first-line treatment in virtually all cases of DED.[4–8] While they do not address the root causes of the condition, a large body of evidence shows that ocular lubricants are effective in reducing DED symptoms, and present only a very low risk of adverse effects or injury. Numerous studies also support their role in reducing clinical signs of DED, protecting the ocular surface, and restoring visual acuity.

2.2.1 Limitations of Currently Available Formulations

Despite their essential role in effective DED treatment, current ocular lubricant formulations suffer from unpredictability of patient response. There is a very large selection of ocular lubricant

products available for patient use, with significant differences in the combination of lubricating polymers, pH, osmolarity, viscosity, and other ingredients used. However, comprehensive analysis of the literature published to date offers no reliable method of predicting which formulation will offer superior results for a given patient.[6] As a result, patients and their clinicians must undertake a trial-and-error approach to find an effective ocular lubricant product,[12,29] which can be frustrating, time-consuming, and result in unnecessary suffering.

Another major limitation of ocular lubricants currently on the market is the trade-off between efficacy and ease of use. The ocular lubricants that are most convenient to use are low-viscosity, multi-use eye drops containing preservatives. These products are packaged in an eye drop dispenser containing many doses (approximately 200 or more), allowing patients to use a single bottle for an extended period (typically a week or longer). During this time, the preservative included in the formulation functions to prevent bacterial growth in the lubricant solution and thus maintain safety for topical administration. The water-like consistency of these products also prevents any blurring of vision or deposition of residue on the perimeter of the eye. However, low viscosity ocular lubricants are typically only effective for patients with mild to moderate dry eye due to their short duration of action. Blinking and tear production cause most low viscosity lubricants to be completely eliminated from the eye within approximately 20-30 minutes of instillation[14]. The beneficial effects of the lubricants are therefore short-lived, and patients with moderate to severe dry eye may need to apply the drops hourly or even more frequently to achieve satisfactory effects. This is an example of the efficacy-ease of use trade-off; while preserved low viscosity ocular lubricants are the easiest to use, they generally have low efficacy.

Preservative-free ocular lubricant formulations are recommended for patients who use eye drops three to six times a day or more.[7,10–13] Preservative agents are known to be cytotoxic and

exacerbate dry eye at sufficient doses, making it critical for patients' preservative exposure to be maintained below toxic limits.[30] This is particularly important for those with comorbidities that require treatment using additional eye drops (e.g. glaucoma), as these medications usually contain preservatives and add to the patients' overall exposure.[30] Preservative-free formulations therefore provide a significant improvement in DED treatment efficacy for these individuals, as they enable ocular lubricants to be administered at the elevated dose required for symptom relief, without the harmful effects of high preservative exposure. However, the trade-off between ease of use and efficacy comes into effect once again, as sterility and safety of the ocular lubricant solution must be maintained using either single-use vials or advanced bottles with in-built sterility filters (recently made available by limited brands). In addition to the inconvenience of frequent administration, ocular lubricants in single use vials are significantly more expensive for patients, and present the added inconvenience of daily transportation and disposal of many plastic vials. Although new bottle designs containing sterility filters are expected to reduce cost to the patient and improve convenience, additional time and increased market adoption is required to accurately assess their impact on DED treatment.

Intermediate or high viscosity ocular lubricants (namely gel drops, gels, and ointments) are necessary to provide sufficient symptom relief and ocular protection to some DED patients. These products have a significantly longer residence time on the surface of the eye, thereby providing superior tear film stabilization, ocular protection, and symptom relief than their low viscosity counterparts.[4] However, blurring of vision and deposition of unwanted residue on the eye perimeter after instillation are known, unavoidable effects of using these formulations. Intermediate viscosity gel drops are therefore be administered at times where temporary reduction in visual acuity does not present a safety hazard, and the patient must tolerate the cosmetic

drawback of residue deposition on the eye perimeter. Blurring of vision and residue deposition are more pronounced for high viscosity gels and ointments, resulting in clinicians typically recommending their use at bedtime only. These drawbacks in ease of use constitute the trade-off for the greater treatment efficacy of intermediate and high viscosity ocular lubricants on the market today.

Ocular inserts are another form of ocular lubricant used by DED patients. The primary example of such a product is Lacrisert, a small rod-shaped device composed entirely of the lubricating polymer hydroxypropyl cellulose in its dry state (no preservatives, solvents, or other ingredients are added).[31] The device is inserted into the inferior cul-de-sac of the eye, where it softens upon absorbing fluid[32] and slowly dissolves over a period of 4 to 8 hours.[33] This presents the eye with an ongoing supply of hydroxypropyl cellulose during this time, providing long-lasting stabilization of the tear film, protection of the ocular surface, and relief from DED symptoms. Most patients achieve effective ocular lubrication with administration of Lacrisert once daily, with many patients not requiring the simultaneous use of any other ocular lubricants. Statistically significant improvements in symptoms and clinical signs of DED have been observed in the majority of patients with moderate to severe dry eye, a population that is often resistant to ocular lubricant treatment. Ocular inserts are therefore advantageous due to their advanced efficacy in treating dry eye and conveniently low administration frequency. However patients face a trade-off in ease of use once again, as ocular inserts are difficult and uncomfortable to place in the inferior cul-de-sac, many patients experience discomfort due to foreign body sensation during use, and a prescription is required to purchase Lacrisert. A significant minority of patients (approximately 10%) also experience blurring of vision during use. As a result of these trade-offs, ocular inserts

are considered an ancillary treatment for DED[7], and are recommended only in patients who do not gain satisfactory benefit from other types of ocular lubricants.

As shown by this summary of ocular lubricants currently available to DED patients, there are significant limitations that demand further research and the development of superior products. A central concern is the stubborn trade-off between efficacy and ease of use for virtually all of the ocular lubricant formulations available today. The improvement of these technologies is an urgent endeavor, as ocular lubricants continue to play a critical role in the treatment of hundreds of millions of DED patients worldwide. The following section describes recent advancements to this end and details the rationale for the area of particular focus chosen for this thesis.

2.2.2 Novel Formulations

Recent research activity in the field of ocular lubricants can be divided into four major categories: formulations containing hyaluronan, lipid-supplementing formulations, novel methods of prolonging ocular retention time, and formulations that use a combination of these approaches.

2.2.2.1 Hyaluronan-containing Formulations

Hyaluronan (also referred to as hyaluronic acid and sodium hyaluronate in its protonated and unprotonated forms) is a glycosaminoglycan naturally found in significant quantities within the human body. Structurally, hyaluronan is an unbranched macromolecule composed of repeating disaccharide units of N-acetylglucosamine and glucuronic acid, with a large molecular weight.[34] It is found within the aqueous and vitreous humours of the eye, synovial fluid that lubricates and protects joints, and serves many functions within epithelial, nerve, and connective tissues.[4] Hyaluronan is a particularly effective material for ocular lubricant applications due to its shear-thinning and wound-healing properties. Shear-thinning is a non-Newtonian fluid property that

imparts low viscosity at high shear rates (e.g. during blinking) but high viscosity at low shear rates (e.g. between blinks). This allows hyaluronan to effectively spread over the entire eye during blinking but remain in place while the eye is open, increasing its ocular residence time and lubrication efficacy.[35] Hyaluronan has also been shown to have wound-healing properties that promote repair of damaged ocular surface tissues.[4]

An intensive research effort has been undertaken by academia and industry in the past decade to explore the benefits of incorporating hyaluronan into ocular lubricant formulations. An early large-scale clinical study was conducted by Dumbleton et al. to compare the efficacy of a formulation containing 0.25% polyethylene glycol and 0.38% sodium hyaluronate (Blink gel tears) to a comparable formulation that has achieved commercial success (1.0% carboxymethyl cellulose, marketed as Refresh Liquigel).[36] The study was a prospective double-masked randomized trial involving 110 participants, with assessment of symptoms and clinical signs at baseline, 7 days, and 30 days, and additional symptom evaluation at 15 days. Results demonstrated a statistically significant superiority of the PEG-HA formulation over the CMC formulation in the metrics of patient-reported end-of-day comfort (71% vs. 57%, $P = 0.012$) and overall improvement in ocular comfort (62% vs. 45%, $P = 0.015$). However, no difference between treatment groups was observed for clinical signs such as visual acuity, ocular staining, tear quality, and tear quantity. Blink gel tears is now commercially available in the US and other markets.

A variety of other hyaluronan-containing ocular lubricants have been prepared through incorporation of various therapeutic agents and/or chemical modification of hyaluronan. She et al. investigated a novel combination of hyaluronan and carboxymethylcellulose, a formulation that has now been commercialized as Refresh Optive Fusion.[37] Pinto-Bonilla et al. investigated a combination of hyaluronan with trehalose, a material previously shown to protect against

dessication and oxidative damage.[38] This formulation was shown to be superior to Systane in symptom relief in a small-scale randomized crossover trial, and further work has led to its commercialization under the tradename Thealoz Duo. Others have also combined hyaluronan with other agents such as the anti-inflammatory epigallocatechin gallate, a formulation which has demonstrated effective anti-inflammatory properties in preclinical trials.[39] Chemical modifications of hyaluronan have also been found to be advantageous for DED treatment. A randomized double-blind clinical trial in dogs found crosslinked hyaluronan to be superior to native hyaluronan in improving signs of ocular health and in owner satisfaction.[35] Crosslinking of hyaluronan has also been shown to increase ocular retention time, while preservation of wound-healing activity has also been demonstrated.[40]

2.2.2.2 Lipid-supplementing Formulations

Recent years have also seen a marked rise in the development of formulations that augment the tear film's lipid layer. This undertaking is strongly supported by epidemiological evidence, as it is known that in most patients, evaporative disease mechanisms make up a greater portion of the underlying disease processes that cause dry eye.[1,20] Lipid-supplementing formulations have been formulated that contain a variety of lipid agents designed to enhance the tear film's natural lipid layer and reduce the rate of evaporation. In turn, this is expected to shift ocular hydration and osmolarity closer to normal levels, resisting and potentially reversing the vicious cycle of dry eye.

Oil-in-water emulsions make up a large portion of the lipid-supplementing formulations developed to date. One of the earliest such formulations to be developed was a mineral oil macroemulsion, named Soothe (currently marketed as "Soothe XP" or "Soothe Restore" in the US and Canada, respectively). A double-blind study was conducted on 40 subjects with tear film lipid deficiency (lipid layer <75 nm in thickness), with Soothe administered to a randomly selected eye

and Systane (a purely aqueous-supplementing formulation containing no lipids) administered to the contralateral eye as a control.[41] The short-term effect on lipid layer thickness was then determined by measurements at baseline, 1 minute, 5 minutes, and 15 minutes after instillation. Results showed that the average lipid layer thickness (LLT) in eyes treated with Soothe was 124.4 nm (107% increase from baseline of 60.0 nm), while average LLT in eyes treated with Systane was 71.6 nm (16% increase from baseline of 61.5 nm). Augmentation of LLT was therefore significantly more effective with the lipid-supplementing Soothe formulation ($p < 0.0001$), indicating that lipid-supplementing ocular lubricants may be effective in reducing tear evaporation.

Recent work in oil-in-water emulsions for ocular lubricants has focused on smaller droplet sizes, as they offer greater storage stability (less need for shaking prior to administration) and longer ocular retention time. Simmons et al. investigated non-inferiority of Refresh Optive Advanced (a microemulsion formulation containing the lipid-supplementing ingredients polysorbate 80 and castor oil, as well as aqueous-supplementing CMC and glycerin), in comparison to Refresh Optive (containing only CMC and glycerin).[42] The study was performed as a prospective, double-masked, multi-centre trial over a period of 30 days, with four randomized treatment groups consisting of a total of 315 patients. Results showed that the lipid-supplementing Optive Advanced formulations was in fact non-inferior to the traditional Optive formulation, according to the primary outcome of DED symptoms (OSDI score) at day 30. There were also no notable differences in TBUT, ocular surface staining, or Schirmer's test results between treatment groups. This study showed that lipid-supplementing ocular lubricants may have similar efficacy as aqueous-supplementing formulations in treating heterogeneous groups of DED patients. This suggests that it may be possible for a wide variety of DED patients to utilize lipid-supplementing formulations without compromising the efficacy of their ocular lubricant treatment.

Nanoemulsion-based ocular lubricants have also generated considerable interest in academia and industry in recent years. These formulations offer the greatest stability and ocular retention time, and can be crafted to contain a wide variety of lipid-supplementing ingredients. A promising preclinical study was reported by Zhang et al. in which a nanoemulsion formulation was synthesized containing petrolatum, lanolin, and medium-chain triglycerides as lipid-supplementing ingredients.[43] The formulation was found to be stable under long-term storage and non-cytotoxic in in-vitro experiments. In vivo experiments in a mouse dry eye model indicated that the formulation was effective, with statistically significant improvements observed in tear break-up time, corneal staining, and histopathology when compared to the untreated group of mice. In addition, the nanoemulsion formulation showed a statistically significant advantage in extending TBUT and reducing corneal staining over Tears Naturale Forte (an aqueous-only ocular lubricant containing no lipid-supplementing ingredients).

Novel materials are also under development for lipid-supplementing ocular lubricants. One such example is the liposome-based formulation prepared by Vicario-de-la-Torre et al., consisting of phosphatidylcholine, cholesterol, and vitamin E (α-tocopherol).[44] The formulation has performed well in characterizational and pre-clinical safety studies, and is a promising candidate for further development. Another novel lipid-supplementing material is perfluorohexyloctane, a semifluorinated alkane marketed under the brand name NovaTears.[45] This formulation is purely composed of perfluorohexyloctane (contains no water), and is an effective lipid supplement that may be particularly effective in treating patients with evaporative dry eye resulting from meibomian gland dysfunction.[46]

2.2.2.3 Novel Methods of Extending Ocular Retention Time

The trade-off between ease of use and efficacy described in Section 2.2.1 also applies to the retention time of most ocular lubricants currently on the market. Low viscosity formulations are generally the easiest for patients to use, but their efficacy is limited by very short residence times on the ocular surface (usually well below 30 minutes).[14] On the other hand, moderate to high viscosity formulations offer superior efficacy due to extended residence times, but are less user-friendly due to blurring of vision and deposition of unwanted residue on the perimeter of the eye. Recent years have seen a considerable dedication of effort towards developing novel methods of prolonging ocular lubricant retention and overcoming this trade-off.

One of the early methods of increasing ocular retention time was in-situ gelling, first developed and commercialized by Alcon Laboratories Inc. The key component of the in-situ gelling system is hydroxypropyl guar, a high molecular weight branched polysaccharide that transforms from a free-flowing solution to a soft gel upon exposure to the tear film (due to the presence of borate ions and increased pH).[47] It was proposed that an ocular lubricant formulation containing hydroxypropyl guar would therefore be a low-viscosity aqueous eye drop prior to instillation, but transform to form a thin mucin-like gel layer when administered to the ocular surface. The ocular lubricants present in the formulation (polyethylene glycol 400 and/or propylene glycol in the case of Alcon's Systane brand) would then become encapsulated within the gel, providing greater lubrication and duration of action than traditional aqueous drops. Although preclinical studies showed promise,[47] clinical studies published to date demonstrate conflicting results, and it is not possible to deduce any definitive advantage of the Systane in-situ gelling formulation over other ocular lubricants.[6] In addition, one study reported a statistically

significant increase in blurring of vision upon use of Systane versus an ocular lubricant with no in-situ gelling activity.[6]

Another method of extending ocular retention time is the incorporation of poly(L-lysine)-graft-poly(ethylene glycol) (PLL-g-PEG) into a formulation.[48] The PLL block of the copolymer is polycationic, and is therefore able to electrostatically bind to the negatively charged glycocalyx, prolonging residence time on the ocular surface. Others have explored modifications to hyaluronan that impart mucoadhesion, enabling extended ocular retention via anchoring to the epithelium-associated mucins of the ocular surface. Laffleur et al. successfully modified hyaluronan with cysteine ethyl ester, enhancing mucoadhesion 30.5-fold and thereby enabling longer residence times for a material that is known to have pronounced therapeutic properties for DED.[49] Mucoadhesive formulations consisting of mixtures of other polymers often used in ocular lubricants have also been formulated, including viscosity-optimized combinations of hydroxypropyl methylcellulose, hydroxyethylcellulose, guar gum (from which hydroxypropyl guar is derived), and chitosan.[50]

Contact lenses that act as delivery vehicles for ocular lubricants are also a growing area of research for dry eye treatment. Contact lens wear is a well-known risk factor for DED, and it is thus important to develop methods of minimizing/treating dry eye development.[1,10,20] Delivery of ocular lubricants from contact lenses is a potential approach, offering the benefits of sustained lubricant delivery and longer residence times.[51] A variety of loading methods have been utilized to achieve a large range of lubricant delivery times, from a few hours (soaking of contact lens in lubricant) to 2 months (molecular imprinting).[51] A wide variety of materials have also been successfully delivered from the prepared lenses, including hyaluronic acid, phospholipids, hydroxypropyl methylcellulose, polyvinylpyrrolidone, and poly(vinyl alcohol).[51–53]

2.2.2.4 Combination Approaches

Several novel formulations have been developed in recent years that combine multiple of the aforementioned approaches. These are particularly promising ocular lubricants, as they combine the advantages of multiple of the novel approaches described above. A prime example is the Novasorb cationic nanoemulsion platform developed by Novagali Pharma, which forms the basis of the novel ocular lubricant Cationorm (now marketed worldwide, but under the tradename Retaine in the United States).[54–56] Cationorm combines the effective lipid-supplementation and enhanced ocular retention of oil-in-water nanoemulsions with the additional extension of ocular surface residence time provided by the positively charged emulsion droplets' attraction to the negatively charged ocular surface. Another method of extending the duration of action of lipid-supplementing formulations was developed by Acar et al., where an in-situ gelation system based on gellan gum extends the ocular retention time of lipid-supplementing liposomes.[57] In-situ gelation was also utilized by Rangarajan et al. to extend the residence time of hyaluronan, thereby increasing the therapeutic benefits of the material by extending its duration of action.[58] In fact, this formulation is now commercially available as the Systane Ultra Hydration line of products.

3.0 Synthesis of Dextran Hydrogel Nanoparticles

3.1 Summary

Based on the literature review in Chapter 2, it can be seen that the incorporation of nanoparticles is a highly promising strategy for improving the efficacy and ease-of-use of ocular lubricants for DED treatment. This chapter describes the synthesis of dextran hydrogel nanoparticles for this purpose, and details the engineering controls that can be used to modify the key properties of both the nanoparticles and the synthesis process. The surfactant/solvent removal method was identified as a key step in the NP synthesis, and affected various properties including diameter, yield, and phase of final purified product. Three surfactant/solvent removal methods were developed in total, namely separatory funnel washing, calcium chloride precipitation, and solvent precipitation; each of these methods were found to have unique advantages and limitations. A subset of synthesis parameters were identified as having a pronounced impact on the properties of DH-NPs produced. These parameters included DexOx concentration, crosslinker-polymer ratio ($R_{H/A}$), reducing agent treatment, and PBA feed quantity. Manipulation of these parameters allowed for the synthesis of a wide range of DH-NP variants, and can thus be used to tailor the properties of DH-NPs to the needs of a particular application or study.

3.2 Introduction

Dextran hydrogel nanoparticles (DH-NPs) are a novel nanomaterial developed by our research group. We based our work upon investigations of the bulk material by Maia et al.,[59,60] and the nanoparticle formation process described by Bharali et al.[61] DH-NPs are spherical in shape, have a diameter on the order of 100 nm, and are composed primarily of a random network of oxidized dextran chains crosslinked by adipic acid dihydrazide. The surface of the particle is

modified with phenylboronic acid moieties in order to impart mucoadhesion and allow the DH-NPs to adhere to the ocular surface. This occurs through covalent bond formation between PBA moieties on the DH-NPs and sialic acid moieties on mucins anchored to the epitheliums of the cornea and conjunctiva.[62–64] This high-strength association has been shown to enable ocular residence times of over 24 hours in comparable nanoparticles.[65]

Previous studies of bulk hydrogels composed of oxidized dextran (DexOx) and adipic acid dihydrazide (ADH) by Maia et al. explored the effects of dextran oxidation degree, DexOx concentration, crosslinking density, and pH on DexOx-ADH hydrogel swelling, mechanical properties, degradation behaviour, and other properties.[59,60] We translated many of these studies to the nanoscale with a focus on degradation rate, and conducted additional investigations on reducing agent treatment and nanoparticle properties. PBA was also conjugated to the surface of the DexOx-ADH nanoparticle cores, and a series of tests were performed to quantify the PBA content of various formulations and their respective mucoadhesion strengths.

Our research group selected the nanoparticle synthesis process described by Bharali et al.[61] as the basis for developing our DH-NP synthesis techniques. The method described by Bharali is unique due to its capability for producing an water-in-oil nanoemulsion without the use of high-energy processes such as sonication or temperature inversion. This presents a significant process engineering advantage for future scale-up considerations. Over the course of our research we developed two additional nanoparticle purification methods tailored to particular DH-NP variants, each with unique advantages and limitations. We also employed additional methods of quantifying synthesis yield, PBA conjugation, and mucoadhesion strength.

3.3 Materials and Methods

3.3.1 Materials and Instrumentation

Sigma-Aldrich (St. Louis, MO, USA) was used as the primary supplier of chemical reagents, including the following: dextran (from *Leuconostoc* spp., average MW of 6,000), sodium periodate, glycerol, deuterium oxide, ethyl carbazate, adipic acid dihydrazide, dioctyl sodium sulfosuccinate, sodium borohydride, sodium cyanoborohydride, potassium chloride, calcium chloride, 3-aminophenylboronic acid, sodium bicarbonate, hydrochloric acid, and sodium hydroxide. Phosphotungstic acid was purchased from Fisher Scientific (Waltham, MA, USA), while sialic acid (N-acetylneuraminic acid) was purchased from Carbosynth (Compton, Berkshire, UK).

All water used for these studies was ultra-pure and prepared by a Millipore reverse osmosis system, unless otherwise specified.

Dialysis tubing used in these studies were purchased from Fisher Scientific, specifically 3.5 kDa MWCO Fisherbrand™ regenerated cellulose and 100 kDa MWCO Spectrum™ Labs Biotech cellulose ester.

Instrumentation used in these studies includes analytical balances (Shimadzu AUW120D), centrifuges (Thermo Scientific Sorvall RT1 and Thermo Scientific Sorvall Legend Mach 1.6), probe sonicator (Fisher Scientific Branson), UV-visible microplate spectrophotometers (BioTek Epoch and Tecan Infinite® 200 PRO M Plex), freeze drier (Labconco), fluorimeters (Tecan Infinite® 200 PRO M Plex and Photon Technology International type LS-100), and NMR spectroscopes (Bruker 300 MHz and 500 MHz).

3.3.2 Oxidized Dextran (DexOx) Preparation

Dextran was oxidized by overnight reaction with sodium periodate, with a targeted oxidation degree of 100% (i.e. two aldehyde groups per glucose monomer). An aqueous solution of 6 kDa dextran (50 mg/mL, 6 g dextran in total) was allowed to react with sodium periodate (1+ times molar excess, 9.1 g total) at room temperature and under protection from ambient light. The reaction was stopped by glycerol addition the following morning. The products were dialyzed for approximately 3 days against deionized water with frequent medium changes (eight in total), using 3,500 MWCO dialysis membranes (regenerated cellulose, Fisherbrand[TM]). The purified product was then concentrated by air-drying and lyophilized before storage.

3.3.3 Oxidized Dextran (DexOx) Characterization

DexOx oxidation degree was characterized primarily by NMR, using the method described by Maia et al.[60] Briefly, samples of DexOx were dissolved in deuterium oxide (heavy water) at a concentration and volume suitable for the NMR instrument being utilized (30 mg/mL, 1.5 mL). A molar excess of ethyl carbazate was then added, and the reaction was allowed to continue for exactly 24 hours. The NMR spectrum was then acquired (1d proton, 300 MHz, 32 scans, with water suppression) and the ratio of the integrated 7.3 ppm carbazone peak to the 4.9 ppm anomeric proton peak was calculated. This ratio represents the average number of aldehyde groups per glucose monomer, and was divided by two to determine the measured degree of oxidation as a percentage (where 100% indicates the presence of two aldehyde groups per glucose monomer). It is critical to note that the degrees of oxidation reported in our study are equivalent to twice that reported by Maia et al. This is because Maia et al. considered 100% oxidation (i.e. maximum degree of oxidation) to be the presence of one aldehyde per DexOx monomer, while we defined complete oxidation to be the presence of two aldehyde groups per DexOx monomer.

Sodium borohydride titration was also used to quantify DexOx oxidation degree, as a method of validating the above ethyl carbazate NMR method. The method of Liu et al.[66] was followed, in which a sample of DexOx was added to a molar excess of $NaBH_4$. The reaction flask was sealed and connected only to a burette filled with water, designed to measure volume of evolved hydrogen gas. The volume of hydrogen produced was recorded at the end of the 2 hour reaction period and again after addition of excess acetic acid to consume the unreacted $NaBH_4$. The sum of the two hydrogen volumes revealed the quantity of $NaBH_4$ that did not react with DexOx. This experiment was then repeated using a pure water solution of identical volume (no DexOx added). The volume of hydrogen gas produced in the DexOx run was subtracted from the volume produced in the blank run to calculate the quantity of $NaBH_4$ that reacted with aldehyde groups in the DexOx sample. Ideal gas law was used to convert volume to moles of hydrogen consumed in the reaction with DexOx and thereby determine the oxidation degree of the DexOx being analyzed.

3.3.4 Nanoparticle Formation

DexOx polymer was crosslinked with adipic acid dihydrazide (ADH) within nanoscale emulsion droplets to create the hydrogel core of DH-NPs. This reaction was conducted within 20 mL septum-capped vials, within a 37°C water bath and under magnetic stirring. A 60 mM solution of dioctyl sodium sulfosuccinate (also referred to as AOT) in n-hexanes was first added to the reaction vial, such that the volume ratio of hexane to water would be 20:1 after completion of DexOx and ADH addition. The desired concentration and volume of DexOx was then added to the vial in a dropwise manner, after which the sample was allowed to re-equilibrate to 37°C. The desired volume of 77 mg/mL ADH was then added to the vial dropwise over a 5-minute interval with the aid of a syringe pump. This marked the initiation of the crosslinking reaction that produced

the hydrogel core of DH-NPs. Sample vials were capped at all times besides during reagent addition in order to minimize hexane evaporation.

Phenylboronic acid (dissolved in DMSO, 400-700 mg/mL concentration) was added at this stage in some experiments. PBA addition was done dropwise by micropipette, after a defined period of uninterrupted hydrogel core crosslinking. High PBA stock solution concentrations were used to minimize DMSO volume added, due to its destabilizing effect upon the nanoemulsion. In other experiments, PBA coating of DH-NP hydrogel cores was carried out in aqueous solution, after completion of core crosslinking and purification from the AOT surfactant and hexane (see 3.3.5). Samples containing PBA were protected from ambient light.

Crosslinking of the hydrogel core was allowed to continue overnight in some experiments. In other studies, crosslinking was terminated after a predetermined time interval by the addition of sodium borohydride (40 mg/mL aqueous solution, prepared immediately before use and stored in ice during waiting periods between samples). Sodium borohydride reduces aldehyde groups in DexOx to form hydroxyls, preventing further hydrazone bond formation with ADH. It also reduces hydrazone crosslinks that have already formed into more stable secondary amines that are less prone to hydrolysis.[67] Sodium cyanoborohydride was also added (directly, in powder form) after a predefined crosslinking time in some studies. This did not cause crosslinking to halt, as sodium cyanoborohydride does not reduce aldehyde groups (it is used solely to reduce crosslinks from hydrazones into their more stable secondary amine form).[67]

3.3.5 Nanoparticle Purification

A number of different methods were utilized to remove surfactant, organic solvents, salts, leftover reagents, and aggregates from newly formed DH-NP samples. The first step in this process

31

was AOT and hexane removal, followed by PBA coating and/or reducing agent treatment (if applicable to the experiment in question), removal of salts and leftover reagents, and finally, aggregate separation.

The method of AOT and hexane removal used most often was separatory funnel washing, as it was found to provide the greatest nanoparticle yield. This method was newly developed for DH-NP synthesis, and is based upon the principle reported by Mazi et al. for protein purification.[68] In essence, the technique involves formation and tuning of a water-in-oil emulsion that encapsulates impurities while causing the desired product (which is larger than the emulsion droplet size) to phase separate into a purified aqueous phase.

In practice, a separatory funnel was filled with fresh n-hexanes such that the volume ratio of hexane to NP sample (unmodified after the NP formation step) would be approximately 3:1. Note that the NP sample was already in water-in-oil emulsion form within the reaction vial, containing a 20:1 ratio of hexane to water. The entire NP sample was then transferred from the reaction vial to the separatory funnel, with 5 mL of water added to each 20 mL vial to achieve a complete transfer. The separatory funnel was then agitated thoroughly to mix the contents, forming an opaque water-in-oil emulsion with large droplet size. Sufficient potassium chloride was then added to achieve near-saturation (approximately 2 M KCl concentration within the aqueous phases). The separatory funnel was thoroughly agitated once again, and a dramatic reduction in emulsion droplet size was realized, marked by a gradual transition to complete transparency. The funnel was allowed to sit undisturbed for 30 minutes to complete this process, during which time an aqueous phase containing DH-NPs also formed at the bottom of the funnel (while AOT and hexane remained in the upper organic phase). The aqueous phase was then collected and transferred to the next stage of purification.

Calcium chloride treatment is a second technique that was used to remove AOT from DH-NP samples, particularly in cases where separatory funnel washing was not fully effective at eliminating all the AOT present. Calcium ions react quantitatively with the anionic surfactant to form the insoluble AOT calcium salt, allowing it to be effectively separated from the DH-NPs in the sample.[61] In practice, aqueous solutions of DH-NPs were mixed with calcium chloride solution in molar excess (relative to the quantity of AOT in the sample), followed by thorough agitation. This caused AOT precipitates to form, which were separated from the DH-NPs by centrifugation. The supernatant (containing the DH-NPs) was then extracted and dialyzed against deionized water to remove residual $CaCl_2$ and other impurities. In our studies, AOT removal by calcium chloride treatment was used primarily as an add-on purification method in cases where separatory funnel washing alone failed to remove all the AOT in a sample.

Solvent precipitation is third method of AOT and hexane removal utilized in our studies. In this technique, DH-NPs were transferred in nanoemulsion form (directly after nanoparticle formation) into 50 mL centrifuge tubes. The tubes were topped up with cold isopropanol (chilled with ice), thoroughly agitated using a vortex mixer, and centrifuged at 680 g for 5 minutes. The supernatant was discarded, and the precipitate was washed two more times in this manner, using chilled isopropanol. This was followed by two washes with hexane, and finally two additional washes using cold isopropanol. After the resulting pellet was dried overnight in a vacuum desiccator overnight, it was resuspended in a minimum quantity of water. The sample was then subjected to ultrasonication for 10 minutes to break up any reversible aggregation. The DH-NPs were then transferred to the next stage of purification.

At this stage, some studies called for DH-NPs to be coated with PBA and/or treated with reducing agent (other studies involved performing these modifications during the NP formation

33

process described in 3.3.4, or required the production of "blank" DH-NPs that were uncoated with PBA and/or untreated by NaBH$_4$ or NaBH$_3$CN). To coat purified DH-NP hydrogel cores with PBA and/or treat with NaBH$_4$/NaBH$_3$CN, samples were first transferred to phosphate buffer (0.15 M NaCl, 0.1 M NaH$_2$PO$_4$, pH adjusted to 7.2) by solvent exchange via overnight dialysis. Samples were then extracted into 20 mL vials and placed under magnetic stirring at room temperature with protection from ambient light. If PBA coating was desired, 3-aminophenylboronic acid (400-700 mg/mL, in DMSO) was added to the vial, and the reaction was allowed to proceed overnight. To treat samples with reducing agent, either sodium borohydride (40 mg/mL dissolved in water) or sodium cyanoborohydride (1 M dissolved in 1 M NaOH) was added to the reaction vial. Either agent was added in molar excess, calculated against the theoretical quantity of DexOx in the sample and assuming each mole of DexOx monomer will react with two moles of reducing agent. Pressure was released periodically from the reaction vials in the early stages of the reaction by venting the hydrogen gas buildup into a fume hood (particularly important for reduction by NaBH$_4$).

The next step in DH-NP purification was removal of salts, unused reagents, and trace water-miscible solvents such as DMSO. This was done by dialysis against deionized water using 100 kDa MWCO membranes for approximately 1 day (controlled tests are recommended to elucidate the detailed dialysis parameters appropriate given the experimenter's specific laboratory equipment and dialysis conditions).

The final step in the DH-NP purification process was the separation of aggregates. Samples were removed from dialysis, transferred into centrifuge tubes, and then centrifuged at 2800 g for 10 minutes. The supernatant was extracted, while the mass of the pellet was weighed to estimate yield loss to aggregation. The supernatant was further filtered by 450 nm syringe filtration (Pall

Acrodisc® with Supor® membrane), yielding the final purified DH-NP sample. The DH-NPs samples were then ready for use in characterization and testing. In some studies, the nanoparticles were precipitated using cold isopropanol to isolate in solid powder form and enable resuspension at any desired concentration.

3.3.6 Mucoadhesive Coating and Reducing Agent Treatment

A portion of our studies involved the synthesis of DH-NPs that were coated with PBA to impart mucoadhesion and/or treated with reducing agent. "Blank" DH-NPs were also synthesized that were exposed to neither of these processes. It should be clarified that both PBA coating and reducing agent treatment could be performed at two different stages of the DH-NP synthesis process. PBA coating and/or reducing agent treatment was not performed twice for any sample; rather one of the two process windows was selected to carry out the necessary reactions.

The first window during which DH-NPs could be coated with PBA and/or treated with reducing agent was within the nanoparticle formation process, as described in Section 3.3.4. PBA and/or reducing agents were added after a defined waiting period to allow for the DexOx-ADH crosslinking reaction to progress. The second stage at which DH-NP samples could be coated with PBA and/or subjected to reducing agent treatment was towards the end of the nanoparticle purification process, as described in Section 3.3.5. The PBA coating and/or reduction reactions were performed after removal of surfactant and organic solvents, and usually prior to aggregate removal.

3.3.7 Nanoparticle Diameter Characterization

The hydrodynamic diameter of DH-NPs was measured by dynamic light scattering, using a Brookhaven 90Plus particle sizer (λ = 659 nm, 90° incidence). The effective diameter reading is reported for all studies herein.

Transmission electron microscopy was also used to verify the morphology of DH-NPs. Samples were prepared on carbon-stabilized Formvar grids (copper, Ted Pella) and stained briefly (15 seconds) with 20 mg/mL phosphotungstic acid to enhance contrast.

3.3.8 Synthesis Yield Quantification

Nanoparticle synthesis yield was defined as the proportion of raw material mass converted to final purified DH-NPs, and calculated using the following formula:

$$NP\ Yield\ (\%) = \frac{Total\ NP\ Mass}{DexOx\ Mass + ADH\ Mass + PBA\ Mass} \times 100$$

The total NP mass was determined directly by analytical balance measurement for studies involving solvent precipitation of the final purified NPs (as the DH-NPs were isolated in solid form). For studies in which DH-NPs were maintained in aqueous suspension, aliquots of known volume were lyophilized in a vial of known mass to determine the concentration of DH-NPs in the suspension. This concentration was then multiplied by the total volume of NP suspension produced by the synthesis process to determine total NP mass.

3.3.9 PBA Conjugation Quantification

The quantity of PBA successfully conjugated to a sample of DH-NPs was estimated by a fluorescence-gravimetric method. Since phenylboronic acid is a fluorescent molecule, the fluorescence intensity of pure DH-NP samples (i.e. no other fluorophores present) of known

concentration could be used to determine the molar concentration of PBA in the sample. Fluorescence measurements were obtained using either a Photon Technology International LS-100 or a Tecan Infinite® 200 PRO M Plex. Standards of known PBA concentration were used to periodically prepare linear calibration functions for each instrument used. 294 nm was used as the excitation wavelength for all measurements, while the emission wavelength was approximately 375 nm (with up to 5 nm shifts in peak wavelength for some samples).

The PBA concentration measured by fluorescence was then divided by the concentration of DH-NPs in the sample to determine PBA conjugation in units of weight/weight (i.e. wt. %). The molar conjugation of PBA (relative to the moles of DexOx) could not be accurately determined because the exact concentration of DexOx in samples was unknown (it could only be roughly estimated from the ratio of initial feeds of DexOx and ADH).

3.3.10   Mucoadhesion (K_{SV}) Quantification

Mucoadhesive strength was quantified in vitro by measuring the Stern-Volmer binding constant between DH-NPs and sialic acid. The fluorescence method previously reported by our group was used.[65] Briefly, aliquots of a given DH-NP sample were added to aqueous solutions of sialic acid such that the DH-NP concentration was constant between mixtures, but the sialic acid concentration varied. After brief agitation, the fluorescence of each mixture was measured on either a Photon Technology International LS-100 or a Tecan Infinite® 200 PRO M Plex fluorometer. The fluorescence intensities of each sialic acid-DH-NP mixture (denoted as I) was divided by the fluorescence intensity of a blank sample (denoted as I_0 and containing an equal concentration of DH-NPs but no sialic acid). The resulting I/I_0 value was plotted against [SA] (sialic acid concentration in moles per litre) and linear regression was used to find the line of best

fit (with the y-intercept defined as 1). The slope of this line was determined to be the Stern-Volmer binding constant (K_{SV}), as per the following equation:

$$\frac{I}{I_0} = 1 + K_{SV} * [SA]$$

The K_{SV} is thought to be analogous to the association constant K_A for this application, and thus represents a widely utilized measurement of binding strength. A greater value of K_{SV} therefore indicates stronger binding between a DH-NP sample and sialic acid, and therefore predicts stronger adhesion of the DH-NPs to the eyes of patients.

3.4 Results and Discussion

3.4.1 Optimization of Oxidation Degree Characterization

The degree of oxidation of the DexOx polymer used in the DH-NP synthesis process is a key factor that influences the properties of the nanoparticles produced. A higher oxidation degree indicates a greater average number of aldehyde groups per monomer, with an oxidation of 100% being equivalent to two aldehyde groups per DexOx residue (specifically at the 2-carbon and 4-carbon of the former glucose monomers).[69] In turn, a greater quantity of aldehydes in DexOx provides more sites of attack for both ADH and PBA, enabling a higher density of crosslinking and/or a more extensive mucoadhesive functionalization. Accurately characterizing and controlling DexOx oxidation degree was therefore a critical step in synthesizing DH-NPs with the desired properties.

To achieve a reliable method of DexOx synthesis and characterization, we based our work upon the detailed studies conducted by Maia et al.[59,60,69] To enable dense crosslinking and mucoadhesive functionalization, we increased the targeted DexOx oxidation degree from 40% (the highest value in the Maia works), and typical values were found to be approximately 70%. The

final method used to achieve this oxidation is described in Section 3.3.2. We selected NMR titration as the primary method of oxidation characterization, but also validated the method using NaBH₄ titration (as outlined in Section 3.3.3). It was also necessary to introduce a slight refinement to the method to optimize it for DexOx samples with higher degrees of oxidation.

Figure 1 below shows a representative NMR spectrum obtained from the ethyl carbazate titration method. The peak at 4.9 ppm corresponds to the anomeric proton (the hydrogen atom bonded to carbon-1 of each monomer unit), while the peak at 7.3 ppm corresponds to the carbazone formed from reaction of an aldehyde with ethyl carbazate (specifically the hydrogen bonded to the carbon form the aldehyde group).[59] The ratio of the 7.3 ppm peak to the 4.9 ppm peak therefore indicates the average number of aldehyde groups per DexOx monomer, and was calculated to be 1.32 in Figure 1. This corresponds to a 66% degree of oxidation (as complete oxidation would result in two aldehyde groups per glucose monomer).
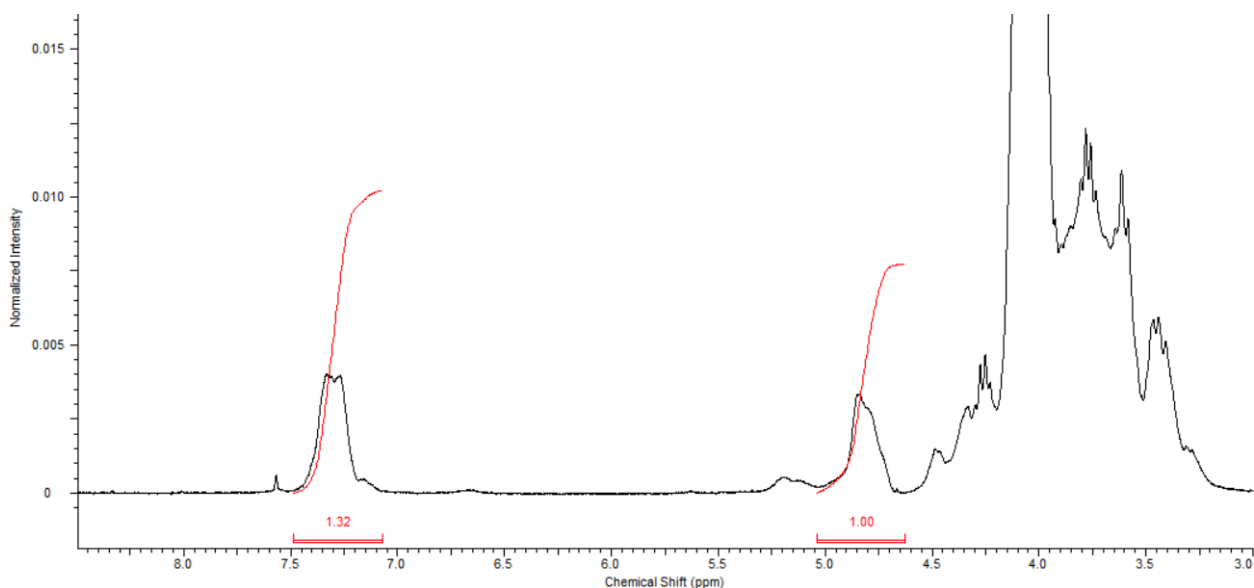
Figure 1: Representative NMR spectrum of DexOx titrated with ethyl carbazate

To validate accuracy, the same batch of DexOx featured in Figure 1 was also analyzed with the NaBH₄ titration method described in Section 3.3.3. The volumes of hydrogen gas measured in

the test indicated an oxidation degree of 70%. This value is in close agreement with the oxidation degree of 66% measured using the ethyl carbazate NMR titration method above. Therefore, it was determined that both methods provide an accurate method of determining DexOx oxidation degree. Since the ethyl carbazate method is more convenient to conduct in the laboratory for multiple samples, it was selected as the primary method of DexOx oxidation characterization for our studies.

An observation of unexpected precipitation led to refinement of the reaction time prescribed for the ethyl carbazate method. Samples of DexOx added to ethyl carbazate were observed to become cloudy and form precipitates after 2-3 days. It thus became evident that the optimum reaction time prior to NMR measurement should be investigated. To this end, a sample of DexOx was titrated with ethyl carbazate as per the method described in Section 3.3.3. NMR measurements were taken after 6 hours, 24 hours, and 72 hours of adding ethyl carbazate to the DexOx sample. The NMR spectra acquired in the experiment indicated an oxidation degree of 53% at 6 hours, 85% at 24 hours, and 51% at 72 hours. A follow-up study found little change in measured oxidation degree within the reaction time window of 18 hours to 45 hours. These results showed that the optimum reaction time for ethyl carbazate reaction with DexOx was 24 hours. Short times such as 6 hours gave oxidation degree measurements that were too low due to incomplete DexOx-ethyl carbazate reaction. On the other hand, long reaction times in excess of 2 days caused measured oxidation degree to decrease once again, likely due to precipitation of DexOx-ethyl carbazate conjugates. A reaction time of 24 hours was optimal because it was a relatively short reaction time that allowed the DexOx-ethyl carbazate reaction to near completion while avoiding precipitation.

Further investigation is warranted to determine the cause of precipitate formation following long reaction times and assess its effect upon the accuracy of oxidation degree determination. A

similar observation of precipitation was reported by Maia et al. for the titration of higher oxidation degree samples with tert-butyl carbazate, and was remedied by the use of the less hydrophobic ethyl carbazate titrant.[60] Since the DexOx samples in our study were oxidized to a significantly greater degree than all samples in the Maia et al. studies, the same mechanism of precipitation may be at play for the DexOx-ethyl carbazate conjugates produced in our studies. It is therefore recommended that methyl carbazate be explored as a superior titrant for further improvement in accuracy of DexOx oxidation degree determination due to its smaller alkyl group and consequent lower hydrophobicity.

3.4.2 Selection of Optimum Purification Methods

DH-NP synthesis involved several purification steps after completion of the nanoparticle formation stage outlined in Section 3.3.4. These purification steps included removal of surfactant and organic solvent, elimination of leftover reagents and salts, and aggregate separation. While a single method was used for most of these process steps, three different techniques were utilized for the removal of surfactant and organic solvent. Over the course of our work, we identified unique advantages and disadvantages for each of these surfactant/solvent removal methods and found that they were each best suited for different studies and DH-NP subtypes. This section outlines the strengths and limitations identified for each method, which may serve as the rationale for other researchers/process engineers to select the most appropriate method for their needs.

The surfactant/solvent purification method utilized most often in our work was separatory funnel washing, as described in Section 3.3.5. This was our preferred method for most studies due to the high nanoparticle yields achievable and a greater efficiency in synthesizing large NP batches.

Table 2 shows the properties of typical nanoparticle batches purified using this surfactant/solvent

separation method.

Table 2: Properties of standard nanoparticles synthesized using the separatory funnel washing method of surfactant and organic solvent removal. The key parameters used in the synthesis of the DH-NPs described in the table are: 200 mg/mL DexOx concentration, 25% crosslinking, no reducing agent treatment, no PBA coating.

Property	Average ± s.e.	n
NP Diameter (nm)	119 ± 12.7	5
NP Yield	23% ± 4.2%	4
Yield Loss to MPs	16% ± 4.4%	5

Despite its utility, the separatory funnel method of surfactant/solvent removal also had

several limitations. A major shortcoming was the inability to purify some DH-NP samples,

particularly those with high levels of PBA conjugation. High levels of AOT (surfactant) were

found to remain in some samples even after the separatory funnel wash, while other samples were

completely lost due to the formation of persistent emulsions within the separatory funnel (Figure

2). The formation of these emulsions may be due to hydrophobic interactions between PBA and

the alkyl chains of AOT. Samples with higher levels of PBA conjugation were observed to form

larger (more stable/persistent) emulsions. This positive correlation between PBA content of the

DH-NPs and emulsion formation during the separatory funnel wash lends credibility to a possible

interaction between PBA and the AOT surfactant.
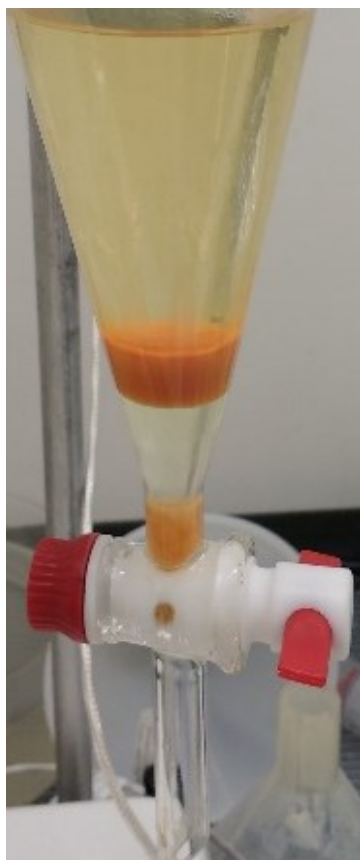
Figure 2: Formation of a third emulsion phase (opaque orange) between the upper organic phase and the lower aqueous phase. Such emulsions were found to be persistent and trapped the DH-NPs within them, causing the loss of almost all the nanoparticle yield in the synthesis batch.

Another major limitation of separatory funnel washing was that it could only produce final purified nanoparticles in the solution state. This presented a challenge for subsequent DH-NP performance testing because nanoparticle concentration could not be precisely controlled by the experimenter. This was due to the minimum quantity of water required to make the separatory process successful (therefore imposing an upper limit on DH-NP concentration), and also due to downstream purification steps such as dialysis that introduced variability in concentration. Because of the variability in DH-NP concentration in final nanoparticle samples, a dedicated synthesis yield quantification step was necessary that sacrificed a significant quantity of the sample and greatly reduced the number of tests that could be performed using each DH-NP synthesis batch. The solution state of the nanoparticles also allowed intrinsic degradation (hydrolysis)

processes to occur during storage, limiting the shelf life of synthesized nanoparticles. In addition, the washing process was relatively time-consuming for synthesis experiments containing many unique samples, limiting the number of unique samples that could be prepared at a time.

Precipitation by calcium chloride was the second method used in our studies for removing surfactant (AOT) from DH-NPs samples. As described in Section 3.3.5, CaC_2 precipitation was carried out after samples had undergone separatory funnel washing but still contained significant quantities of AOT. The key strength of the $CaCl_2$ precipitation method was effective removal of AOT for samples that could not be purified using separatory funnel washing alone (Table 3). The method was confirmed to be capable of successfully purifying DH-NPs with a wide range of PBA coating levels, as shown in Table 4. However, DH-NP purification by $CaCl_2$ precipitation also faced several limitations. Because samples were washed using the separatory funnel method prior to $CaCl_2$ treatment, this was a time-consuming process that further limited the number of unique samples that could be prepared in a given synthesis experiment. Since the final purified DH-NP sample was produced in the solution state, the method also shared the drawbacks of shorter shelf life, lack of nanoparticle concentration control, and loss of usable DH-NPs to synthesis yield determination with the separatory funnel washing method. Preliminary trials were conducted to assess the feasibility of eliminating the separatory funnel washing step by using $CaCl_2$ precipitation to remove AOT and simple evaporation to remove organic solvent (as hexane is highly volatile). However, these trials resulted in impractically low nanoparticle yields, potentially due to excessive aggregation. It is recommended that future studies be conducted to investigate whether the addition of an additional aliquot of water prior to hexane evaporation limits aggregation to an acceptable level.

Table 3: Yield data for two DH-NP samples ("34.5" and "34.6") after separatory funnel washing only ("untreated") and after both separatory funnel washing and CaCl₂ precipitation ("CaCl₂ treated"). The measured NP yield is observed to decrease dramatically from an uncharacteristically high value due to elimination of residual AOT that artificially increased the mass measurement. Note that both samples were synthesized with 7% theoretical PBA conjugation.

	Sample			
	34.5, untreated	**34.6, untreated**	**34.5, CaCl₂ treated**	**34.6, CaCl₂ treated**
NP Yield	86%	83%	28%	20%
Yield Loss to Aggregates	19%	21%	19%	21%
Total Yield	104%	103%	47%	41%

Table 4: Yield data for DH-NPs with various PBA conjugation levels, purified using the CaCl₂ precipitation method (after first completing separatory funnel wash)

Theoretical PBA Conjugation	**NP Yield (average ± s.e.)**	**n**
0%	69%	1
7%	24% ± 1.2%	2
11%	26% ± 7.1%	2
15%	31% ± 1.6%	2

The third method of solvent/surfactant removal utilized in our studies was solvent precipitation (as described in Section 3.3.5). This method was developed due to its unique advantage of producing purified nanoparticles in the solid state. This enabled precise control of nanoparticle concentration during subsequent DH-NP testing and characterization, provided longer shelf life by halting hydrolysis, and eliminated the need to sacrifice a large portion of the NP sample for synthesis yield quantification. The method was also capable of successfully purifying DH-NP samples with extensive PBA coatings that could not be purified using separatory funnel washing alone. Solvent precipitation also offered the opportunity to synthesize a greater number of unique samples in a single synthesis experiment, as it was possible to work with many

more centrifuge tubes simultaneously than separatory funnels. Table 5 shows the typical properties of DH-NPs purified using the solvent precipitation method.

Table 5: Properties of standard nanoparticles synthesized using the solvent precipitation method of surfactant and organic solvent removal. The key parameters used in the synthesis of the DH-NPs described in the table are: 200 mg/mL DexOx concentration, 50% crosslinking, treatment by either $NaBH_4$ or $NaBH_3CN$, no PBA coating.

Property	Average ± s.e.	n
NP Diameter (nm)	136 ± 10.4	4
NP Yield	15% ± 1.0%	4
Yield Loss to MPs	29% ± 0.6%	4

The solvent precipitation method of surfactant/solvent removal was also found to have certain limitations that made it ill-suited for purifying some DH-NP variants. In particular, low-density DH-NPs were found to be highly susceptible to irreversible aggregation when subjected to solvent precipitation. This is shown in Table 6 for DH-NPs synthesized using two different DexOx concentrations (thus resulting in two different crosslinked hydrogel densities) purified using either separatory funnel washing (F) or solvent precipitation (P). The use of solvent precipitation instead of separatory funnel washing resulted in lower synthesis yield and greater aggregation (shown by larger average NP diameter and greater yield loss in the form of aggregates) for both low-density (50 mg/mL DexOx) and high density (200 mg/mL DexOx) DH-NPs. However, aggregation and synthesis yield reduction were more pronounced for lower density DH-NPs, with average synthesis yields becoming impractically low. This challenge of aggregation can likely be explained by the similarity between solvent precipitation and drying processes such as lyophilization and spray drying. The challenges of safely drying polymeric nanoparticles without causing irreversible aggregation are well-known, and various methods have been developed in recent years to combat this phenomenon.[70,71] Specifically, the risk of irreversible aggregation has been shown to be higher for softer nanoparticles,[71] and we believe this lesser mechanical resilience is what made the low-

density DH-NPs highly susceptible to irreversible aggregation in our studies. It was therefore concluded that solvent precipitation is not a feasible method of removing solvent and surfactant from low-density DH-NPs.

Table 6: Key metrics for the synthesis of DH-NPs using DexOx concentrations of either 50 mg/mL (low density) or 200 mg/mL, and either separatory funnel washing (F) or solvent precipitation (P) as the method of surfactant/solvent purification. NP yield was found to be lower due to aggregation for all samples purified by solvent precipitation, particularly for low-density DH-NPs.

| DexOx Concentration (mg/mL) | Purification Method | Synthesis Property (average ± s.e.) | | | n |
		NP Diameter (nm)	NP Yield (%)	Yield Loss to Aggregates (%)	
50	F	111 ± 14.0	40 ± 6.9	8.9 ± 2.7	4
50	P	161 ± 13.6	2.1 ± 1.0	38 ± 2.5	4
200	F	112 ± 12.2	29 ± 6.8	20 ± 3.1	5
200	P	153 ± 12.4	12 ± 1.4	38 ± 3.3	10

It should also be noted that the solvent precipitation method generally carries the disadvantage of lower nanoparticle yield for higher-density DH-NPs as well, as shown in Table 6. The exception to this rule was found to be in the synthesis of DH-NPs treated with reducing agent, for which solvent precipitation and separatory funnel washing were found to have approximately equal performance (Table 7). The large number of solvent washes is another limitation of this method, as it decreases efficiency and results in the use of a large volume of organic solvent. In fact, solvent precipitation produces approximately six times more organic solvent waste than separatory funnel washing. The number of samples that can be processed simultaneously is also limited by the availability of centrifugation equipment at the synthesis facility. These are important considerations and potential areas for improvement in future scale-up considerations.

Table 7: Key synthesis metrics for DH-NPs treated with reducing agent and subjected to either separatory funnel washing or solvent precipitation for surfactant/solvent removal.

Purification Method	Reducing Agent Treatment	Average ± s.e.			n
		NP Diameter (nm)	NP Yield (%)	Yield Loss to Aggregates (%)	
F	Yes	129 ± 28.9	16 ± 4.6	21 ± 3.3	2
P	Yes	136 ± 10.4	15 ± 1.0	29 ± 0.6	4

3.4.3 Effect of DexOx Concentration on DH-NP Properties

A series of studies was conducted to examine the effect of various synthesis parameters on the properties of DH-NPs produced. The first of theses studies tested the impact of the concentration of DexOx used to produce hydrogel cores in the nanoparticle formation stage of synthesis. It was expected that increasing DexOx concentration would increase the polymer network density of the DH-NP core and potentially impact nanoparticle diameter (due to the more extensive crosslinking reaction) and nanoparticle yield. The study was conducted by changing DexOx concentration to either 50 mg/mL or 200 mg/mL while keeping all other parameters constant (hydrazide to aldehyde ratio of 0.5 ($R_{H/A}$), no reducing agent treatment, no PBA coating, surfactant/solvent removal method).

Figure 3 shows the effect of DexOx concentration on nanoparticle yield and diameter for DH-NPs purified using separatory funnel washing. Figure 4 investigates the same effects but focuses on DH-NPs purified using solvent precipitation instead. Both figures show that DexOx concentration does not have a major impact on nanoparticle diameter. However, higher DexOx concentration was found to cause a significant decrease in nanoparticle yield for DH-NPs purified using separatory funnel washing (Figure 3). This large drop in yield (from 49% to 29%) may have been caused by lower stability of the DexOx nanoemulsion during nanoparticle formation. The

AOT/hexane surfactant/solvent system is known to form a lower stability water-in-oil emulsion with 200 mg/mL DexOx than 50 mg/mL DexOx, as shown by the substantial increase in emulsion light scattering (white translucent colour versus complete colourless transparency of the 50 mg/mL DexOx emulsion). Increased emulsion translucency indicates larger droplet size,[72] which may in turn lead to faster particle growth upon intrinsic micelle coalescence processes[73] and increase the formation of aggregates. This may explain the majority of the observed yield loss, as aggregates contributed $8.0\% \pm 6.4\%$ of yield loss for 50 mg/mL DexOx samples but $25\% \pm 2.5\%$ of yield loss for 200 mg/mL DexOx samples (n = 2).
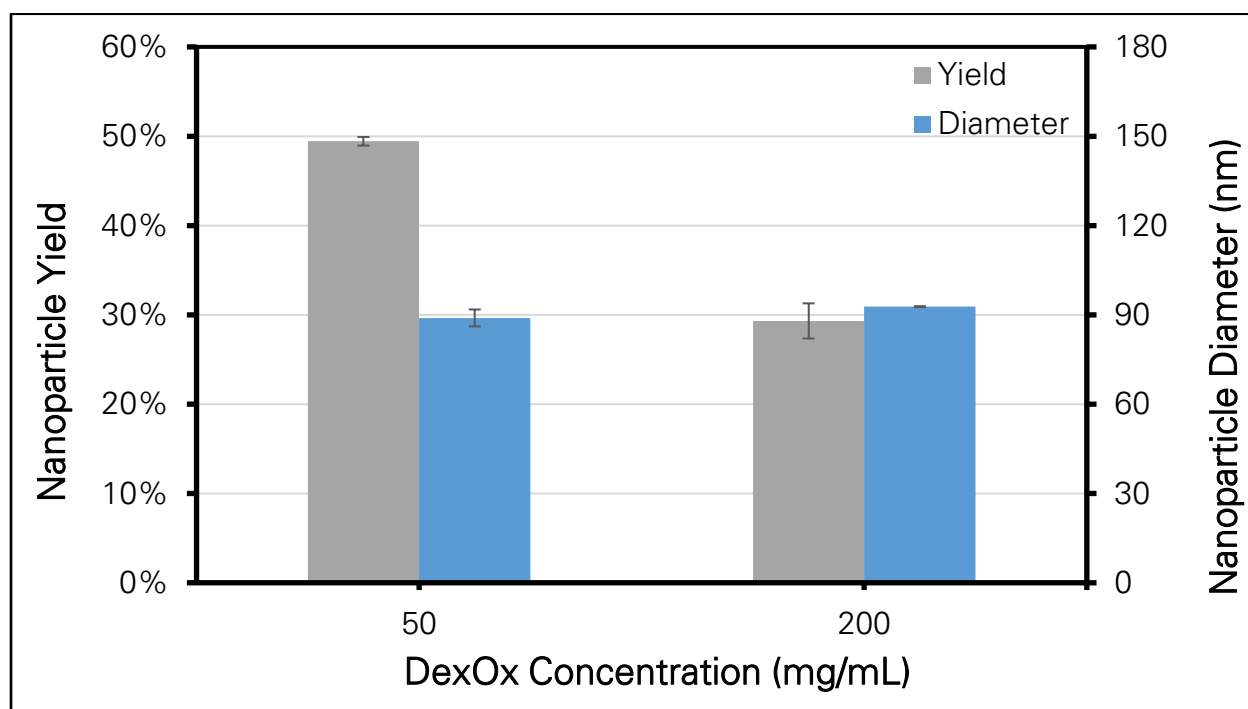
Figure 3: Effect of DexOx concentration on nanoparticle yield and diameter, for DH-NPs purified by separatory funnel washing. n = 2 for all data.

Synthesis yield of solvent precipitated DH-NPs increased significantly when DexOx concentration was increased from 50 mg/mL to 200 mg/mL. As explained in Section 3.4.2 and shown in Table 6, this is likely due to the increased mechanical resiliency of the higher-density 200 mg/mL DH-NPs. Because the solvent precipitation method is analogous to a drying process

and higher density DH-NPs are more resistant against irreversible aggregation, DH-NPs synthesized using 200 mg/mL DexOx achieved higher synthesis yields.
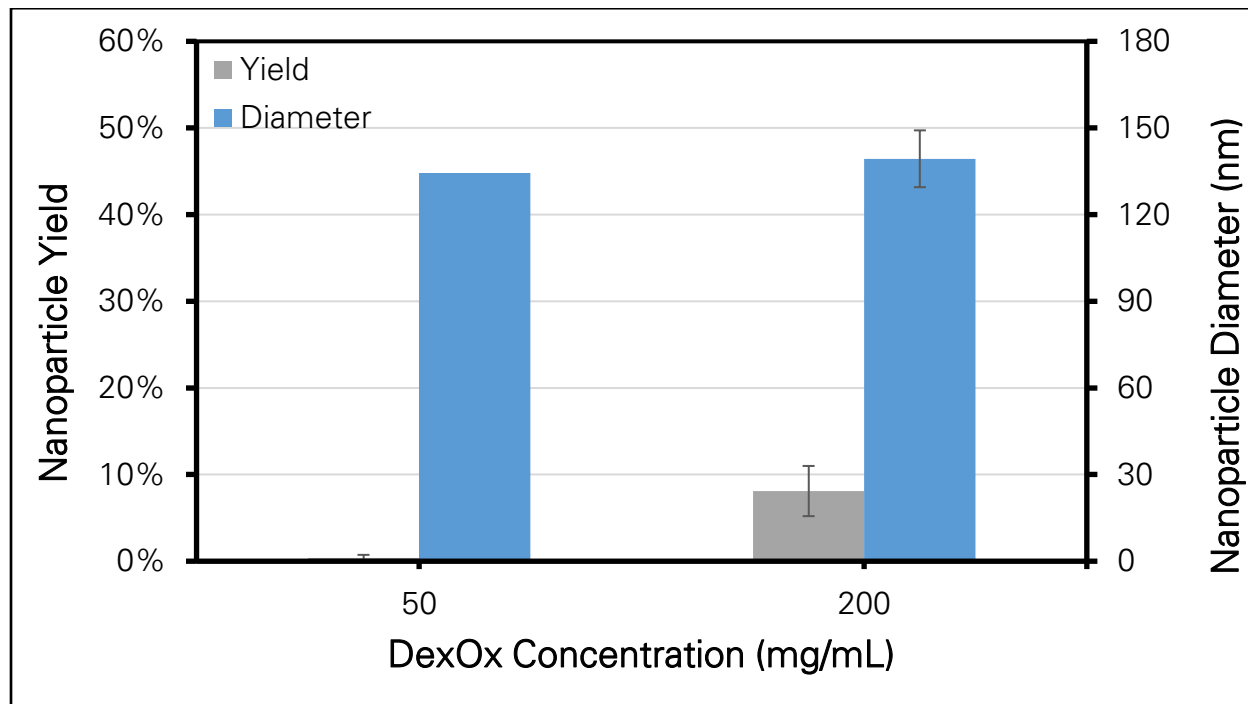
Figure 4: Effect of DexOx concentration on nanoparticle yield and diameter, for DH-NPs purified by solvent precipitation. n = 2 for 50 mg/mL DexOx and n = 3 for 200 mg/mL DexOx.

3.4.4 Effect of Crosslinker-Polymer Ratio on DH-NP Properties

The crosslinker-polymer ratio ($R_{H/A}$) was defined as the moles of hydrazides used to synthesize the hydrogel core of a given DH-NP sample divided by the moles of aldehydes. This was calculated by multiplying the molar quantity of adipic acid dihydrazide by two (as each ADH molecule contains two hydrazide groups) and dividing by the moles of aldehydes present in the DexOx used to synthesize the sample (assuming 100% oxidation and thus the presence of two aldehyde groups per DexOx monomer of molecular weight 146.11 g/mol). This definition is summarized in the following formula, where n_x represents the number of moles of reagent x, and m_x represents the mass of reagent x in grams:

$$R_{H/A} = \frac{n_{hydrazides}}{n_{aldehydes}} \times 100 = \frac{m_{ADH} \cdot \dfrac{2\ hydrazides/mol}{174.22\ g/mol}}{m_{DexOx} \cdot \dfrac{2\ aldehydes/mol}{146.11\ g/mol}} \times 100$$

In practice, the $R_{H/A}$ was adjusted by changing the ratio of ADH to DexOx added to the initial nanoemulsion during the formation of DH-NP hydrogel cores. The effect of this change in crosslinker-polymer ratio on nanoparticle yield, diameter, and mucoadhesion strength was investigated and is presented in Figures 5-7. The other key synthesis parameters were kept constant, namely 50 mg/mL DexOx concentration, no reducing agent treatment, and surfactant/solvent removal by separatory funnel washing. All samples were prepared with no PBA coating with the exception of those used to study the effect of $R_{H/A}$ on mucoadhesion, which were prepared using a theoretical PBA conjugation of 25%.

Figure 5 shows that crosslinker-polymer ratio had little effect on nanoparticle synthesis yield over the range tested. This indicates that use of additional crosslinker did not reduce the stability of the primary nanoemulsion or increase the level of aggregate formation in any other way (this is supported by values of yield loss to aggregates, which also show no relationship with $R_{H/A}$). Observations of the DH-NP synthesis process correlated well with this result, as nanoemulsions consisting of pure ADH were observed to be highly stable and transparent (even more so than standard DexOx nanoemulsions). Therefore, it was an expected result that increasing $R_{H/A}$ would not destabilize the primary nanoemulsion to increase and aggregate formation.

However, theory did suggest a potential increase in aggregate formation (and thus reduction in nanoparticle yield) due to a higher level of inter-particle crosslinking. Since each reverse micelle within the primary nanoemulsion contained a larger quantity of ADH when $R_{H/A}$ was increased, the probability of inter-particle crosslinking reactions during regular micelle coalescence events

may have been greater, potentially leading to excessive particle growth and aggregate formation. However, this phenomenon was not observed within the $R_{H/A}$ range tested in Figure 5. It is hypothesized that since the nanoemulsion droplets still contain a large excess of aldehydes in comparison to hydrazides, inter-particle crosslinking was limited by the much greater likelihood of intraparticle crosslink formation. Further studies on DH-NPs synthesized with $R_{H/A}$ in excess of 50% are recommended to better understand this phenomenon and evaluate the viability of the proposed mechanism.
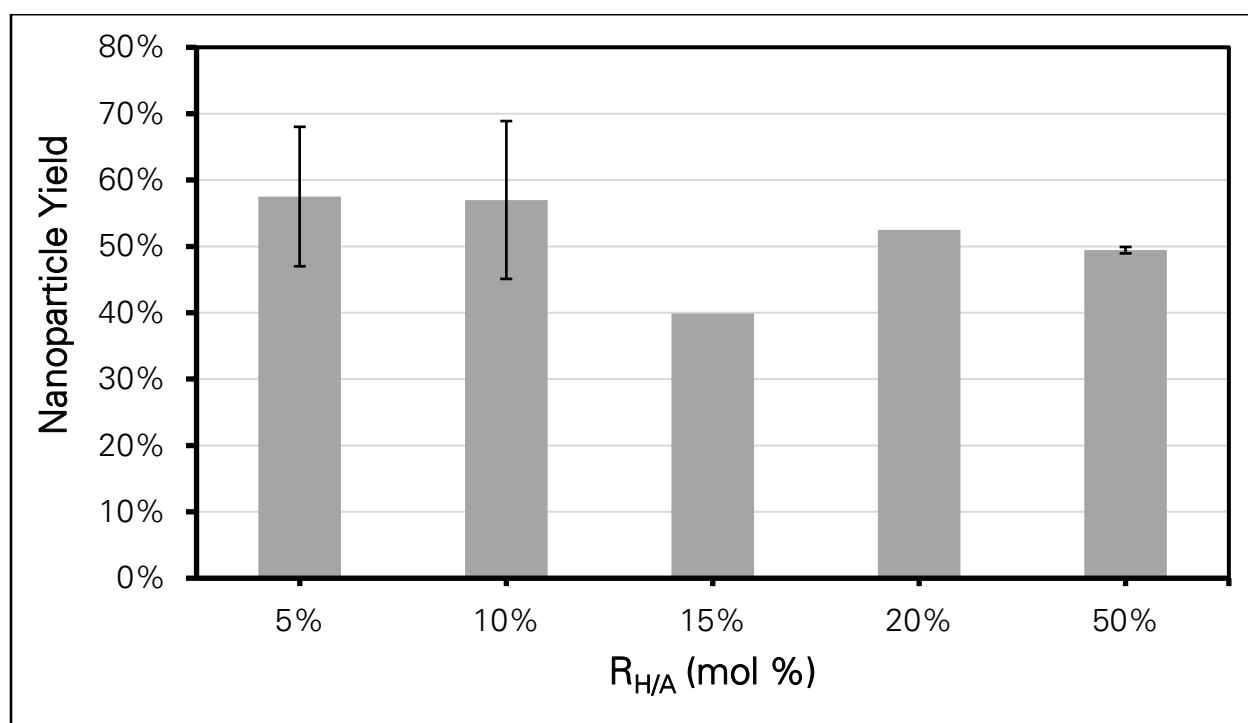
Figure 5: Effect of crosslinker-polymer ratio ($R_{H/A}$) on nanoparticle yield.

Figure 6 examines the effect of $R_{H/A}$ on DH-NP diameter. As can be seen, the trend in the data is somewhat unclear, and further trials are recommended to increase the statistical significance of data points and draw a more reliable conclusion. From the preliminary results displayed in the figure, it appears that DH-NP diameter may decrease to a minimum level around 50% $R_{H/A}$ and subsequently increase once again at greater $R_{H/A}$ levels. The initial decrease in diameter may be a

result of increased polymer network density due to the higher degree of crosslinking. It has been shown in literature that tighter DexOx-ADH crosslinking leads to a reduction in swelling capacity,[59] and the observed shrinking of DH-NP diameter as $R_{H/A}$ increased to 50% may be a result of this compaction in the hydrogel network. The subsequent increase in diameter as $R_{H/A}$ increased above 50% may have been due to a greater level of inter-particle crosslinking as explained in the discussion of Figure 5. As the relative abundance of aldehydes decreases with higher $R_{H/A}$, the increased quantity of singly-crosslinked ADH molecules may increase the likelihood of inter-particle crosslinking during micelle coalescence events, leading to an increase in nanoparticle diameter.
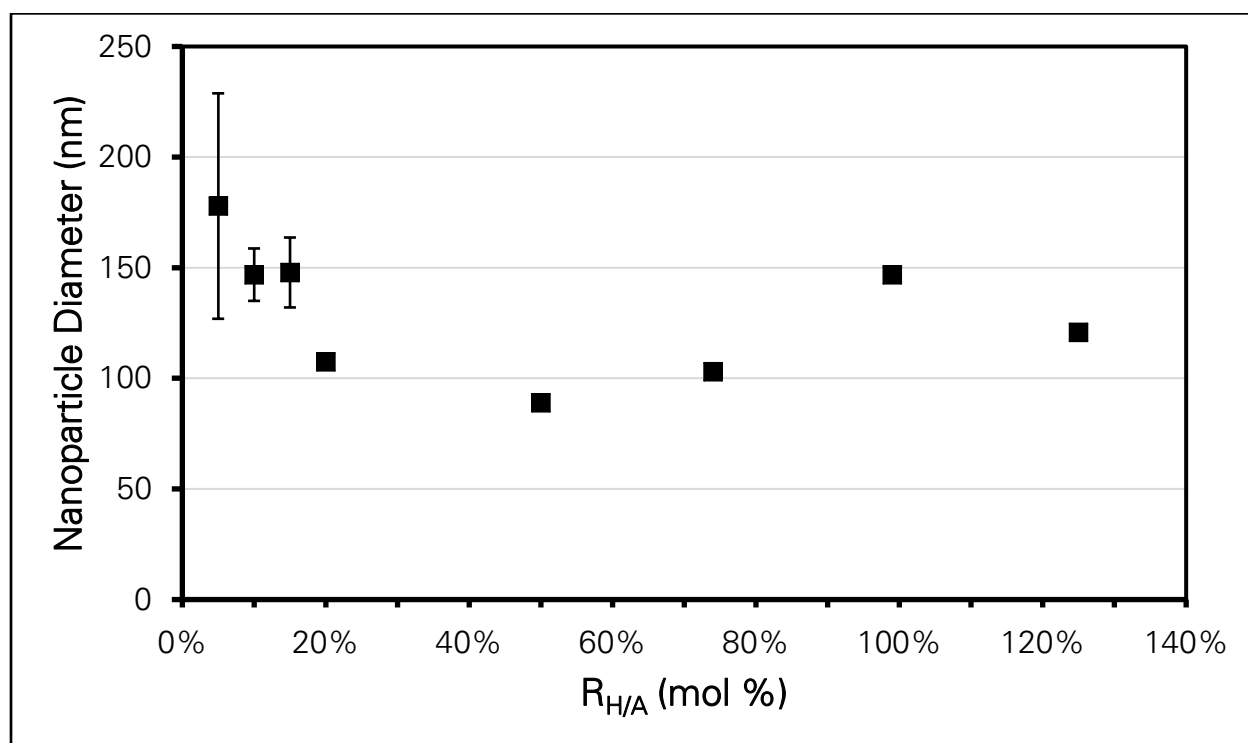
Figure 6: The effect of crosslinker-polymer ratio ($R_{H/A}$) on nanoparticle diameter.

A strong relationship was observed between $R_{H/A}$ and mucoadhesion strength as measured by the Stern-Volmer constant (K_{SV}). As shown in Figure 7, increasing $R_{H/A}$ resulted in an approximately linear increase in K_{SV}. Since the initial PBA feed was designed for 25% theoretical

PBA conjugation for all samples, this study showed that an equal quantity of PBA can impart greater mucoadhesive strength when applied to a more tightly crosslinked hydrogel core. A possible mechanistic explanation of this trend may be a reduction in penetration of PBA molecules to the core of the DH-NP, where they may become inactive with regards to mucoadhesion. Because DH-NP hydrogel cores are in fact porous polymer networks, it is expected that a portion of the PBA molecules introduced during mucoadhesive coating would diffuse into the interior of the hydrogel core before covalently binding to a DexOx chain. Once affixed to the interior of the DH-NP, these interior PBA moieties would be less likely to bind to sialic acid molecules during K_{SV} testing, and completely unavailable to bind to ocular surface mucins during real-world use as an ocular lubricant. Since it is expected that increasing $R_{H/A}$ tightens the polymer network of the hydrogel core, PBA molecules were likely obstructed form diffusing to the interior of the high $R_{H/A}$ DH-NPs as easily, leading to a larger portion of the PBA moieties attaching to the surface of the nanoparticle. It is proposed that this mechanism led to more extensive PBA coatings on DH-NPs with higher crosslinker-polymer ratio, and thus stronger mucoadhesion and higher K_{SV} value.
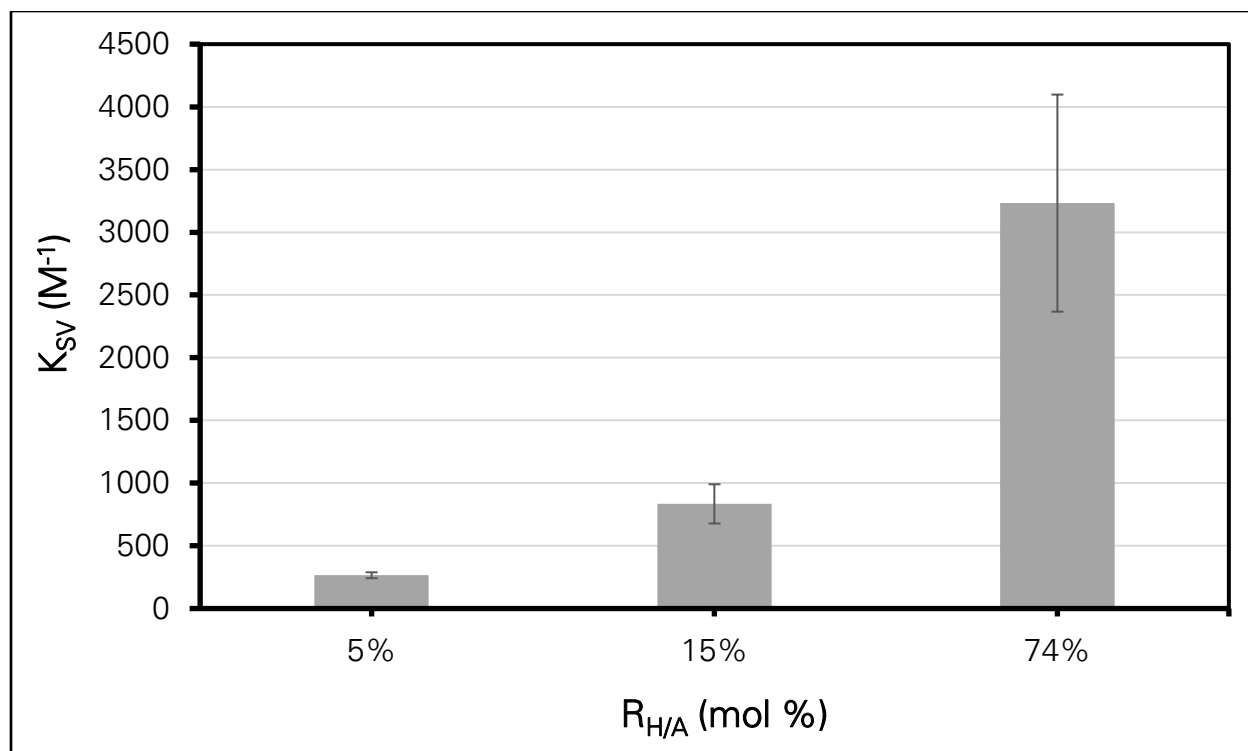
Figure 7: Relationship between $R_{H/A}$ (crosslinker-polymer ratio) and mucoadhesion strength as measured by K_{SV} (Stern-Volmer constant).

3.4.5 Effect of Reducing Agent Treatment on DH-NP Properties

The reducing agents sodium borohydride and sodium cyanoborohydride serve important roles in the synthesis of DH-NPs. Both reduce the hydrazone bonds that form between DexOx and ADH to their more stable secondary amine form, increasing the robustness of the nanoparticle and reducing the rate of crosslink hydrolysis. As discussed in detail in Section 4.4.2, this crosslink reduction is one of the methods of modifying DH-NP degradation and thus helps regulate the polymer release behaviour that is critical for use as an ocular lubricant. Sodium cyanoborohydride is also used as a reaction enhancement agent to drive crosslinking and PBA conjugation reactions further towards completion, as it selectively reduces hydrazone and amide bonds without modifying aldehydes.[67] Since sodium borohydride reduces hydrazones, amides, and aldehydes

however, it is used to terminate crosslinking/PBA conjugation reactions and convert residual aldehyde groups into more biologically inert hydroxyl groups.

The studies presented in Figures 8-9 investigated potential effects of reducing agent treatment on DH-NP properties such as nanoparticle size, yield, and mucoadhesion performance. Blank DH-NPs used to determine the effects of reducing agent on NP yield and diameter (Figure 8) were synthesized using 200 mg/mL DexOx, 50% $R_{H/A}$, no PBA coating, and surfactant/solvent removal by separatory funnel washing. PBA-coated DH-NPs (Figure 9) were synthesized using 50 mg/mL DexOx, 15% $R_{H/A}$, theoretical PBA conjugation of either 15% or 25%, and purified using an as-needed combination of separatory funnel washing (primary method), $CaCl_2$ precipitation, and solvent precipitation. When desired, reducing agent treatment was applied at one of two times during the synthesis process: in the primary nanoemulsion during the nanoparticle formation stage, or during the nanoparticle purification process (after completion of surfactant/solvent removal). Samples designated for reduction were treated with either sodium borohydride or sodium cyanoborohydride, in a molar excess quantity in all cases.

Figure 8 shows the effect of sodium borohydride treatment on nanoparticle yield and diameter. Synthesis yield was found to decrease significantly for samples treated with $NaBH_4$, but the mechanism for this observation is unknown. A possible explanation may be a premature termination of the DexOx-ADH crosslinking reaction due to $NaBH_4$ addition, but further trials are required to evaluate this hypothesis and confirm the true mechanism(s) of yield loss. $NaBH_4$ treatment was also found to cause an increase in DH-NP diameter, but the large standard error places the significance of the result into question. In fact, aggregate mass measurements revealed that the quantity of aggregate production was approximately equal, with $NaBH_4$-treated samples losing 21% $\pm$ 3% yield to aggregates while the reducing agent-free samples saw 25% $\pm$ 3% yield

loss. In addition, the DH-NP sample in which NaBH$_4$ treatment was applied after surfactant/solvent precipitation had an average nanoparticle diameter of 100 nm, very close to that of the samples synthesized without reducing agent treatment. Further trials are therefore recommended to more clearly determine the effect of reducing agent treatment on nanoparticle yield and diameter.
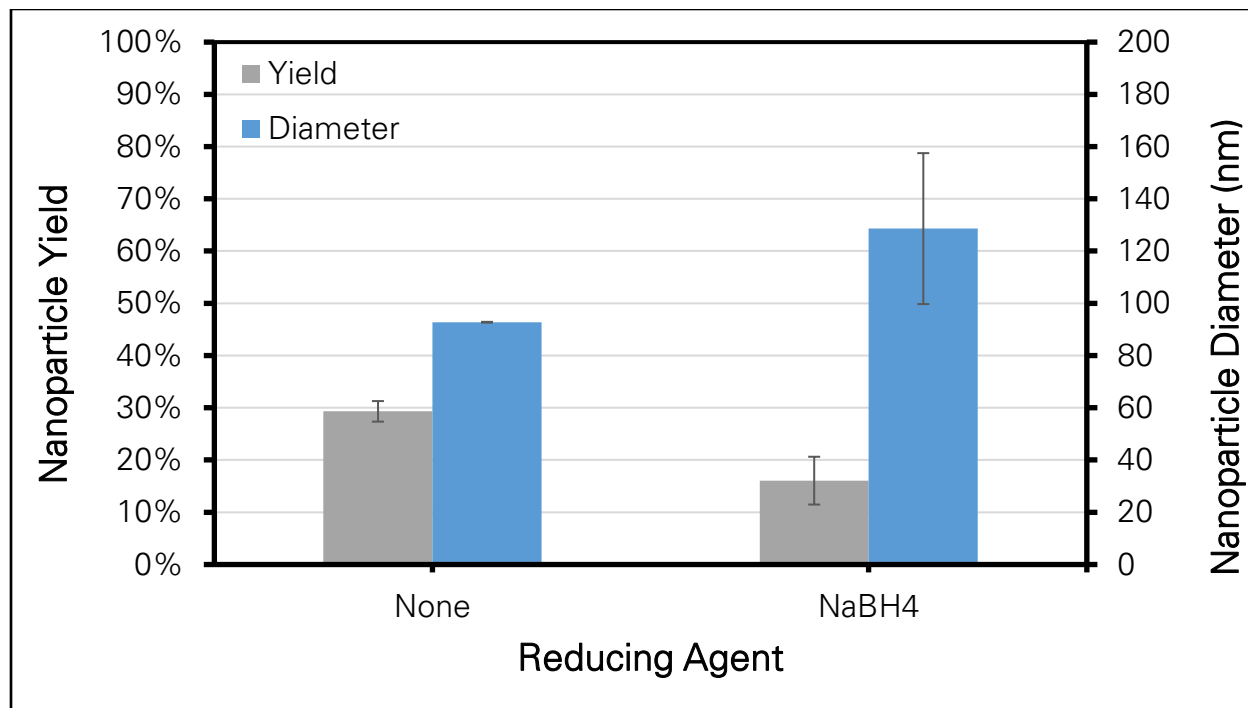
Figure 8: Effect of sodium borohydride treatment on DH-NP yield and diameter.

The effects on mucoadhesion of the duration of reducing agent treatment ("reduction time") are shown in Figure 9. In this study, the reducing agents (primarily NaBH$_4$) were allowed to react with DH-NP samples for varying periods of time, including 0 hours (i.e. no reducing agent added), 2 hours, and overnight (16+ hours). It was found that longer reduction times resulted in lower K_{SV} values for DH-NPs coated with both 15% and 25% theoretical PBA conjugation. This is an important observation for the design of the DH-NP synthesis process, as it shows the dramatic impact reduction time can have on mucoadhesive strength. The data shows that any reducing agent

treatment (regardless of duration) causes a significant decrease in mucoadhesion strength. In addition, a simple increase in reduction time from 2 hours to overnight can decrease K_{SV} by more than 4-fold, with all other parameters kept unchanged. Therefore while reducing agent treatment is required for enhancing DH-NP safety and other purposes, the results of our study suggest its use should be limited in order to maintain the high K_{SV} values critical for ocular surface adhesion. It is important to note that the mechanism by which reducing agent reaction decreased K_{SV} in our study is unknown, and the use of reducing agents such as sodium cyanoborohydride with PBA is commonly found in literature.[74–76] Further studies are therefore recommended to understand the true mechanistic origin of this phenomenon in order to maximize the benefits of both reducing agent treatment and mucoadhesion.

Figure 9: Effect of reaction time with reducing agent ("reduction time") on mucoadhesive strength of DH-NPs, as measured by K_{SV}.

3.4.6 Effect of PBA Feed Quantity on DH-NP Properties

The quantity of PBA added during DH-NP synthesis ("PBA feed quantity") was also found to have significant effects on the properties of nanoparticles produced. The PBA feed quantity w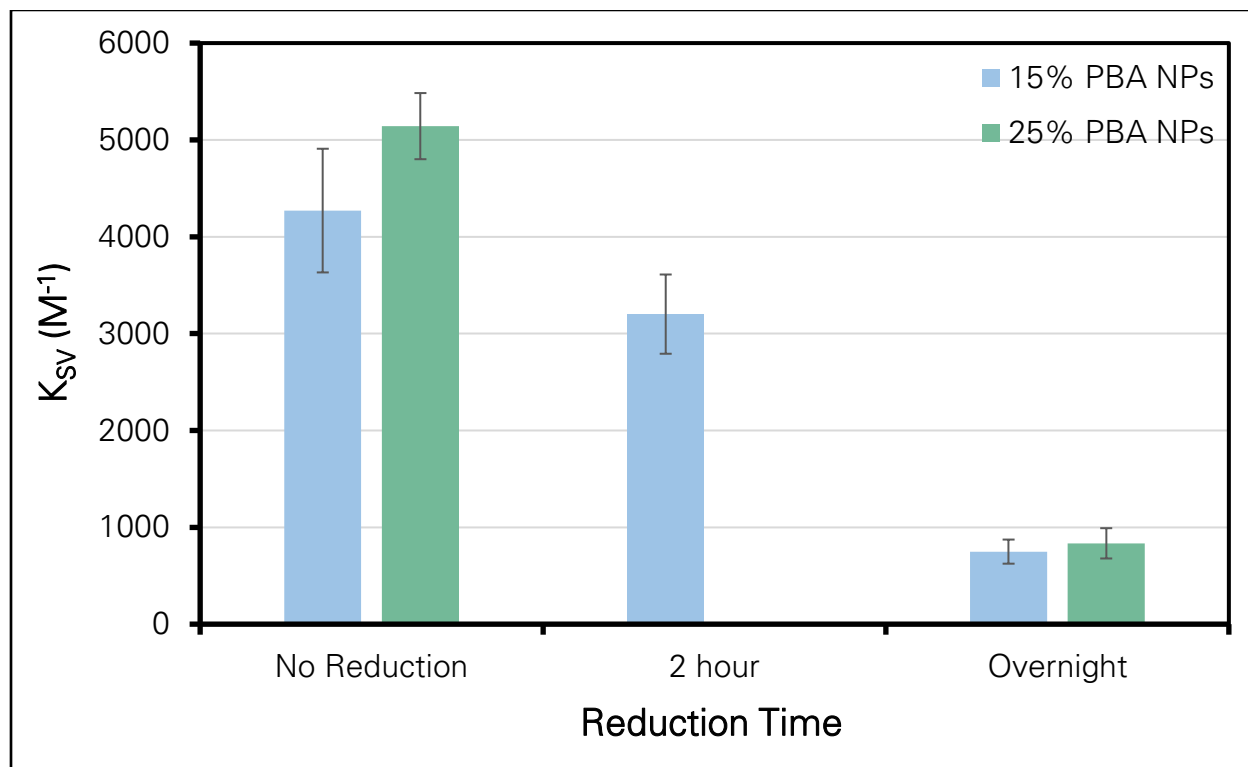as calculated according to the desired "theoretical PBA conjugation," defined as the moles of PBA added divided by the total moles of aldehyde groups in the DexOx added during synthesis (assuming 2 aldehyde groups per monomer and without subtracting any groups that may react with ADH or other reagents). The synthesis parameters maintained at a constant value in the studies presented in this section are DexOx concentration (50 mg/mL), crosslinker-polymer ratio ($R_{H/A}$ = 15%), and surfactant/solvent removal method (separatory funnel washing, with additional $CaCl_2$ precipitation if required). With regards to reducing agent treatment, the study of NP diameter used only untreated DH-NPs, while the yield and mucoadhesion studies used equal quantities of treated and untreated samples.

Figure 10 shows the effect of PBA feed quantity on nanoparticle yield and yield loss to aggregates. In general, it was found that higher PBA feed resulted in lower DH-NP yield due to increased aggregation. The largest drop in yield (over 35%) and increase in aggregate formation (almost 30%) was between 5% and 15% PBA conjugation, after which little change occurred upon increasing PBA conjugation to 30%. It is hypothesized that aggregate formation occurred in this study due to loss of water solubility upon excessive PBA conjugation. It is thought that the 5% theoretical PBA conjugation enabled the DH-NPs to remain largely hydrophilic, but 15% conjugation resulted in a more extensive PBA coating (and thus increase in hydrophobicity) sufficient to cause precipitation. It is likely that since the DH-NPs had already reached the threshold of PBA coating density that results in precipitation at 15% PBA conjugation, increasing to 30% conjugation did not induce a large decrease in yield or increase in aggregation. This result

shows that the extent of DH-NP surface modification with PBA should be limited to prevent excessive hydrophobicity and yield loss due to poor solubility.
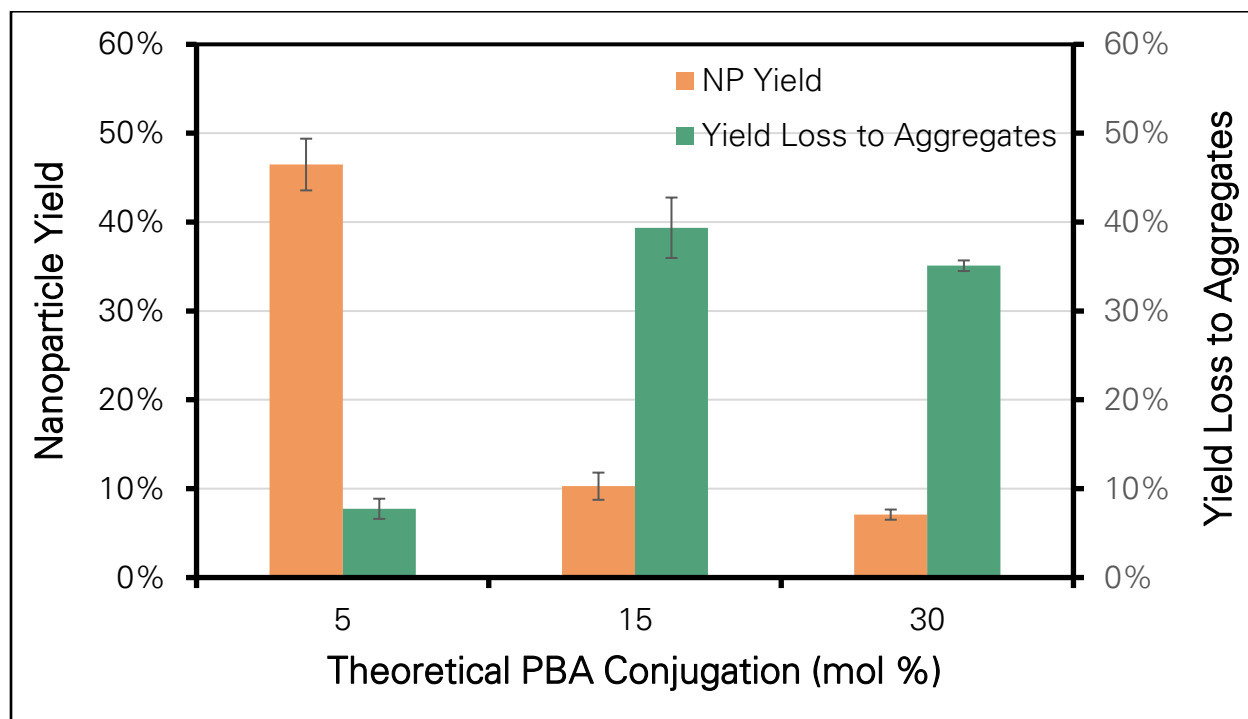
Figure 10: Effect of theoretical PBA conjugation on final DH-NP yield (orange) and yield loss to aggregate formation (green).

As part of the same study presented in Figure 10, the true PBA conjugation and K_{SV} were measured for each DH-NP variant, and are shown in Figure 11. The PBA conjugation values shown in this figure were measured by the method described in Section 3.3.9, which provides an approximation of the quantity of PBA truly conjugated to a DH-NP sample by measuring the fluorescence signal produced by PBA moieties. The measured PBA conjugation values are thus derived from the true PBA content of a sample, whereas theoretical PBA conjugation simply corresponds to the quantity of PBA added during synthesis. As expected, Figure 11 shows that increasing PBA feed caused the measured PBA conjugation to increase, in a continuous fashion between 5% and 30% theoretical conjugations. However, this increase in the quantity of PBA conjugated to the surface of the DH-NPs did not result in a proportional increase in mucoadhesion

strength according to K_{SV} measurements. Rather, there was a large rise in K_{SV} from 5% to 15%

PBA feed, but the K_{SV} remained almost the same when PBA feed was increased further to 30%.

This indicates that while the 30% feed DH-NP samples do contain a greater quantity of PBA in

comparison to the 15% feed DH-NPs, the additional PBA is likely not mucoadhesively active.

Drawing from our hypothesis in Section 3.4.4, the lack of mucoadhesive activity of these PBA

moieties may be a result of conjugation within the interior of the DH-NP due to the surface

becoming saturated with PBA. Since the interior of the DH-NP is likely less accessible to sialic

acids during K_{SV} measurement and certainly inaccessible to ocular surface mucins during real-

world use as an ocular lubricant, the interior PBA moieties do not contribute to mucoadhesion

strength. PBA feed quantity should therefore be selected such that most PBA moieties remain on

the surface of the DH-NP and little to no moieties are conjugated to the NP interior due to surface

saturation.
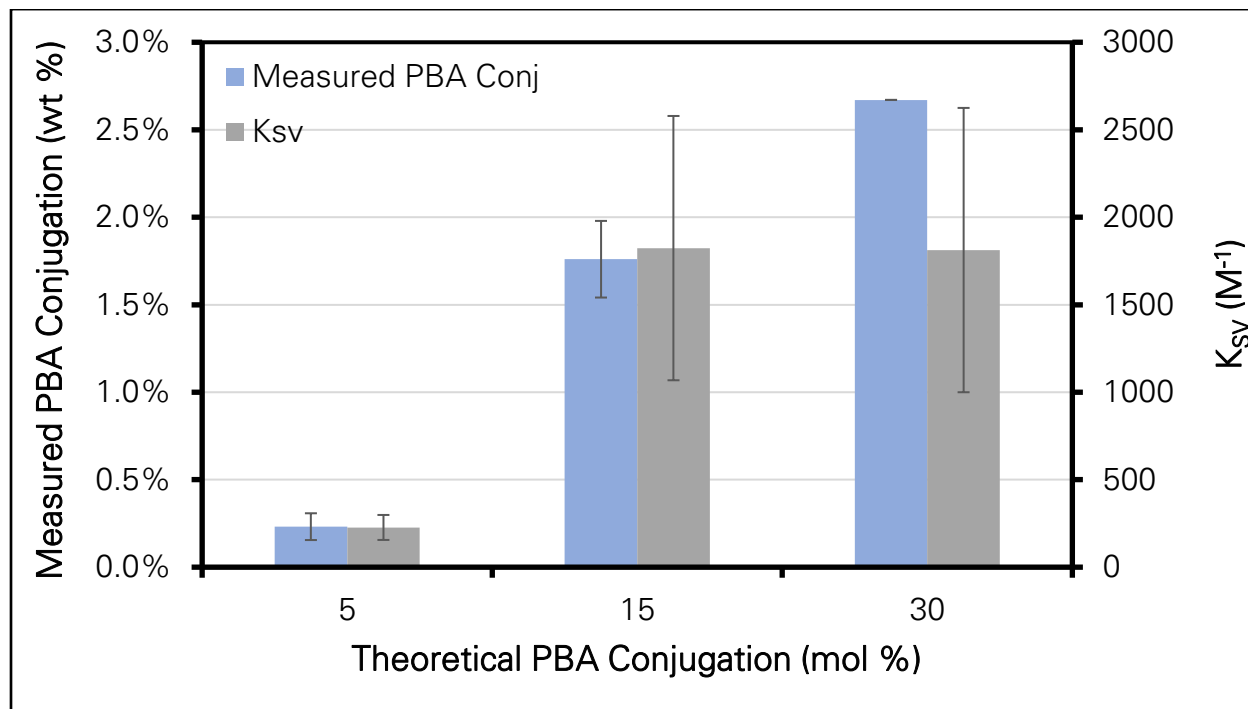
Figure 11: Relationship between PBA feed quantity ("theoretical PBA conjugation") and measured PBA conjugation (as measured by fluorescence) and mucoadhesion (as measured by K_{SV}).

PBA feed quantity was found to have negligible effect on nanoparticle diameter, as shown by the data in Figure 12. Although there is a significant difference between the diameters of 0% PBA and 15% PBA nanoparticles, the almost identical diameters of 0% PBA and 11% PBA shows that DH-NP diameter is largely unchanged within the most likely operating range of PBA feed quantities (since other experiments show that 15% PBA feed causes a large decrease in nanoparticle yield, and also offers no advantage in mucoadhesion strength). This finding shows that the coating of DH-NP hydrogel cores with PBA does not cause an appreciable increase in size. This is an expected result, since the small size of PBA moieties and their chemical inability to form more than a single monolayer coating on the surface of DH-NPs makes it unlikely for any increase in nanoparticle diameter to be observed. Due to the high porosity of hydrogels, the interior core of DH-NPs is also expected to contain a large amount of empty space where PBA moieties could bind without an increase in diameter being observed. Nevertheless, sufficient conjugation of PBA on the interior of DH-NPs may eventually cause diameter to increase, although this phenomenon was not clearly observed in this study.
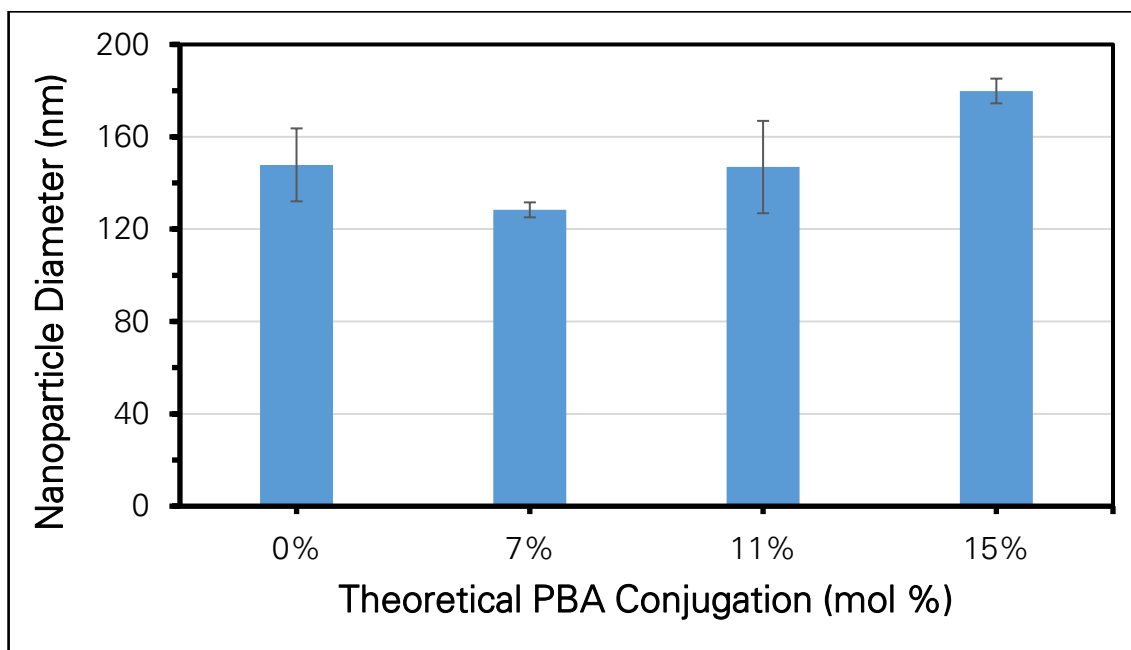
Figure 12: Relationship between PBA feed (theoretical PBA conjugation) and DH-NP diameter.

3.5 Conclusion

This chapter described the synthesis and characterization of various forms of DH-NPs. The synthesis method presented was adapted from multiple literature sources and found to be reliable and repeatable. A suite of characterization methods was developed to analyze several key properties of the nanoparticles produced, including nanoparticle diameter, PBA conjugation, mucoadhesion strength (K_{SV}), synthesis process yield, and DexOx oxidation degree.

A number of variations in the nanoparticle purification process were also developed, each offering unique advantages. The separatory funnel washing method of surfactant/solvent removal was found to be best suited for large-scale DH-NP synthesis, as it offered the greatest nanoparticle yield and was the most resource-efficient for large DH-NP batches. The addition of calcium chloride precipitation to the separatory funnel washing process provided the opportunity for DH-NPs with high PBA content to be successfully synthesized and purified. The solvent precipitation method of surfactant/solvent removal was also found to have unique strengths, particularly in its ability to produce DH-NPs in the dry powder state. This provided the advantage of longer shelf life, precise DH-NP concentration control, and reduced yield wastage (as synthesis yield measurements were no longer required). The relative importance of each of these factors in a given study allows the experimenter to select the most suitable surfactant/solvent purification method for each experiment.

A number of critical synthesis parameters were also identified that enable the tuning of DH-NP properties to meet the needs of a range of use cases and applications. An increase in the parameter of crosslinker-polymer ratio ($R_{H/A}$) has been found to strengthen mucoadhesion (increase K_{SV}) without affecting nanoparticle diameter or yield. A lower DexOx concentration (in

the nanoparticle formation stage) was found to produce higher nanoparticle yields when solvent washing was used as the surfactant/solvent removal method, while higher DexOx concentration resulted in greater yields when the solvent precipitation method was utilized. No clear changes in nanoparticle diameter were observed, although further trials are recommended for confirmation. Reducing agent treatment was found to have a pronounced negative effect on mucoadhesion strength, and also led to a decrease in NP yield and potential increase in NP diameter. It is therefore recommended that reducing agent treatment be utilized at the minimum level that provides acceptable degradation kinetics and ensuring patient safety. Higher quantities of PBA feed were found to steadily increase PBA conjugation, but resulted in dramatic loss of nanoparticle yield due to aggregate formation. Mucoadhesion strength was found to initially increase with higher PBA feed quantity, but reached plateau at a maximum value.

These findings provide a foundation that can be used to synthesize a wide range of DH-NP variants, each with a unique combination of properties. The optimum combination of properties can then be selected by testing for optimum efficacy in an ocular lubricant formulation or other applications.

4.0 Application of DH-NPs in Ocular Lubricant Formulations for DED Treatment

4.1 Summary

The feasibility of developing a next-generation ocular lubricant eye drop based upon DH-NPs was investigated through testing of lubricant delivery and biocompatibility. The sustained delivery of lubricating polymer from DH-NPs was envisioned through two distinct mechanisms, degradation and diffusion. Degradation-controlled release was characterized in detail, with pronounced rate-regulating effects observed for the parameters of crosslinker-polymer ratio, reducing agent treatment, and temperature. The maximum lubricant release rate achieved was 1.8 mg/(day*10 mg NPs), which approaches the clinically effective target of 5 mg/(day*10 mg NPs). Further work is required to characterize and evaluate the diffusion-controlled release mechanism due to challenges with method development. Biocompatibility was studied in a 5-day acute in vivo trial with three rabbits, and indicated excellent tolerance through slit lamp and histopathological evaluation.

4.2 Introduction

DH-NPs are an excellent candidate for incorporation into a next-generation ocular lubricant formulation due to several factors. Firstly, the materials of which they are composed have been shown to be biocompatible in a number of pre-clinical studies.[76,77] The dextran-based composition of DH-NPs also closely mimics the glycocalyx (the polysaccharide coating of corneal and conjunctival epithelial cells that plays a critical role in ocular hydration and protection),[1,20,26] and therefore may act to reinforce it amidst the damages caused by DED mechanisms. Since glycocalyx damage has been identified as a critical factor in the development and propagation of

DED, reinforcement with DH-NPs may serve a pivotal role in managing and treating the condition in patients. The dextran base of DH-NPs also provides an abundance of aldehyde and hydroxyl functional groups, facilitating chemical modifications that enhance lubricating properties (such as PBA coating for mucoadhesion). Hydrogel nanoparticles (also known as nanogels) such as DH-NPs are also known to have high drug loading capacity and greater stability in physiological environments than other types of nanocarriers used in ocular lubricants today, such as liposomes and nanoemulsions.[78] This allows the unique strengths of nanoparticles (including long duration of action and no impairment of visual acuity) to be harnessed in an efficient and reliable manner. DH-NPs also have an intrinsic, highly controlled degradation behaviour that can be used directly as a method of ocular lubrication. The polymers released during degradation are dextran-based (composed of DexOx and ADH) and thus highly hydrophilic. It is anticipated that they may also share lubricating properties with dextran 70, a type of dextran that has been granted GRASE (generally recognized as safe and effective) designation by the US Food and Drug Administration (FDA) for use within ocular lubricant (i.e. ophthalmic demulcent) formulations.[79] It is therefore expected that the degradation of DH-NPs can serve as effective and sustained source of ocular lubricant release (specifically DexOx-ADH polymers) upon administration to the eye.

The role of DH-NPs in a next-generation ocular lubricant formulation is envisioned to primarily be as a vehicle for sustained release of lubricating polymer, and also as a glycocalyx-reinforcing material. A primary feature of DH-NPs that has been designed to enable these roles is mucoadhesion, which causes the nanoparticles to adhere to the ocular surface through mucins bound to corneal and conjunctival epithelial cells (and thus part of the glycocalyx). Various studies performed to develop and test this feature are described in Section 3.4.6. It is hypothesized that DH-NPs will serve to reinforce the glycocalyx of DED patients simply by virtue of their

hydrophilic polysaccharide-based composition and their mucoadhesion to the native glycocalyx. The following sections in Chapter 4 describe two proposed approaches of achieving sustained lubricating polymer release from the DH-NPs (Figure 13). The first approach (degradation-based release) leverages the intrinsic composition and degradation behaviour of the DH-NPs, and releases lubricating fragments of DexOx-ADH polymer (blue chains in Figure 13) through a sustained hydrolysis process. The second approach is diffusion-based, and uses physical encapsulation and controlled release of other lubricating polymers (red chains in Figure 13) from within the nanoparticle cores to lubricate patient eyes over a sustained period of time. DH-NPs are modified in the diffusion-based release approach to prevent hydrogel core degradation, and the DexOx-ADH material is thus not released onto the patient's eye.
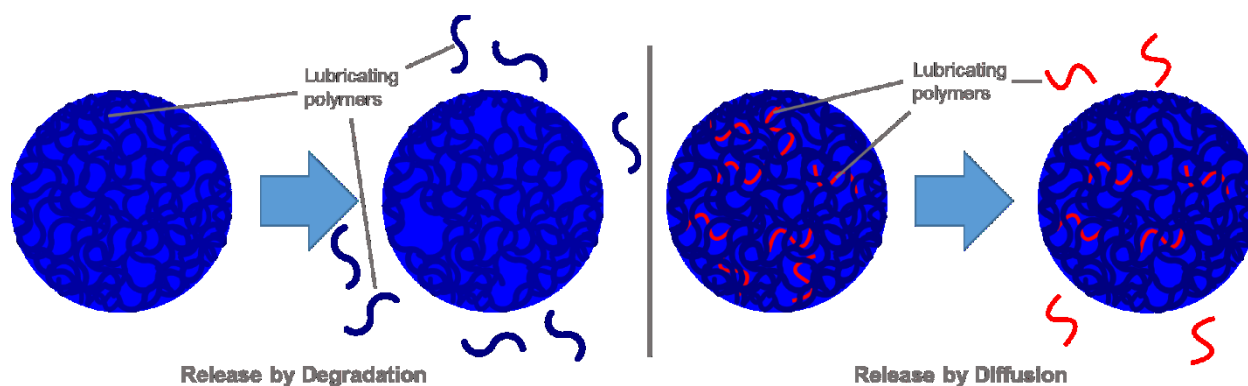
Figure 13: The two proposed approaches to sustained release of lubricating polymer from DH-NPs. The first method is based upon degradation of the DH-NP core itself (blue chains in the figure), while the second is based upon diffusion-controlled release of other lubricating polymers (red chains in the figure) encapsulated within the DH-NPs. DH-NPs are modified to prevent degradation of the hydrogel core in the diffusion-controlled release method.

4.3 Materials and Methods

4.3.1 Materials and Instrumentation

Details regarding any materials or instrumentation also used in studies from Chapter 3 can be found in Section 3.3.1.

New materials utilized for studies in this chapter include Amicon^{TM} centrifugal filter units (MilliporeSigma, 30 kDa MWCO, 15 mL capacity), and Pall Acrodisc® 0.2 μm sterile syringe filters with Supor® membrane.

Instruments uniquely used in Chapter 4 include the S4Optik SL-Z3 slit lamp, and Waters HPLC system (2690 separations module and 2996 photodiode array detector) with Agilent Zorbax SB-C18 column with 5 μm particle size, 4.6 mm internal diameter, 150 mm length.

4.3.2 Polymer Release Characterization

The release of lubricating polymer from DH-NPs was tracked over time using a novel method developed for our studies. DH-NPs were separated from the lubricating polymers they had released by placing them in the top chamber of an Amicon^{TM} centrifugal filtration device. The membrane that separates the top chamber from the lower chamber was selected to have a pore size of 30 kDa, sufficiently small to retain DH-NPs in the top chamber while allowing released polymers to pass into the lower chamber. Note that the material remaining in the top chamber after centrifugal filtration is termed the "retentate" while the material that filters into the lower chamber is referred to as the "filtrate."

To characterize release of lubricating polymer by DH-NP degradation, four equal aliquots (containing >10 mg of DH-NPs each) were stored in a 37°C incubator. The first aliquot was

immediately taken to measure polymer release. Polymer release was quantified by centrifugal filtration using a 15 mL Amicon device with a 30kDa MWCO membrane. The device was first cleansed of membrane preservation agents and other possible contaminants by washing with 0.1 M NaOH and then Millipore water. The NP sample was then added to the top chamber of the device, and filtered by centrifugation at 2800 g for 15-30 minutes (until >80% of the sample had entered the lower chamber). This constituted the first wash. The top chamber was then topped up to the original volume with Millipore water and pipette mixed to resuspend any NPs that had settled or become lodged in the filtration membrane. Centrifugation was then repeated as was done for the first wash. NP resuspension and centrifugation were then repeated once more, for a total of three washes. Finally, NP resuspension in the top chamber was performed once more, after which the resuspended solution was lyophilized to determine the retentate mass. The filtrate was also lyophilized to determine its mass. This process of repeated centrifugal filtration of the DH-NP aliquots was repeated at each time point, namely after 24 hours, 48 hours, and 72 hours. Polymer release at a given time was quantified by calculating the difference between the mass of filtrate measured at that time point and the mass of filtrate measured at the beginning of the study (0 hours).

4.3.3 In Vivo Acute Ocular Biocompatibility

The acute ocular tolerance of DH-NPs was tested through a 5-day high dosage study using a rabbit model. The study design was reviewed and approved by the University of Waterloo Office of Research Ethics Animal Care Committee, and was in accordance with the Canadian Council on Animal Care standards and guidelines, and the Ontario Animals for Research Act. The study was conducted using three male New Zealand White rabbits (Charles River Laboratories, Wilmington, Unites States) housed in individual cages and provided with standard laboratory diets. The rabbits

were acclimatized to the University of Waterloo animal facility for at least one week prior to study commencement.

Each rabbit was administered both a solution of DH-NPs on either the right or left eye (randomly assigned) while the contralateral eye was administered physiological saline (0.9% NaCl) as a control. Administration of each treatment was done with two 25 μL drops added to each eye six times daily (at 1 hour intervals and at the same times each day). Both eyes of each rabbit were examined by slit lamp microscopy at the end of each day during the study period, and also at the beginning of the first day as a control. Slit lamp examination was performed using a S4Optik SL-Z3, and findings were quantified using the Draize scoring method for conjunctival redness, secretion, corneal opacity, and iris involvement. The rabbits were euthanized at the end of the fifth day, after which the ocular globes and eyelids were extracted and fixed in 10% neutral buffered formalin for histopathological analysis.

Samples of the full ocular globe and eyelids were processed for histopathological analysis. Tissues were embedded in paraffin wax and sectioned of 5 μm thickness were then prepared. Sections were stained with hematoxylin and eosin before evaluation using bright field microscopy (Leica DM1000, ICC50 HD, Leica Microsystems Inc, Concord, ON, Canada).

4.4 Results and Discussion

4.4.1 Need for Specialized Polymer Release Characterization Method

In designing the polymer release characteristics of DH-NPs, it was necessary to select a release characterization method that was accurate in the short-term. In particular, the polymer release within the first 24 hours was critical to measure precisely and accurately, as it is for this quantity of time that DH-NPs are expected to remain on the ocular surface.[65] Traditional release characterization methods based upon dialysis were found to be inadequate, as they introduced artificial delays in release time of almost 24 hours (Figure 14). Other traditional release characterization methods relied upon centrifugation to cause settling of nanoparticles from solution, after which the supernatant could be analyzed to determine the quantity of polymers released from the nanoparticles. However this method was also unsuitable for our studies, as the available centrifugation equipment (capable of achieving up to 21,130 g of centrifugal force) could not effectively settle DH-NPs due to their relatively low density. As a result, it was necessary to develop a new method of release characterization to meet the needs of our studies, and this technique was described in Section 4.3.2.

It should be noted that in our studies, polymer release characterization was chosen to serve as a key in vitro efficacy test for the use of DH-NPs in ocular lubricant formulations. Other methods of demonstrating in vitro efficacy were also explored, particularly the water retention method described by Zheng et al.[80] However, after extensive investigation the method was found to be incapable of reliably assessing the ocular hydration capability of an ocular lubricant formulation. Statistically significant differences between positive controls (commercial ocular lubricants) and negative controls (pure water) could not be identified, while non-meaningful artifacts such as differences in masses of formulations used in the experiment created noticeable

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differences in water retention time. It was therefore concluded that this approach is not a reliable method of assessing efficacy of DH-NPs, and it is recommended that this method be avoided for future investigations.
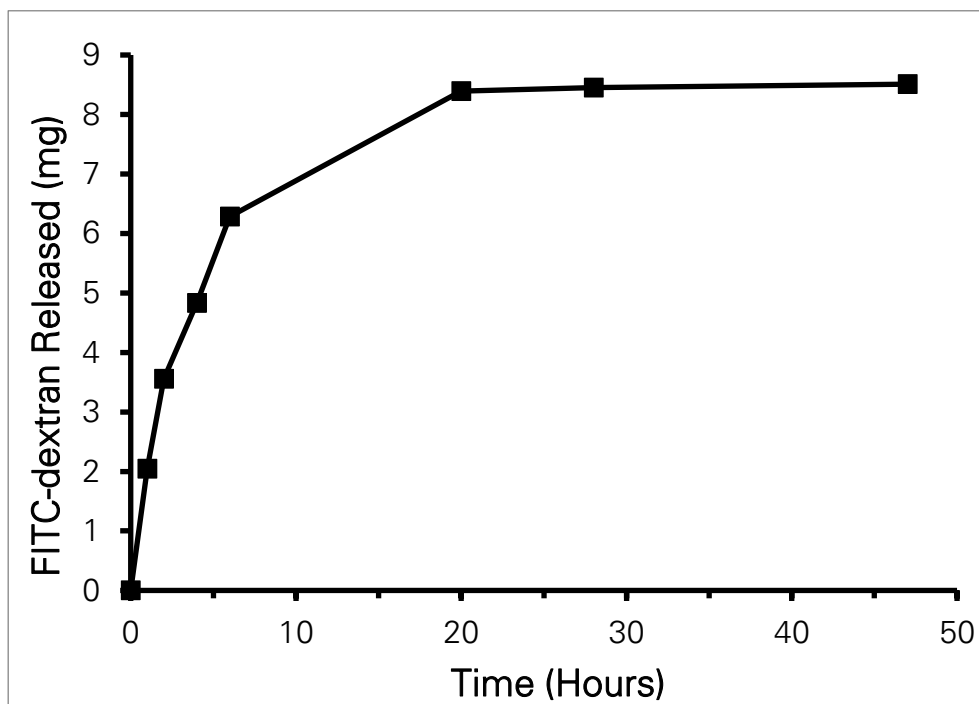
Figure 14: Release of (unmodified) 10 kDa FITC-dextran through a 100 kDa dialysis membrane. Although all the FITC-dextran within the dialysis bag was unencapsulated (i.e. equivalent to polymers released from DH-NPs), approximately 20 hours elapsed before a sufficient quantity was able to diffuse out to establish equilibrium. Polymer release measured using this method will therefore suffer from a delay of almost 1 day between release and detection of release (i.e. exiting from the dialysis bag by diffusion).

4.4.2 Lubricant Release by Degradation

The controlled degradation of DH-NPs was a phenomenon we observed early in our work with the technology. Since the hydrazone-forming crosslinking reaction between ADH and DexOx is readily reversible,[67] fragments of DexOx-ADH polymer are released as the reversal of sufficient crosslinks causes the polymer fragments to detach from the remainder of the nanoparticle. This degradation-controlled polymer release was found to proceed at a near-linear rate for a prolonged

period of time (several months), as shown in Figure 15. Since the DexOx-ADH polymer closely resembles the chemical structure of dextran (a material already designated as GRASE for use in ocular   lubricants by the FDA), it has considerable potential for providing effective ocular lubrication for DED patients.
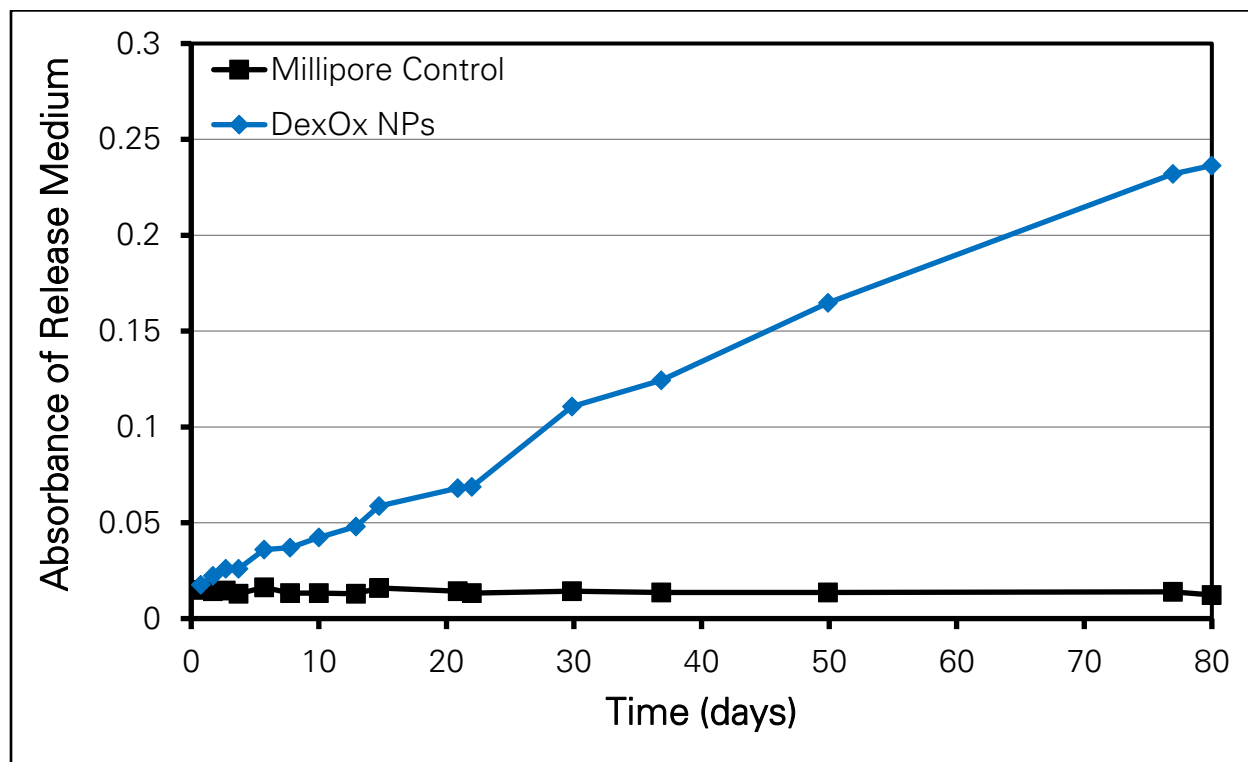
Figure 15: Release of DexOx-ADH polymer from DH-NPs (blue line) through their intrinsic degradation behaviour. The black line shows the absorbance of a control sample that was processed and analyzed identically but contained pure Millipore water in place of an aqueous suspension of DH-NPs. Polymer release was found to continue for over 2 months.

A series of experiments were thus conducted to further understand the degradation-controlled release properties of DH-NPs and its potential efficacy as a method of providing continuous ocular lubrication. The rate of DexOx-ADH polymer release was quantified, and several parameters were identified that allow this release rate to be optimized.

Figure 16 shows data from a set of experiments that examined the effect of crosslinker-polymer ratio and reducing agent treatment on DH-NP degradation rate. The effect of crosslinker-polymer ratio can be seen by comparing the 5% $R_{H/A}$ unreduced (blue) and 15% $R_{H/A}$ unreduced (orange) lines. An increased $R_{H/A}$ value caused the rate of DexOx-ADH polymer release to decrease significantly, especially during the critical first 24 hours of the study. This is the expected result, as an increased $R_{H/A}$ value indicates the formation of an increased number of DexOx-ADH crosslinks. As a result, each fragment of polymer is attached to the nanoparticle through a greater number of anchoring points, each of which must be broken in order to cause the polymer fragment to be released. Since the rate of crosslink hydrolysis remains the same, the sample with higher crosslinker-polymer ratio should release polymer more slowly because a larger number of crosslinks must be hydrolyzed. The significantly lower lubricant release observed for the 15% $R_{H/A}$ sample in comparison to the 5% $R_{H/A}$ sample is in accordance with this mechanistic prediction.

The second finding to be taken from the results in Figure 16 is related to the effect of reducing agent treatment. The expected release profile of an unreduced DH-NP sample with an $R_{H/A}$ value of 10% is shown by the dashed grey line without data markers, and represents a release rate that is greater than that of 15% $R_{H/A}$ NPs, but less than the rate observed for 5% $R_{H/A}$ NPs (according to the $R_{H/A}$ principle described in the previous paragraph). However, Figure 16 shows that treatment with reducing agent caused the DH-NPs represented by the solid grey line (labelled "10% $R_{H/A}$, R") to have the lowest lubricant release rate of all samples tested. The reduction in lubricant release rate is represented visually by the difference between the dashed grey line and solid grey line, and is caused by the decrease in hydrolysis rate induced by reducing agent treatment. This is the expected result, as reducing agent treatment converts hydrazone bonds into secondary amines which are substantially more resistant to hydrolysis. In this way, reducing agent

treatment makes DH-NPs more resistant to hydrolysis and reduces the rate of release of lubricating DexOx-ADH polymer.
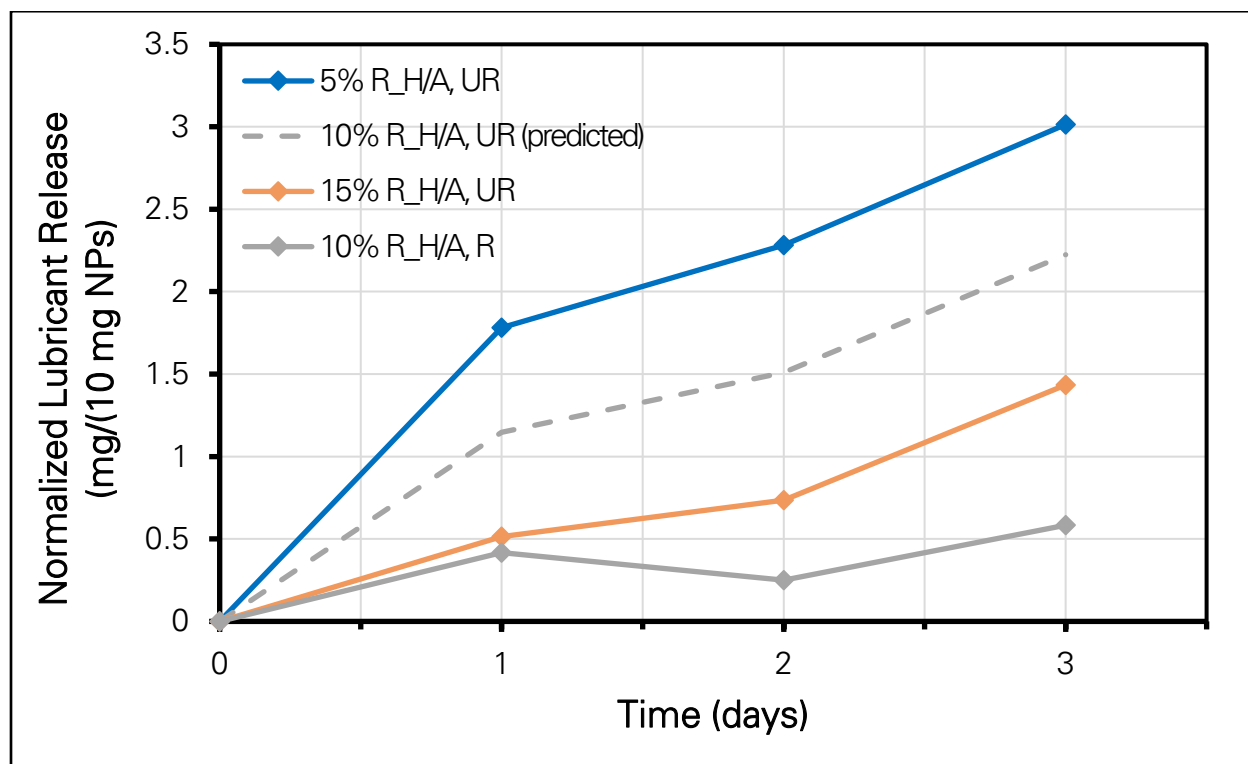
Figure 16: Effect of crosslinker-polymer ratio (R_H/A) and reduction (UR=unreduced, R=treated with reducing agent) on the rate of lubricant (DexOx-ADH polymer) release by DHNP degradation. Data point markers with solid lines indicate measured data points while the dashed line is a predicted data series shown for illustrative purposes. Mass of lubricant released has been normlaized by mass of DH-NPs used in the release study. A value of 1 indicates the release of 1 mg of DexOx-ADH polymer for every 10 mg of DH-NPs present at the outset of the release study.

Lubricant release rate from DH-NP degradation was also found to be greater at higher temperatures (Figure 17). Incubation at room temperature (19°C) instead of body temperature (37°C) caused the average degradation rate over a 3-day period to decrease by 43%. This result agrees with theory, as hydrolysis processes are known to typically accelerate at higher temperatures. It is expected that this property can be extended further, and that storage of DH-NPs at even lower temperatures (such as 4°C refrigeration) will slow degradation further. The potentially large reduction in degradation rate that may result would be advantageous for practical

purposes in the final ocular lubricant product, as it would extend shelf life without impairing on-eye performance. Further studies are therefore recommended to fully explore the range of degradation rate adjustment possible through temperature variation.
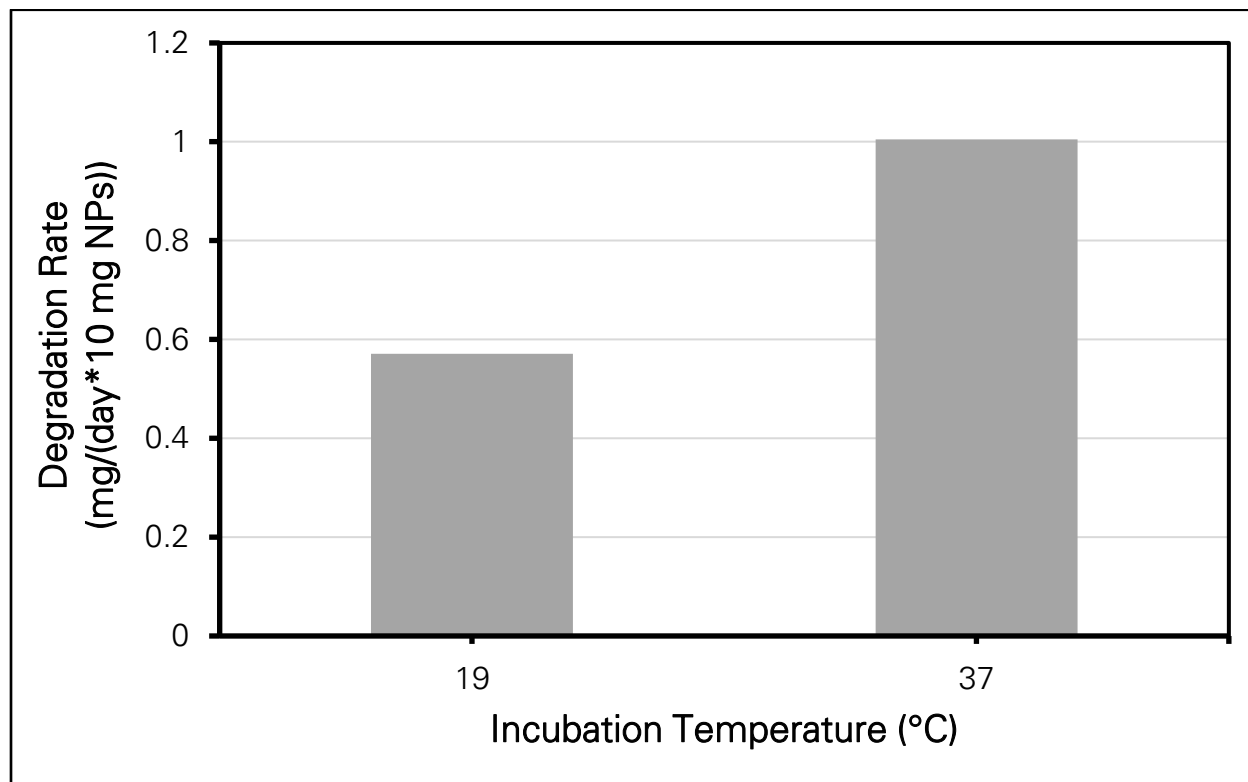
Figure 17: Rates of lubricating polymer release by degradation from DH-NPs stored at different temperatures. Higher temperature was found to induce faster DH-NP degradation.

The composition of materials released from DH-NPs during degradation was investigated using nuclear magnetic resonance. The filtrates of a release study (as described in Section 4.3.2) were resuspended in D_2O after lyophilization, and proton NMR spectra were acquired. The results contained peaks specific to both DexOx and ADH, revealing that the degradation products must be DexOx-ADH polymer fragments. The degradation products were further analyzed by ethyl carbazate titration, as described in Section 3.3.3. The results indicated the presence of aldehyde groups in the DexOx-ADH polymer fragments released from DH-NPs not treated with reducing agent (Figure 18). Reducing agent treatment was found to be 100% effective in eliminating all

aldehyde groups by reduction to hydroxyls. This is an important finding for safety considerations, as aldehyde groups are inherently prone to reaction with amines and other nucleophilic functional groups common in the human body. The use of reducing agent treatment to convert aldehydes into hydroxyls is therefore an effective and important method of enhancing the safety and biocompatibility of DH-NP formulations.
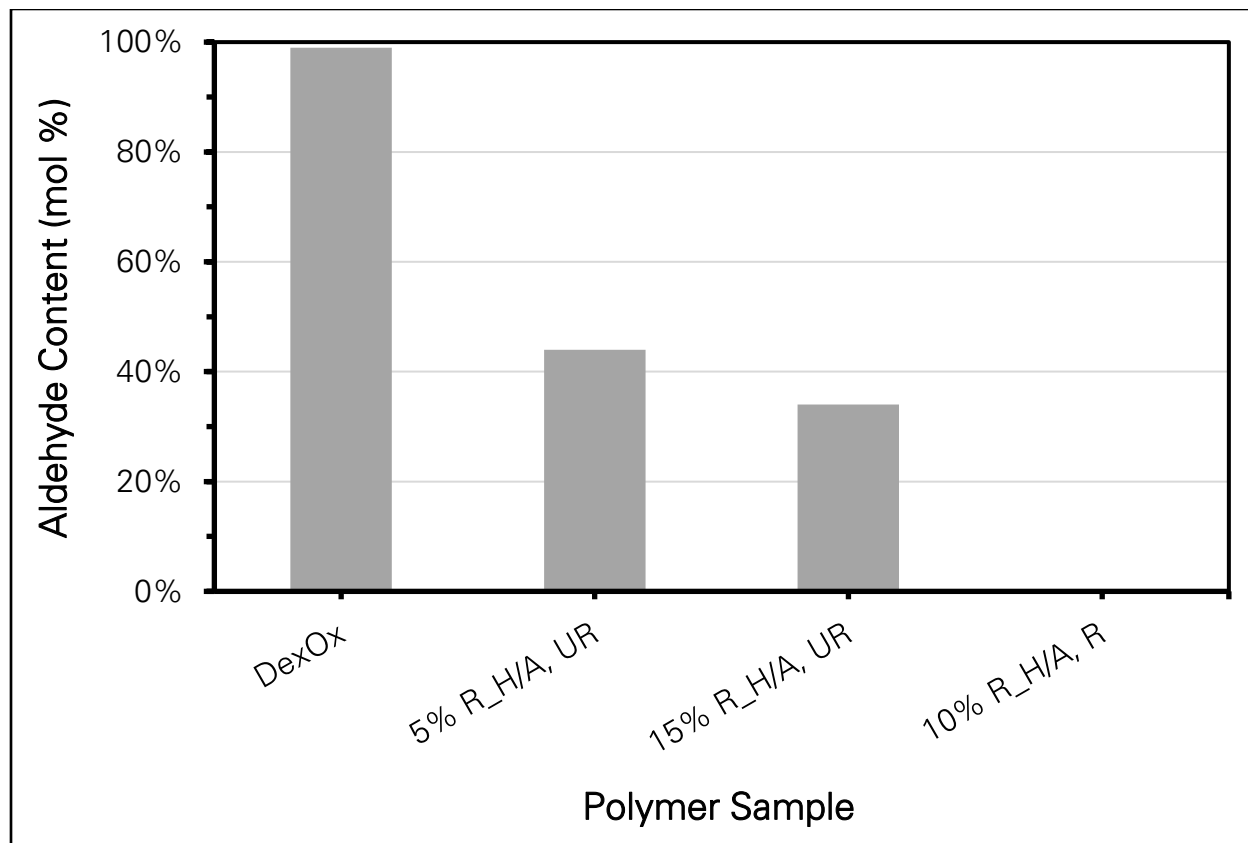
Figure 18: Aldehyde content of pure DexOx (prepared as described in Section 3.3.2) and DexOx-ADH polymers released from the degradation of various DH-NP variants, as measured by ethyl carbazate NMR titration. DH-NP variants tested include unreduced 5% $R_{H/A}$ NPs, unreduced 15% $R_{H/A}$ NPs, and 10% $R_{H/A}$ NPs that were treated with reducing agent.

Figure 19 shows daily release rates of DexOx-ADH lubricant for the studies presented in Figure 16. It can be seen once again that the unreduced NPs with 5% $R_{H/A}$ degraded the fastest while 10% $R_{H/A}$ NPs treated with reducing agent had the lowest rate of degradation. It is also interesting to note that the differences between DH-NP samples were most pronounced at different

times. The greatest enhancement in release rate was observed for the unreduced 5% $R_{H/A}$ sample on day 1, while the greatest attenuation in release rate was for the reducing agent-treated 10% $R_{H/A}$ sample on days 2 and 3. Additional trials are recommended to fully explore the daily release rates of other DH-NP variants and confirm consistency. The lubricant release rate in the first 24 hours is especially critical, as this is an approximation of the quantity of polymer likely to be delivered to a patient's eye (since the residence time of DH-NPs on the ocular surface is expected to be approximately one day).
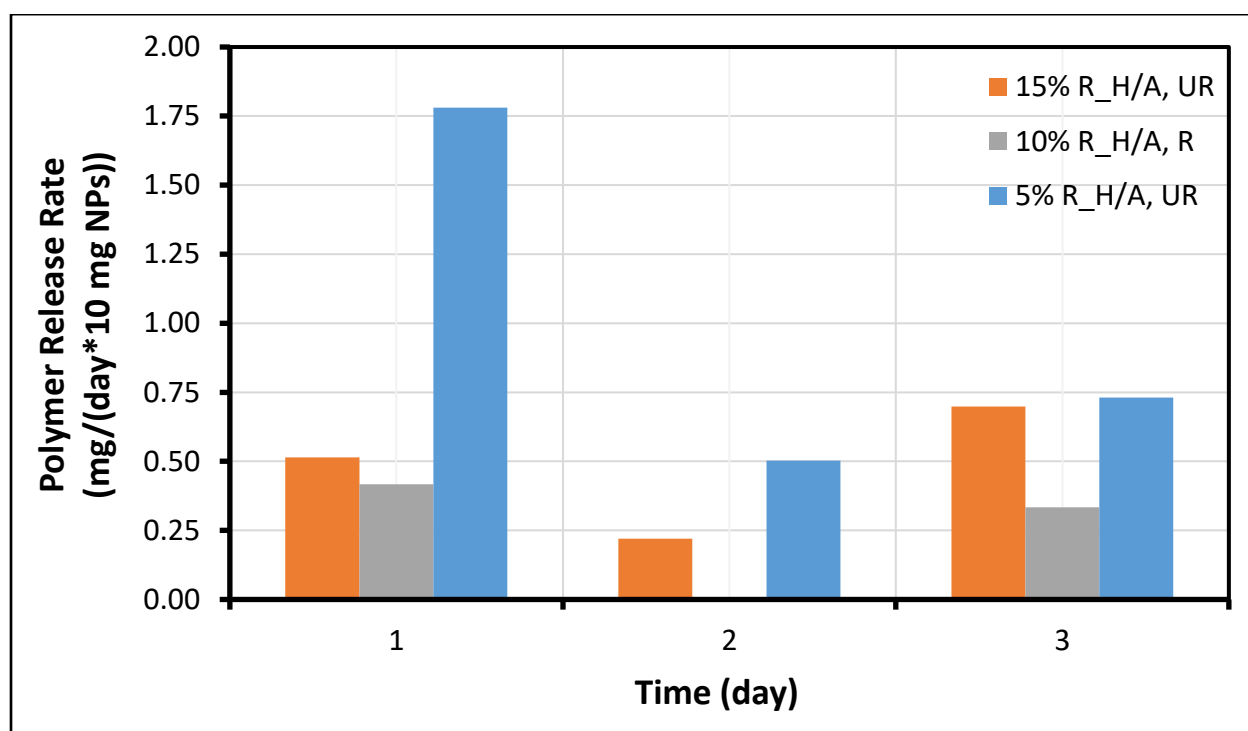
Figure 19: Rate of daily DexOx-ADH polymer release for the 3-day studies represented in Figure 16. DH-NP variants include unreduced 15% $R_{H/A}$ NPs (orange), 10% $R_{H/A}$ NPs that were treated with reducing agent (grey), and unreduced 5% $R_{H/A}$ NPs (blue).

A preliminary assessment of the efficacy of DH-NPs as an ocular lubricant formulation can be made using the normalized release rates in Figure 19. A rate of 5 mg/(day*10mg NPs) would allow for 5 mg of lubricating polymer to be delivered to a patient's eye from a single 50 µL drop over the course of 24 hours. Note that this metric assumes that 100% of the DH-NPs in the eye

drop will adhere to the patient's eye and that a 20% w/v solution of DH-NPs can be prepared with physical properties appropriate for an eye drop (viscosity, osmolarity, etc). Achieving 5 mg/(day*10mg NPs) release would approximate the properties of Lacrisert, an ocular insert that releases 5 mg of lubricant over the course of its dissolution period (patients usually administer one insert per day which dissolves over a 4 to 8 hour period). Since Lacrisert has been shown to be effective in numerous clinical studies, the 5 mg/(day*10mg NPs) rate was targeted for DH-NP lubricant release with the goal of achieving the same clinical efficacy as Lacrisert.

As shown in Figure 19, the maximum daily release rate achieved was 1.8 mg/(day*10 mg NPs), and thus 36% of the target rate of 5 mg/(day*10 mg NPs). Further work is therefore required to engineer DH-NP variants capable of achieving the target lubricant release rate. Since the preliminary work described in this thesis was sufficient to reach a degradation rate within the same order of magnitude as the target, it is anticipated that further engineering efforts will be successful in reaching the targeted lubricant release rate. Beyond fully optimizing the rate-affecting parameters identified above, a method of further increasing degradation rate may be the use of higher molecular weight DexOx in DH-NP synthesis. It is predicted that higher molecular weight DexOx would enable successful hydrogel formation at lower crosslinker-polymer ratios (since a longer DexOx chain can be incorporated into the nanoparticle with the same number of ADH molecules). The lower $R_{H/A}$ values would then allow faster degradation-controlled release of DexOx-ADH lubricating polymer, bringing the technology closer to its target lubricant release rate.

A number of other challenges remain to be overcome for the successful development of a next-generation ocular lubricant based on DH-NPs. One of the major concerns is the potential safety risk due to presence of aldehyde groups in the DH-NPs. As shown in Figure 18, aldehydes

can be quantitatively converted to benign hydroxyl groups by treatment with sodium borohydride. However, reducing agent treatment has a number of implications on other DH-NP properties, and it remains to be seen whether sufficient aldehyde reduction can be achieved while maintaining DH-NP yield, mucoadhesion, degradation rate, and other properties within satisfactory ranges. A prudent method of minimizing use of reducing agents while also minimizing residual aldehyde content may be to synthesize DH-NPs using DexOx with lower degrees of oxidation. Instead of always synthesizing DexOx with oxidation degrees approaching 100%, dextran can be oxidized less extensively such that the degree of oxidation is only slightly higher than the sum of the targeted $R_{H/A}$ and PBA conjugation values. This would ensure that all the aldehyde groups participate in either crosslink bonds or are used for PBA conjugation, leaving a minimal level of residual aldehyde groups. However, it should be noted that the DexOx-ADH polymers released during degradation are likely have some residual level of aldehydes due to the crosslink bonds that had to be broken/reversed to allow the polymer fragment to be released.

Another limitation of the degradation-controlled lubricant release behaviour of DH-NPs is the unknown clinical lubrication efficacy of DexOx-ADH polymers. Although the efficacy is expected to be quite high due to close resemblance in chemical structure with dextran (an FDA-certified GRASE lubricant), this must be verified through further testing. Some recent literature studies featuring ocular lubricants with similar composition show promising results.[66] A recent authoritative review also found that the efficacy of ocular lubricants does not have a significant association with the chemical structure of the lubricating agent. This provides further confidence that the DexOx-ADH polymers released as lubricants from DH-NPs will prove effective in relieving treating DED. However, thorough testing must be completed to demonstrate this efficacy,

a process that may present cause a significant prolongation of the regulatory approval process for providing DH-NPs to DED patients.

Shelf life considerations present another challenge for the application of DH-NPs in next-generation ocular lubricant products. The lubricant release studies in this section were carried out on DH-NPs synthesized immediately prior to beginning the study (storage time of less than one week). During real-world use however, an ocular lubricant batch must retain its potency and lubricant release rate for several months after manufacture (to allow sufficient time for delivery to the patient, as well as satisfactory duration of use after the patient has the product in their possession). Although some methods of prolonging shelf life have been identified above (such as storage at low temperatures), it remains to be seen whether these strategies are sufficient to extend shelf life to the required level.

Lubricant release by DH-NP degradation is therefore a highly promising method of achieving the targeted sustained release properties for application in next-generation ocular lubricant formulations. However, a number of challenges remain which must be addressed in future studies.

4.4.3 Lubricant Release by Diffusion

An alternate approach to achieving sustained release of lubricating polymer on the ocular surface is to encapsulate a separate ocular lubricant within the DH-NPs. The NPs can be loaded with this ocular lubricant by soaking them in a high concentration solution of the material (referred to as the "storage solution"). This causes the lubricating polymer to diffuse into the interior spaces of the DH-NPs, and an equilibrium is established with the storage solution that remains intact for the duration of the lifetime of the DH-NPs. In the case of real-world patient use, the eye drop

dispenser (the same design of container as is commonly used currently for artificial tears) would

contain this same mixture of storage solution and NPs. When the patient administers a drop of the

formulation onto their eye, the ocular lubricant storage solution is washed away (by blinking and

tear production), while the loaded DH-NPs remain on the ocular surface due to their mucoadhesive

property. The chains of lubricating polymer that had diffused into the NPs then slowly diffuse out,

hydrating the patient's eye over the designated period of time (right-hand side of Figure 13; the

blue lines represent the DexOx-ADH polymer of which the DH-NPs are composed while the red

lines represent the separate lubricating polymer that has diffused into the DH-NPs from the storage

solution).

This diffusion-controlled approach is distinct from the degradation controlled lubricant

release described in Section 4.4.2 in several ways. Firstly, the active lubricating agent released

onto the patient's eye is not the DexOx-ADH polymer released from DH-NP degradation. Rather,

the separate lubricating polymer (such as hyaluronan or polyvinylpyrrolidone or others) found

within the storage solution is the active ingredient that diffuses into the DH-NPs and is then

released onto the patient's eye after administration. The use of this storage solution (containing a

high concentration of the lubricating polymer of interest) is also a unique characteristic of

diffusion-controlled delivery, as degradation-controlled lubricant release does not require the use

of any specialized storage solution. Because the mechanism of release is diffusion instead of

nanoparticle degradation, the DH-NPs used for diffusion-controlled lubricant release are

synthesized to minimize degradation (thereby prolonging shelf life and allowing aldehyde content

to be minimized). The DH-NPs synthesized for diffusion-controlled lubricant delivery are also

designed to have greater density (through a higher DexOx concentration and larger $R_{H/A}$ value) in

order to achieve longer diffusion times (and thereby extend the duration of delivery and ocular lubrication).

The unique characteristics of diffusion-controlled lubricant release confer several advantages over degradation-controlled release. Firstly, the diffusion-controlled release approach provides even better patient safety as the DH-NPs are designed to undergo minimal degradation. It is thus possible to fully reduce DH-NPs to ensure they contain no residual aldehyde groups, virtually eliminating the possibility of patient exposure to aldehydes.

Lubricant release by diffusion also provides greater versatility in the design of the formulation. Any water-soluble ocular lubricant (or combination of multiple lubricants) can potentially be delivered to the eye in a continuous manner by incorporating the desired lubricant(s) into the storage solution. This allows DH-NPs to release lubricants that already been tested extensively for their efficacy in treating DED and possess the FDA Generally Recognized as Safe and Effective (GRASE) designation. This may increase the efficacy of the DH-NP ocular lubricant formulation further, since the lubrication efficacy of GRASE ocular lubricants may be higher than that of DexOx-ADH polymer. Because the GRASE lubricants have already undergone extensive preclinical and clinical testing for safety and efficacy, their use as the active ingredients in a diffusion-controlled DH-NP ocular lubricant product would also simplify the pathway to regulatory approval. This is because fewer components of the formulation would be novel and require safety and efficacy testing, and the accelerated approval route for over-the-counter products could potentially be utilized.

The diffusion-controlled release mechanism may also provide greater shelf life for the final ocular lubricant product. This is because there is no lubricant release during storage, since an

equilibrium in lubricant distribution is achieved between the DH-NPs and the storage solution in which they are suspended. This may enable the shelf life to be considerably longer than in the case of DH-NP formulations that utilize degradation-controlled lubricant release, and also eliminate the requirement for storage in cold conditions or dry form. These considerations may simplify the manufacturing process and make the final product more practical for commercial distribution.

Overall, the diffusion-based approach to sustained release of lubricant from DH-NPs is expected to offer several advantages over the degradation-based method. However certain limitations are also anticipated, such as a potential reduction in lubricant delivery capacity. Since the concentration of the storage solution must be limited to levels that are comfortable to the patient (important considerations include viscosity, optical clarity, safety, etc), the quantity of lubricant that can be loaded into the DH-NPs will also be limited. It remains to be seen whether the achievable lubricant loadings are sufficient to offer effective treatment for DED patients.

While a number of efforts were undertaken to evaluate the in vitro efficacy of DH-NPs designed to release ocular lubricant by diffusion, further method development and experimentation is still required to obtain a reliable assessment. The experiments conducted to date aimed to quantify the release of lubricant from DH-NPs, but were unsuccessful due to an inability to remove unencapsulated lubricant while keeping the lubricant-loaded DH-NPs intact. The method of choice for our studies was solvent precipitation; isopropyl alcohol was found to be capable of precipitating DH-NPs while solubilizing polyvinylpyrrolidone (PVP), a common GRASE ocular lubricant. A series of experiments were performed in which DH-NPs were suspended in PVP solutions of varying concentration (the "storage solution") for several days. The solution containing PVP and DH-NPs (now loaded with PVP) was then added to a sufficient volume of chilled IPA to precipitate the DH-NPs, while the PVP would remain in the supernatant. The ratio of IPA volume to PVP

mass had to be maintained above a threshold value to allow for successful precipitation of the DH-NPs (due to the surfactant-like properties of PVP). Upon successful precipitation, the DH-NPs were thoroughly dried and then resuspended in pure water through stirring and probe sonication. Resuspension in water marked the beginning of the release of encapsulated PVP polymers from within the DH-NPs, and release samples were taken at regular intervals thereafter using the centrifugal filtration method described in Section 4.3.2. Figure 20 shows a sample result, which showed that the continuous release of some DexOx-ADH polymer was interfering with the PVP measurement (by UV-visible absorbance at 228 nm) and therefore making the measurements increase even after PVP release had ended (the study was continued beyond what is shown in Figure 20, and release was found to continue at an approximately linear rate for at least 24 days).
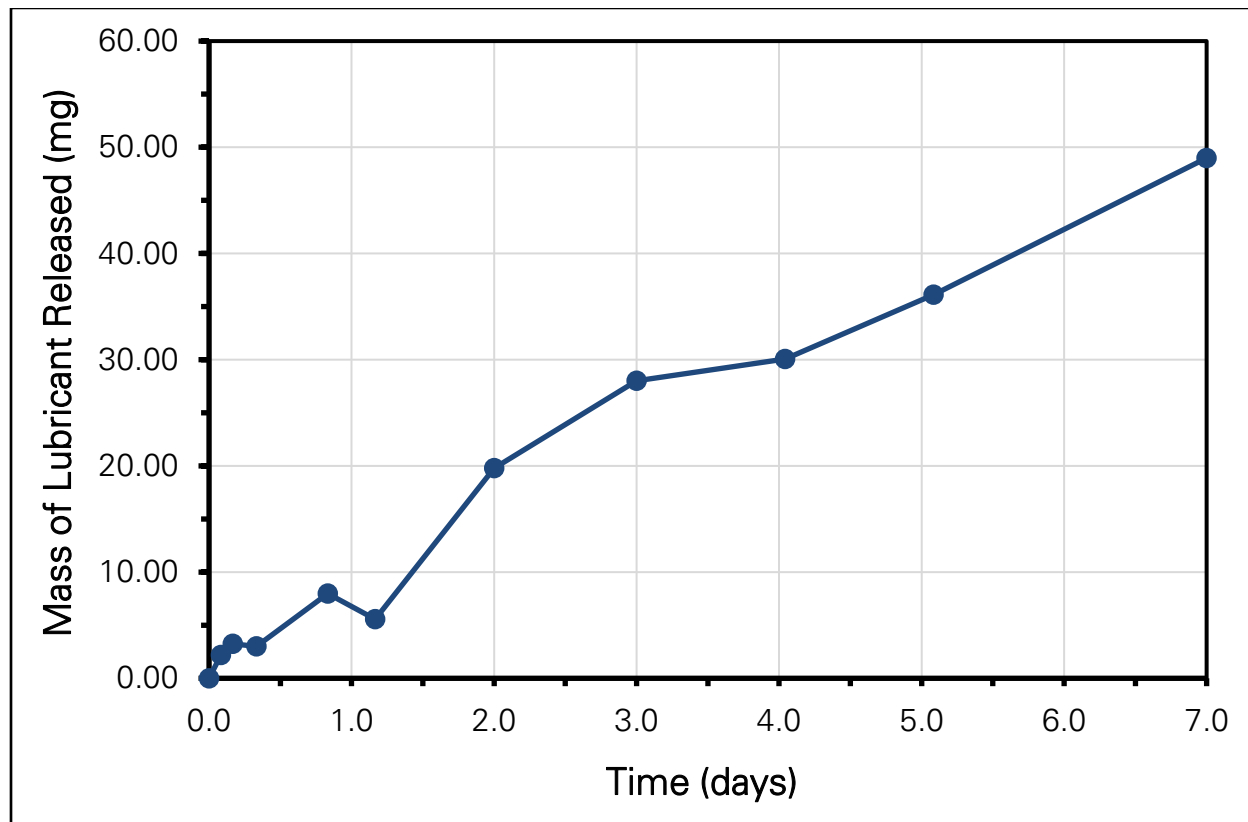

Figure 20: Results of release study attempting to measure PVP release by direct 228 nm absorbance measurement. Small levels of continuous DexOx-ADH polymer release were found to interfere with PVP measurements and thus make the measurements increase despite no PVP release.

To eliminate the interference of DexOx-ADH polymers in PVP measurements, a high-performance liquid chromatography (HPLC) method was developed to separate DexOx-ADH from PVP prior to absorbance measurement. The method utilized a Waters HPLC instrument with an Agilent C-18 column as the stationary phase and a 2% IPA – 98% water mixture as the mobile phase. The method provided sufficient data to draw qualitative conclusions but had some difficulty in resolving the DexOx-ADH and PVP peaks, with the majority of DexOx-ADH polymer eluting at a retention time of less than 3 minutes, but small peaks also appearing at retention times of 4.5 and 5.2 minutes. The PVP peak was found to elute at 4.8 minutes, which caused overlap with the smaller DexOx-ADH peaks on some occasions.

Despite the lack of resolution, it became clear after several experiments that some DH-NP samples tested contained almost no PVP despite being soaked in a PVP storage solution for several days. The quantity of PVP released from the DH-NPs was also found to be relatively low and inconsistent. Each of these findings indicated that the solvent washing method of removing unencapsulated PVP was likely also extracting PVP from the interior of the DH-NPs, leaving almost no PVP within the DH-NPs to be measured during the release study. The inability of IPA washing to remove unencapsulated PVP without also extracting encapsulated PVP chains is therefore a critical flaw in the release study method we developed. The method must be changed at a fundamental level to enable effective and gentle isolation of the loaded DH-NPs, followed by accurate measurement of PVP release. A potential solution is to utilize a sialic acid-coated chromatography column that will bind PBA-coated DH-NPs while allowing unencapsulated PVP of to elute immediately. Allowing the column to run for an additional 24 hours and collecting aliquots of eluent at regular intervals would reveal the quantity of PVP released at each time

interval. HPLC may be required to separate PVP from any DexOx-ADH released for accurate measurement.

4.5 In Vivo Acute Biocompatibility Assessment

Observation of the rabbits in the acute biocompatibility study was done by visual inspection and also slit lamp examination. A trained experimenter used a scale of 0 to 4 to rate conjunctival redness, level of ocular secretions, corneal opacity, and iris involvement. The 5-day mean of these measurements is shown in Figure 21. The results show excellent tolerance of the DH-NP formulation, with all four rating categories yielding average values below one. In fact, corneal opacity and iris involvement were found to be zero at all time points, while conjunctival redness and secretion usually yield values of zero or one. In all cases, no significant difference was found between the control and the DH-NP formulation. It is highly likely that the non-zero values of conjunctival redness and secretion are attributable to the environmental and procedural stresses of the study upon the animals.
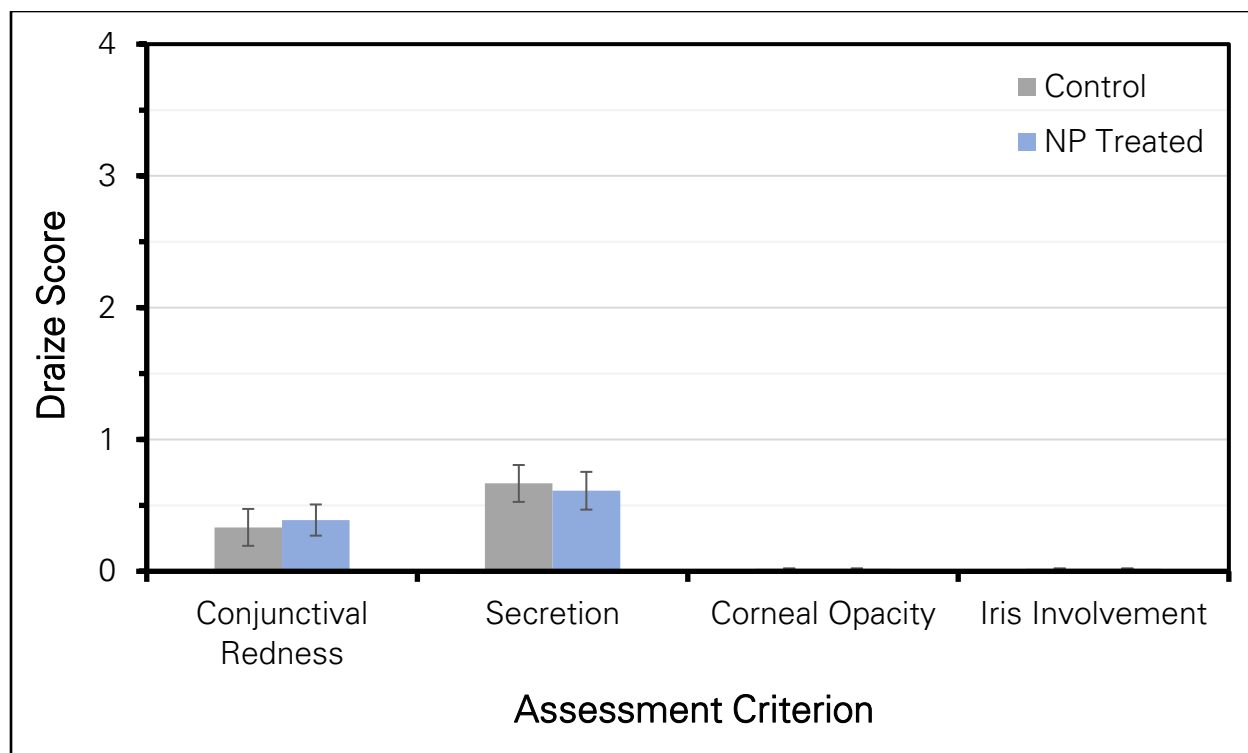
Figure 21: Acute biocompatibility Draize scores averaged over the 5-day study period

Histopathological analysis of the ocular tissues of the animals in the study also indicated excellent tolerance of the DH-NP formulation (Figure 22). All tissues in both control and DH-NP-treated eyes retained normal morphology and showed no signs of abnormal inflammation or other concerning changes such as hyperplasia, hypervascularization, or hyperkeratosis. In the corneal tissues (Figure 22 slides A and B), a healthy cell layer structure was observed with normal epithelial maturation and renewal. No signs of inflammation were observed. Conjunctival tissues (Figure 22 slides C and D) featured a healthy quantity of goblet cells with plentiful secretory mucin production. Isolated lymphocytes and eosinophils were observed in the bulbar conjunctival epithelium and stroma of both untreated and treated eyes, but these findings were consistent with the characteristics of healthy mucous membranes exposed to the outside environment. No edema or abnormalities in vasculature or lymphoid tissues was noted.
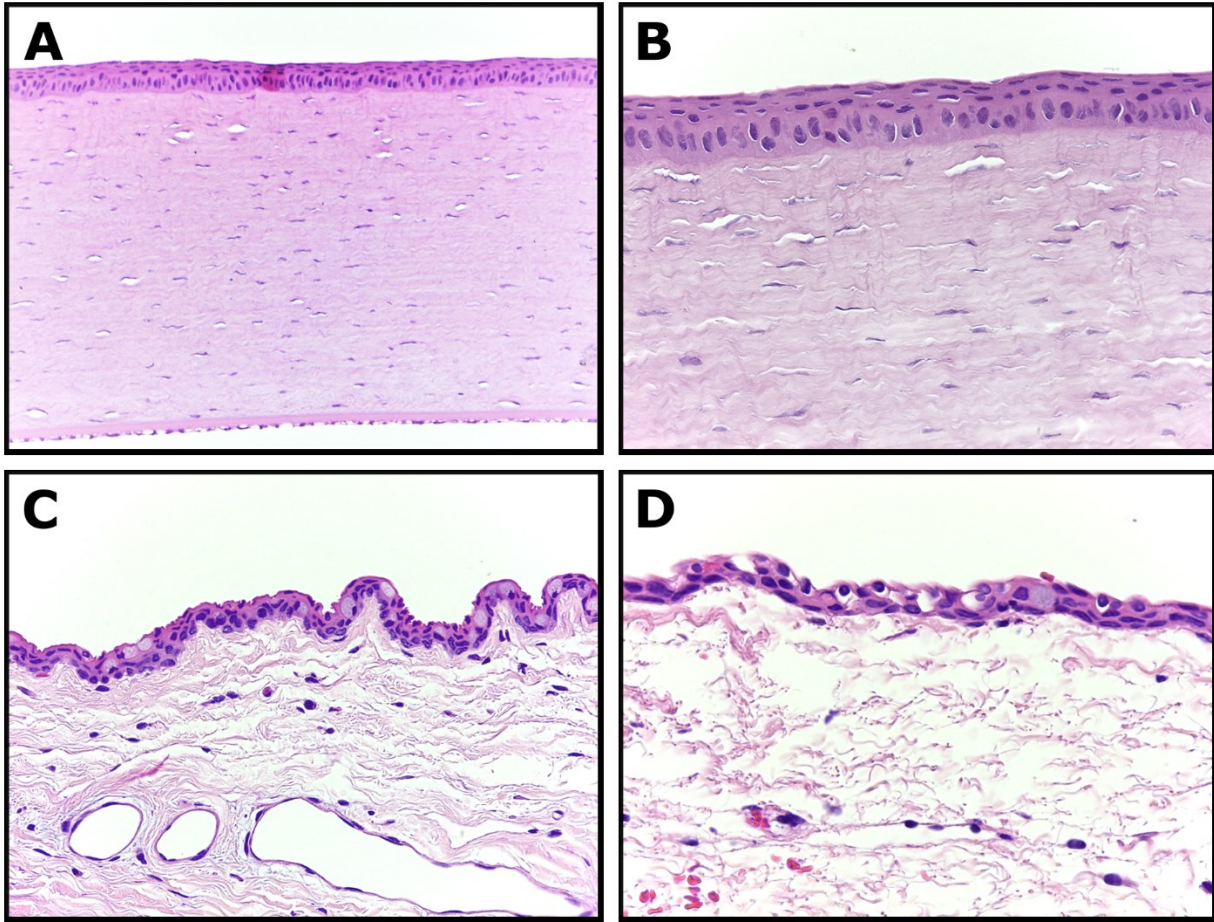
Figure 22: Histopathological slides showing ocular tissues of rabbits used in the acute biocompatibility study. All slides show tissues of the nanoparticle-treated eye. Slides A and B show the cornea (low vs. high magnification), while slides C and D show the bulbar conjunctiva.

4.6 Conclusion

DH-NPs are anticipated to have excellent efficacy as part of ocular lubricant formulations due to their ability to provide sustained release of ocular lubricant and their glycocalyx-mimicking composition. In this chapter, two distinct mechanisms of sustained lubricant release were proposed: degradation-controlled release and diffusion-controlled release. Biocompatibility of DH-NPs was also tested in an acute study using a rabbit model.

In the degradation-controlled mechanism, the patient's eye is lubricated by the release of DexOx-ADH polymer fragments from controlled degradation of DH-NPs. This release method is advantageous due to a potentially higher capacity of lubricant delivery. It was also successfully investigated in further detail through a series of studies that identified key parameters that regulate DH-NP degradation rate (and consequently the rate of ocular lubricate delivery to the eye). It was determined that lower crosslinker-polymer ratios ($R_{H/A}$) lead to faster degradation, while both reducing agent treatment and lower temperatures cause degradation rate to decrease. The final degradation rates achieved in our studies were below the target of 5 mg/(day*(10 mg NPs)), but within the same order of magnitude. It is therefore projected that additional optimization of the degradation-regulating parameters (including both parameters already tested and novel ideas such as DexOx molecular weight) will enable the targeted lubricant release rate to be met.

Diffusion-controlled release is an alternative mechanism of ocular lubricant delivery that may surpass even degradation-controlled release in efficacy within ocular lubricant formulations. In the diffusion-controlled mechanism, ocular lubrication is achieved by the release of encapsulated lubricating polymers (such as hyaluronan, PVP, etc) from within the hydrogel core of DH-NPs (see Figure 13 in Section 4.2). Controlled release of the encapsulated lubricants occurs

by diffusion, while degradation of the DH-NP is minimized as much as possible. Diffusion-controlled release of ocular lubricant is expected to offer improved safety (due to more complete elimination of aldehydes), greater efficacy (due to ability to deliver GRASE lubricant(s)), longer shelf life, greater versatility of lubricant selection, and potentially streamlined regulatory approval. However, these expectations could not be tested due failure of the method developed for quantifying diffusion-controlled lubricant release. Future studies should place a high level of priority on developing a method of removing unencapsulated lubricant from DH-NP samples while keeping the loaded DH-NPs intact. This would enable accurate characterization of diffusion-controlled release from DH-NPs and allow the merit of the release mechanism to be evaluated against degradation-controlled lubricant release.

Biocompatibility of the DH-NPs was also tested in an acute in vivo study with three rabbits as the subjects. Despite administration of the DH-NPs at an exaggerated frequency of six times daily, both Draize score (i.e. observation of symptoms by slit lamp) and histopathology indicated excellent tolerance and no adverse effects over the course of the study. The DH-NPs were thus shown to be biocompatible in the preliminary in vivo acute tolerance study.

5.0 Conclusion and Future Work

5.1 Summary and Conclusion

This thesis described the development of novel dextran hydrogel nanoparticles (DH-NPs) for incorporation into next-generation ocular lubricant formulations for dry eye disease (DED). The nanoparticles are based upon a hydrogel core consisting of oxidized dextran chains (DexOx) crosslinked with adipic acid dihydrazide (ADH). The surface of this hydrogel core is coated with phenylboronic acid (PBA) to make the nanoparticles mucoadhesive.

When administered by a patient in the form of an ocular lubricant eye drop, the DH-NPs are designed to diffuse to the ocular surface and bind to transmembrane mucins on the epithelial cells of the cornea and conjunctiva. Once anchored in place the DH-NPs are expected to remain on the eye for approximately 24 hours, far longer than the less than 30 minute retention time of today's traditional ocular lubricant eye drops. Throughout this extended ocular residence time, DH-NPs are designed to continuously release lubricating polymers, stabilizing the tear film and providing lubrication to the eye. Due to their polysaccharide-based composition, the DH-NPs are also expected to act as a reinforcement for the glycocalyx, the polysaccharide coating of the ocular surface's epithelial cells that is critical for hydrating and protecting the eye (but sustains significant damage due to dry eye disease). When taken together, DH-NPs' dual modes of action of lubricant release and glycocalyx reinforcement are expected to provide substantial long-lasting therapeutic benefit for DED patients.

Chapter 2 of this thesis provided a review of the literature relevant to the this technology, including a detailed overview of dry eye disease and some recent developments in the ocular lubricant formulations used for its treatment. The literature provides strong evidence in support of

the proposed DH-NP technology, as glycocalyx damage has been found to be one of the fundamental etiological driving forces behind dry eye disease. Successful reinforcement using DH-NPs would therefore enhance ocular lubrication while also hampering the vicious cycle that is responsible for the propagation of DED. Literature also highlights short ocular residence times as one of the main shortcomings of current ocular lubricant formulations, with most products entirely eliminated from the eye within 20-30 minutes of administration. Nanotechnologies have generated considerable interest in the area of ocular lubricants due to their enhanced ocular residence times and inconspicuous nature. The covalent bond mediated mucoadhesion of DH-NPs is particularly well-suited to enhance ocular retention because it is stronger than the electrostatic mucoadhesion implemented in most nanotechnologies investigated for use in ocular lubricants thus far. Sustained release of ocular lubricant throughout the prolonged residence time of DH-NPs makes the technology particularly promising for enhancing the efficacy and duration of action of ocular lubricants in DED treatment.

DH-NP synthesis, characterization, and methods of tuning key properties of the synthesis process and resulting nanoparticles were presented in Chapter 3. The synthesis process began with the production of oxidized dextran, followed by formation of hydrogel nanoparticle cores via crosslinking with ADH within a water-in-oil nanoemulsion. If desired, reducing agent treatment and/or PBA coating was then performed at this stage or after removal of surfactant and organic solvents. Three methods of surfactant/solvent removal were presented, each with unique advantages and limitations. The method used in a given study was selected based upon the required nanoparticle yield, special needs of the DH-NP subtype (such as low density or extensive PBA coating), importance of controlling NP concentration in subsequent studies, and shelf life considerations. A set of key synthesis process parameters were also identified that allowed the

final properties of the DH-NPs to be tuned. Higher crosslinker-polymer ratio increased mucoadhesion strength (K_{SV}), while lower DexOx concentration produced higher nanoparticle yields (unless solvent precipitation used for purification), and less reducing agent treatment led to both greater mucoadhesion strength and higher NP yields. Additionally, higher PBA feed quantity was found to decrease NP yield due to higher aggregation but result in higher PBA conjugation and mucoadhesion strength (until plateau was achieved). These parameters formed a basis for the customization of DH-NP properties according to the application needs determined by the experimenter.

Our studies on the suitability of DH-NPs for use in ocular lubricants are presented in Chapter 4. Two distinct methods were proposed for the sustained delivery of ocular lubricant, namely degradation and diffusion. In degradation-controlled lubricant release, the hydrogel cores of the DH-NPs undergo controlled hydrolysis to release fragments of DexOx-ADH polymer that lubricate the patient's eye. Our studies achieved a maximum lubricant release rate (i.e. degradation rate) of 1.8 mg/(day*10 mg NPs), which approaches the targeted clinically-effective rate of 5 mg/day belonging to the Lacrisert ocular insert. It is therefore expected that some additional optimization will enable this target to be reached with DH-NP technology. A series of parameters capable of altering this release rate were also identified in our studies, which serve as a basis for further optimization efforts. It was found that lower crosslinker-polymer ratios ($R_{H/A}$) cause degradation to occur more rapidly, while both reducing agent treatment and lower temperatures lead to slower degradation. Various trials were also conducted to explore the substantial anticipated merits of diffusion-controlled lubricant release, but were unsuccessful due to a lack of suitable methods. This is recommended to be a major area of future work due to the significant projected advantages of the diffusion mechanism over degradation. Lastly, an in vivo

biocompatibility study was conducted over the course of 5 days to assess acute toxicity and tolerance of DH-NPs. Despite the exaggerated administration frequency, both slit lamp examinations and histopathological analysis indicated excellent tolerance and no substantial areas of concern.

Overall, it is believed that DH-NP technology holds a high level of promise as the basis of a next-generation ocular lubricant formulation. Work on DH-NP technology completed to date has enabled the development of a reliable method of synthesizing the nanoparticles with considerable flexibility in key properties such as nanoparticle diameter, density, crosslinker-polymer ratio, PBA conjugation degree, mucoadhesion strength (K_{SV}), and degradation rate. Critically, all our studies to date indicate excellent biocompatibility of the DH-NP platform, with our in vivo acute tolerance study showing no signs of concern for patient safety in the future. The lubricant release properties of DH-NPs have been successfully tuned to approach that of the clinically-effective Lacrisert product, and it is expected that this target can be fully achieved with further optimization. Effective mucoadhesion has been demonstrated in vitro, and it is expected that 24-hour ocular retention times can be achieved in vivo with some optimization. Realization of these lubricant release and ocular retention targets are expected to provide DH-NPs with powerful ocular lubrication properties unmatched by any product currently on the market. When combined with the substantial potential benefits of glycocalyx reinforcement, the DH-NP platform presents a great deal of promise to improve the lives of millions of DED patients around the world.

5.2 Recommendations for Future Work

In reviewing the progress made in the development of DH-NP technology, it should be noted that the majority of research objectives outlined in Section 1.2 have been achieved. Outstanding items include a portion of objective 1a (maximizing NP yield), a portion of 2a (measuring crosslinking degree of DH-NP hydrogel cores), a portion of 3b (in vitro assessment of mucoadhesion), and objective 3c (in vivo evaluation of lubrication efficacy). The majority of these objectives are included in the revised recommendations below, in rough order of priority. These suggestions were formulated based on the current state of knowledge for the DH-NP platform, with the goal of solidifying the pre-clinical data portfolio. Subsequent stages of development (clinical trials and commercialization efforts) are not included in this summary.

1. Characterize and optimize diffusion-controlled lubricant release. This is highest priority due to the many projected additional benefits over degradation-controlled release. A method of separating unencapsulated lubricant and then characterizing release kinetics must be developed.

2. Continue studies on optimizing degradation-controlled lubricant release. Investigate methods of further accelerating release such as use of higher molecular weight DexOx. Investigate methods of enhancing shelf life in parallel, such as further reduction in storage temperature (refrigeration and freezing)

3. Conduct in vivo ocular retention trials to further optimize mucoadhesive property. Conjugate fluorescent marker onto DH-NPs to enable tracking by fluorescent detector in confocal scanning laser ophthalmoscope

4. Verify lubrication capacity of DexOx-ADH polymers released by DH-NP degradation. In vitro cell line work is likely most suitable.

5. Further refinement of DH-NP synthesis process. Areas for improvement include understanding why K_{SV} decreases upon reducing agent treatment, developing calcium chloride precipitation as a stand-alone method of surfactant/solvent removal, scale-up, and others

6. Explore methods of synthesizing higher density DH-NPs, to enable greater resiliency against aggregation and potentially prolong diffusion-controlled release of lubricants. One strategy for achieving this may be to increase the molecular weight of DexOx used during nanoparticle formation

7. Conduct in vivo trials to assess the efficacy of the most promising DH-NP-based lubricant formulation (after extensive in vitro testing).

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