Surface Modification of Model pHEMA Contact Lenses with Aptamers for Controlled Drug Release

SURFACE MODIFICATION OF MODEL pHEMA CONTACT LENSES WITH APTAMERS FOR CONTROLLED DRUG RELEASE

By

AAKASH SHAW, B.Sc

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AUTHOR: Aakash Shaw, B.Sc (McMaster University)

SUPERVISOR: Professor Heather Sheardown

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ABSTRACT

An efficient delivery system and patient compliance are two of the most important factors for any drug delivery system design to be successful. The current standard, particularly to the ocular anterior segment, are topical applications including eye drops. However, due to ocular physical barriers including blinking, the varying tear film layers, and the structure of the corneal epithelium, less that 5% of drug reach the target tissue from a single eye drop dose. While most treatment regiments combat this with increased frequency of dosage and higher than needed concentrations, the need for a more efficient and controlled system has been recognized to reduce the risk of possible side effects. Contact lenses (CL) have been a widely discussed potential drug delivery device given their accepted use in the population, their ability to hold drug, as well as their placement on the ocular surface.

The current work focuses on testing a novel delivery system using CLs with the incorporation of drug specific oligonucleotide chains known as aptamers on the surface of the lenses. This application of contact lenses is aimed at capitalizing on the strong affinity of aptamers to hold drug on the surface of the lenses until they are applied to the eyes. The aptamers were covalently attached to the surface via the activation of the hydroxyl groups on pHEMA as a model lens material using 1'1-carbonyldiimidazone CDI chemistry and subsequent reaction with the amine group on the 5' end of the aptamer. The presence of aptamers was confirmed using 6-carbofluorescein (6-FAM) fluorescence detection and x-ray photoelectron spectroscopy (XPS). The release of kanamycin B in comparison to regular pHEMA gels using a soaking uptake method was assessed.

In this work, aptamers were confirmed through fluorescence to have been successfully reacted onto the surface, however XPS was not able to confirm a consistent reading. This may have been due to low initial amounts of aptamer or uneven distributions along the surface. The efficiency of the aptamer reaction was not tested and would need to be further investigated. The contact angle had a significant change with increased hydrophilicity at 60.7 ± 1.55° compared to 66.6 ± 0.67°, however physically it should not affect wettability. The lower aptamer amounts resulted in no significant difference during drug release. Kanamycin B was detected using liquid chromatography mass spectroscopy (LCMS) with a reverse phase method using a C_{18} column however quite a few errors in the methodology led to the conclusion that this method of drug release requires further investigation. It is recommended an aptamer-surface reaction efficiency be determined with the use of a much larger starting aptamer amount, as well as a follow up drug release.

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LIST OF ABBREVIATIONS

CDI	1,1'-carbonyldiimidazole
ACN	Acetonitrile
AMD	Age-related macular degeneration
CL	Contact lens
EDGMA	Ethylene glycol dimethacrylate
EMS	Enhanced Mass Scan
FA	Formic Acid
FTIR	Fourier-transform infrared spectroscopy
HIV	Human immunodeficiency virus
HILIC	Hydrophilic-Interaction Chromatography
HEMA	Hydroxyethyl methacrylate
LC-MS	Liquid Chromatography-Mass Spectroscopy
LOD	Limit of detection
LOQ	Limit of quantification
TRIS	Methacryloxypropyl tris(trimethylsiloxy)silane
PDMS	monomethacrylated polydimethylsiloxane
MRM	Multiple reaction monitoring
MS	Mass Spectroscopy
PEG	Polyethylene glycol
PBS	Phosphate buffered saline
pHEMA	Polyhydroxyethyl methacrylate
PMMA	Polymethyl methacrylate
PoLTF	Post-lens tear film

PreLTF	Pre-lens tear film
RGP	Rigid gas permeable
RP	Reverse Phase
SiHy	Silicone hydrogels
SELEX	Systemic evolution of ligands through an exponential enrichment
VEGF	Vascular endothelial growth factor
XPS	X-ray Photoelectron Spectroscopy

1. INTRODUCTION

1.1 Ocular drug delivery

Ocular disease prevalence continues to rise with projections for some of the most common ocular diseases affecting both the anterior and posterior segments show a doubling of disease prevalence by 2050 (National Eye Institute, 2019). One of the most important considerations for treatment of these diseases is the route of administration which determines the effectiveness of drug delivery to target tissue.

Optimal methods of administration are still being developed due to the highly isolated structure of the eye. The ocular tissue contains blood-aqueous and blood-retinal barriers which regulates molecular exchange, specifically preventing drug absorption from the blood (Xu et al., 2018). High concentrations of drug would be needed to reach therapeutic levels, however this increases the risk of significant systemic side effects. From the external route of administration such as eye drops, physical barriers including blinking, the tear film, and the highly effective barrier functional performed by the multilayer corneal epithelium result in a very short residence time in the precorneal space, significantly impacting the amount of drug that reaches the target tissue (Reimondez-Troitiño et al., 2015). While eyedrops are a commonly used method for delivery of drugs to the eye, tear drainage and rapid removal from the precorneal space results in less than 5% of the drug reaching the target tissue (Bachu et al., 2018). Thus, highly concentrated doses and frequent dosing are required overcome the limitations (Stone et al., 2009). While this does combat the inefficiency of eye drops, various case studies have identified that the increased patient responsibility with proper instillation of drops and multiple administrations a day results in decreased

patient compliance, ultimately resulting in missed doses, which is particularly problematic in cases of chronic diseases (Stone et al., 2009). Thus, within the context of anterior delivery, extending the residence time of drugs on the eye may be an optimal strategy to developing safer and more efficient treatment regimens to reduce patient administration and thus increased patience compliance.

Numerous strategies are being tested to enhance the residence time of drugs, while maintaining or improving patient comfort. These include the use of contact lenses or nanocarrier emulsions such as liposomes or micelles which entrap drug, making penetration through the lipid layers and corneal cell multilayers more effective. Alternatively, the number of individuals adapting to contact lenses are growing every day and they provide the benefit of bypassing several surface physical barriers of the eye. From a drug delivery perspective, this would result in delivery of a larger portion of the drug to the corneal epithelium. Contact lens drug delivery has mainly involved soaking the lens in a drug containing solution. However, while relatively straightforward, this method is generally characterized by an initial burst followed by rapid drug release. Furthermore, it is mainly applicable to low molecular weight and more hydrophilic drugs which excludes a great number of ocular drugs (Maulvi et al., 2016). Cationic coated liposomes entrapping drug have been incorporated into the lens structure and have shown prolonged residence times and higher corneal penetration, as a result of attractive electrostatic interactions with the negatively charged cornea and conjunctiva (Abdelbary, 2011). In addition, contact lenses have also been entrapped with colloidal drug containing particles. Hydroxyethyl methacrylate (HEMA) models have been used to show that polymerization with these carriers does not affect the structural integrity of

the lenses, thus enabling release from the lenses in a controlled manner (Maulvi et al., 2016). While these have been more effective at increasing the residence time, a burst release is still present indicating large portions of the drug are released too early. More recently, the use of DNA micelles have also been investigated to hold drug and extend the residence time of carrier molecules, however they have not yet been applied to contact lenses (Willem de Vries et al., 2018).

Aptamers have been a revolutionary use of DNA and RNA for molecular detection, biological marking, and drug delivery. Aptamers are synthetic single stranded chains of nucleotides, which form a three-dimensional structure highly specific to a target molecule or compound. The origins of the use of aptamers began with the understanding of viral vectors, specifically pertaining to the human immunodeficiency virus (HIV), which showed RNA sequences could bind to endogenous proteins (Nimjee et al., 2017). Over the last decade, research has been able to shape a synthetic selection method for RNA to be specific to targets and are now being investigated for a variety of biomedical applications including drug delivery. Within the ocular space, aptamers have only been used to hold drug within nanocarriers and thus until they are delivered to the target tissue, these aptamers serve no greater benefit.

1.2 Research Objectives

The overall objective of this research was to synthesize a model contact lens system utilizing the high specificity of aptamers to improve controlled drug release from contact lenses. The aptamers were incorporated along the backbone of the polyhydroxyethyl methacrylate (pHEMA) model lenses. Aptamers should be able to form a peptide bond after surface activation of pHEMA which contains a high

concentration of hydroxyl groups. Aptamers are also quite small and hydrophilic, so no significant changes to the surface physical characteristics were expected as contact lens applications require optical clarity and little irritation to the eye.

The second goal of this research was to determine if the addition of aptamers along the surface of a lenses could reduce the burst release and thus prevent large amounts of drug from being released too early as seen in most cases with soaked lenses. It was hypothesized that the use of the aptamers would result in greater drug retention on the surface, reducing the rate of diffusion of drug from the inner matrix due to surface interference.

1.3 Thesis Outline

This thesis presents the information of this research in five chapters. Chapter 1 discusses the general concepts pertaining to the research topic. Chapter 2 is a literature review of the main topics, materials, and reactions discussed and utilized in this research including the ocular anatomy, the use of pHEMA for model contact lenses, silicone lenses for future applications, and aptamers and their applications. Chapter 3 includes the materials and methods used for the experiments in this research. Chapter 4 combines the results and discussion, assessing the outcomes for the two primary goals of this research. Chapter 5 includes the conclusion and future directions for this research.

2. LITERATURE REVIEW

2.1 Exterior Ocular Anatomy

The eye has a well protected structure, broadly defined by the two sections: the anterior segment and posterior segment. The anterior part is the most relevant for contact lens (CL) and any topical drug delivery for anterior segment diseases. It consists of the cornea and opaque sclera, joined by the limbus, layered on top with a multilayer (Kels et al., 2015). One of the biggest structural barriers any topically administered drug will encounter is the cornea, a connective tissue designed to protect the eye from foreign objects while enabling the passage of light (DelMonte & Kim, 2011). The cornea is covered by a thin fluid layer known as the tear film. Together, these two structures protect the outer surface of the eye from foreign objects while absorbing nutrients through diffusion (Bron et al., 2004). While the cornea is a transparent barrier, the surrounding structure of the exterior eye is an opaque, fibrous layer known as the sclera, designed to prevent light passage and thus centralize light entrance to the cornea. The limbus is the area where the cornea and sclera join and harbours pathways for the outflow of aqueous humour (Buskirk, 1989). Finally, beyond the limbus is a tissue that resides above the sclera known as the conjunctiva. This tissue forms a cavity which is responsible for releasing lubricating fluids, electrolytes, and mucins to maintain the tear film, keep the ocular surface moist and reduce the friction between the ocular surface and the eyelids (Dixon et al., 2015; Foster & Lee, 2013).

A contact lens interacts with the tear film, specifically residing within the aqueous layer making it an ideal location for drug to be released topically as most molecules

enter the eye through the tear film. The interaction between the surface of a contact lens and the tear film are thus of high importance both in lens design and when considering a drug release system.

2.1.1 The Tear Film

The tear film has a three-layer composition and serves a variety of functions including but not limited to ocular surface lubrication, mechanical and immune protection, suppling nutrients for the corneal epithelium, removal of metabolic by-products, and maintaining a smooth surface for light refraction (Foster & Lee, 2013; Prydal & Campbell, 1996). The three layers are distinguished by their unique compositions, the outer being mainly lipid based, the middle is an aqueous layer, and the inner is a mucous layer which provides an interface between the water layer and the more hydrophobic cellular layer of the corneal epithelium (Figure 1). The tear film is roughly between 4 – 8 μm thick in healthy individuals with a tear volume of 6 μL and a 16% fluid turnover rate per minute (Farkouh et al., 2016; Foster & Lee, 2013). A healthy tear film generally maintains a pH of 7.3 – 7.7, similar to human plasma and thus is ideal for many drug applications (Yamada et al., 1997). The tear film itself is composed of a variety of enzymes including DNAse, lipids, salts, proteins including growth factors, electrolytes, and antioxidants (Foster & Lee, 2013; Sonawane et al., 2012).

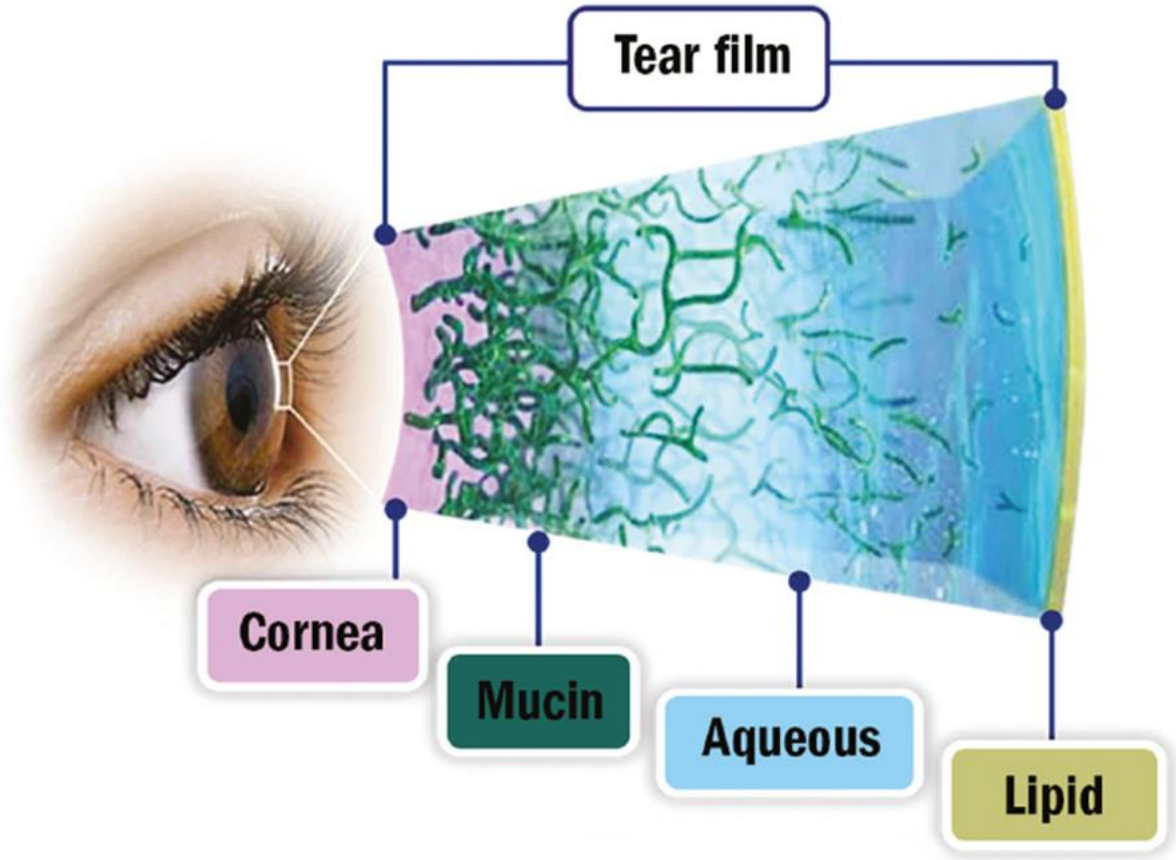

Figure 1: *The structure of the tear film composed of three main layers including the outer lipid layer, middle aqueous layer, and inner mucin layer which then sit above the corneal epithelium. Adapted from https://www.opticianonline.net/cet-archive/5865*

The lipid layer is a coating on the superficial area of the ocular surface and is comprised of a lipid bilayer formed from lipids produced by the Meibomian glands (Georgiev et al., 2017; Rohit et al., 2013). It is primarily known to control or prevent tear evaporation and also reduces tear spillage and debris from entering the film (Rohit et al., 2013). The two main types of lipids secreted by the Meibomian glands including non-polar lipids which appear on the outer layer and polar lipids which interact at the border to the aqueous layer (McCulley & Shine, 2003; Rohit et al., 2013). The non-polar lipids act a first line of defense for the eye and keep out debris. The aqueous layer makes up about 98% of the tear film volume containing but not limited to electrolytes,

proteins, peptides, growth factors, vitamins, and antimicrobial agents (Stahl et al., 2012). This layer enables the transfer of necessary compounds for the nourishing of the cornea including proteins, oxygen, electrolytes, vitamins, and inorganic salts. Contained within this layer are also numerous protective enzymes and antimicrobial agents including lysozyme, lactoferrin, and various immunoglobulins which are responsible for the protection of the corneal surface (Stahl et al., 2012). Finally, the posterior layer is the mucosal layer, primarily comprised of mucins released from goblet cells of the conjunctiva with various mucins released from the corneal epithelium (Gipson & Argueso, 2003; Nichols et al., 1985). This hydrophobic mucin layer helps to retain water and keep the corneal surface lubricated (Gipson & Argueso, 2003). It also stands as the last physical barrier for the cornea against pathogens and other particulates including drugs released from topical applications.

2.2 Contact Lens

In 2016, it was estimated that 41 million adults wore contact lenses in the United States alone, with a global market reaching $7.6 billion (Cope et al., 2017; Jones et al., 2016). Currently, there are two common types of contact lens found in the market, specifically rigid gas permeable (RGP) and soft contact lenses. The initial RGP lenses were made from polymethyl methacrylate (PMMA) which were optically transparent, light, and thin. However, PMMA lenses had low hydrophilicity and more importantly had low oxygen transmissibility (Musgrave & Fang, 2019). This limitation resulted in low gas permeability and presented a very rigid structure, both of which within the sensitive and hydrophilic tear film resulted in several ocular ailments including corneal swelling and hypoxia leading to ailments such as neovascularization and cell damage (Ansari & Cho,

2016; Chen et al., 2012; Moezzi et al., 2004). More recently, these rigid lenses have been mixed with silicone acrylates to create slightly more flexible materials, but more importantly much more gas permeable lenses for oxygen influx. While being quite effective lenses, soft lenses are more comfortable and thus more commonly worn and prescribed. Soft contact lenses today are known as either conventional hydrogels or silicone containing hydrogels.

2.2.1 Conventional Hydrogel Lenses

Soft lenses were designed with the goal of providing more comfort and longer lasting wear and thus polymer hydrogels were quickly adopted and are now widely used in the medical and pharmaceutical industry. These lenses are crafted from hydrophilic monomers such as HEMA crosslinked to form a three-dimensional network which can absorb and retain large amounts of water (Seo et al., 2017). Hydrogel materials in general have been used for multiple biomedical applications because of their high hydrophilicity including as drug delivery systems, scaffolds for tissue engineering, biosensors, and contact lenses (Caló & Khutoryanskiy, 2015).

Within the ocular space, polyhydroxyethyl methacrylate (pHEMA) has been one of the main hydrogels used due to the high hydrophilicity which makes for more flexible and comfortable wear within the tear film. However, even with greater water levels, symptoms of dryness from extended use and hypoxic conditions has still been a concern for conventional lenses (Brennan & Efron, 1989; Musgrave & Fang, 2019). In addition to hypoxic conditions, bacterial buildup has also been noted from extended wear, specifically microbial keratitis (Borazjani et al., 2004). Thus, these conventional hydrogels were not viable for yet again for extended wear but are ideal for daily

applications and have been the market standard for daily lenses. This problem has been by and large overcome with the introduction of silicone containing hydrogel materials which have excellent oxygen permeability and high enough water permeability for comfort.

2.2.2 Silicone Containing Hydrogels Lenses

Silicone hydrogels (SiHy) contact lenses were first introduced in the late 1990s with the breakthrough of increasing oxygen permeability. The addition of silicone domains through monomers such as monomethacrylated polydimethylsiloxane (PDMS) or methacryloxypropyl tris(trimethylsiloxy)silane (TRIS) to HEMA or other hydrogel monomers enabled a higher solubility of oxygen from the aqueous phase of the tear film (Kim et al., 2008). The siloxane domains present hydrophobic regions for gas permeability while the water content is still governed by the hydrophilic monomer backbone of the hydrogel (Tran & Yang, 2019). The increased oxygen content has been significant for extended wear however first-generation silicone hydrogels did have several challenges. The hydrophobic domains near the surface result in lower wettability altering the properties of potential lens and their interactions within the tear film (Chekina et al., 2006; Tran & Yang, 2019). Since then, various attempts have been made to improve surface wettability such as adding internal wetting agents, applying external solutions containing surfactants, or functionalizing hydrophilic compounds to the silicone domains (Keir & Jones, 2013). In addition, variations of silicone ratios should produce lenses with varying degrees of water solubility and oxygen solubility which is where the market stands today. Silicone lenses have been tailored to specific

wear times with a large portion sold as either daily wear or monthly lenses and can thus be used for a variety of applications outside of corrective vision.

2.3 Drug Delivery for Anterior Ocular Diseases

2.3.1 Conventional Topical Eye Drop Delivery

Many anterior segment ocular disorders require treatment using drugs over a period ranging from days to weeks to a consistent lifetime of treatment. Systemic administration has never been an ideal route of delivery as the blood-ocular barrier isolates the eye. The barriers consist of two sections including the blood-aqueous and blood-retinal barriers. Both contain tight junctions which limit the transport of drugs from the systemic circulation (Xu et al., 2018). Thus, for anterior segment diseases, over 90% of treatments are applied topically though dosages in solutions, suspensions, or ointments (Bachu et al., 2018; Hu et al., 2011). The goal of topical treatments is to deliver drug to the corneal surface where it can then be absorbed by the corneal epithelium to be delivered within the ocular structure. However, with the multiple anatomical and physical barriers present on the surface of the eye, less than 5% of the dosage is effective in the target tissue (Bachu et al., 2018; Hu et al., 2011). The rapid tear fluid clearance through the lacrimal system is a major source of drug loss with delivery via eye drops applications. Typically, the human eye tear volume is approximately 6 µL whereas with eye drop formulations, the instilled volume is between 20 - 50 µL (Farkouh et al., 2016). A small portion of these volumes interact with the tear film long enough for drug to diffuse due to high turnover. This turnover has a rate of 0.5-2.2 µL/min which is responsible for the low residence time and thus loss of a single dosage within the first 2 – 5 minutes after administration (Bachu et al., 2018; Maulvi et al., 2016; Xu et al., 2018). The remaining solutions drain into the conjunctival sac (10µL

capacity) after which the excess fluids are spilled onto the cheek or are drained through the nasolacrimal duct (Farkouh et al., 2016). For drugs that reach the tear film, the initial lipid layer also acts as a barrier with the polar and non-polar lipid layers which adds to reduced diffusion of hydrophilic and/or hydrophobic drugs. Generally polar drugs have a greater difficulty diffusing through the initial non-polar layer and thus drug absorption is reliant on lipid bilayer transporters to reach the aqueous layer (Farkouh et al., 2016). Additionally, blinking, low corneal penetration, and patient compliance are also common sources of lost drug when applying eye drop solutions (Bachu et al., 2018; Maulvi et al., 2016; Newman-Casey et al., 2015). The conjunctiva generally absorbs hydrophilic drugs, but significant losses occur in this case as these drugs are unlikely to be carried to the ocular site of treatment as they enter the systemic circulation (Dixon et al., 2015; Hosoya et al., 2005). As a result, high frequency administrations are required for these topical methods to reach appropriate levels of dosage. Frequent dosages can lead to inconsistent drug concentrations, through a treatment period including either too low before another dose or too high after one. Side effects can also be presented in the nasal cavity or systemic circulation depending on how much drug was removed from the dosage (Xu et al., 2018). About 50% of most dosages end up in the systemic circulation for clearance. Additionally, frequent dosages usually result in lower patient compliance, particularly for patients with chronic diseases such as glaucoma and dry eye syndrome (Ciolino et al., 2018; Maulvi et al., 2016; Xu et al., 2018).

Thus, while eye drops are inexpensive to manufacture and are quick to administer, they are not an efficient delivery system for consistent therapeutic effect. In order to reach therapeutic levels, eye drops require high frequency of dosage, and

contain much higher drug concentrations which can aggravate side effects and reduce patient compliance.

2.3.2 Drug Delivery from Hydrogel Contact Lenses

Contact lenses have been suggested as having the potential to overcome some of the limitations of eyedrops for the delivery of drugs to the anterior segment. One of the main objectives with these lenses is to increase the resident time of drugs on the ocular surface which has been observed to rise up to 30 minutes compared to 5 minutes from eye drops (Xu et al., 2018). Additionally, contact lenses reside in between the tear film in the aqueous layer thus by passing the lipid bilayer and enabling drugs to be released much closer to the corneal epithelium layers. Lenses split the aqueous layer into two main sections pre-lens tear film (PreLTF) and post-lens tear film (PoLTF). Release from the PoLTF reduces the distance drug has to diffuse across and it known that drug absorbs rather quickly by the corneal epithelium whereas the release from a hydrogel into the PoLTF is much slower (Prausnitz & Noonan, 1998). Thus, release into the PoLTF can be seen as the rate limiting step and research has focused on hydrogel modifications which can enhance or control this release. Furthermore, with silicone hydrogels, the possibility for extended drug delivery is also an option which could reduce the frequency of administration required for treatment.

With the use of any delivery system, loading efficiency and capacity are also important characteristics since they affect the total release and possible dosage amounts. One of the most cost effective and simplest loading mechanisms is soaking which occurs through exposure of the lens to the drug solution for drug uptake (Maulvi et al., 2016). Typically, upon administration, soaked lenses demonstrate an immediate

and rapid burst of a large portion of drug within the first couple of hours, as a rate which depends on the physical properties of the drug compared to the properties of the hydrogel (Kim et al., 2008; Maulvi et al., 2016). Smaller, hydrophilic drugs generally release more quickly from unmodified hydrogels while larger and/or more hydrophobic drugs are retained relatively longer (Kim et al., 2008). This initial burst release is one of the major problems with the soaking method as a large portion of the loaded drug is lost at initial application (Kim et al., 2008; Phan et al., 2014). Various researchers have thus delved into enhancing CL drug delivery, either by enhancing the surface properties of CLs or employing a secondary delivery system.

2.4 Modifications of CL for Enhancing Drug Delivery

The use of modifications for drug delivery from CLs includes several factors which must be considered. These include how the method selected can affect the drug release rate, or whether it will alter the therapeutic efficacy of the drug as well as how it will impact the properties of the CL. The hydrogels must maintain their transparency to be used for CL application and cannot cause irritation to the eye meaning that the lens surface must maintain a level of hydrophilicity. Keeping those in mind, modifications have been made to both the bulk structure of hydrogels, as well as the surface.

2.4.1 Bulk Modifications

Bulk modifications include any changes which alter the structure of the hydrogel to accommodate more drugs or alter their release. Molecular imprinting is one such common technique which involves the molding of hydrogels with cavities to fit a specific drug (Gupta & Aqil, 2012). The addition of microparticles containing drug such as liposomes or nanoparticles during the formation of a hydrogel is another common

technique used to embed a drug which will release at the degradation rate of the secondary carrier (Gulsen & Chauhan, 2005; Gupta & Aqil, 2012).

Despite bulk modifications being useful for embedding drugs within the hydrogel as opposed to diffusive loading, they do have a higher risk of altering the mechanical properties of the hydrogel as well as potentially altering the opacity and durability over a long term release (Hu et al., 2016). Introducing bulk modifications can also change the structural integrity depending on the pH and temperature of the environment and while this is not a major issue in the isolated ocular system, it must be considered and can complicate the manufacturing of the CL (Hu et al., 2016). Additionally, many gels are molded through free radical polymerization where these radicals could be a risk to cause degradation of some of the drugs or carrier systems (Dixon et al., 2015). Thus, research has considered various surface modifications in order to maintain the integrity of CLs and the therapeutic effect of the drugs (Dixon et al., 2015).

2.4.2 Surface Modifications

Surface modification generally does not affect the structural integrity of the hydrogel; however, they still can affect such physical characteristics as opacity and hydrophilicity, making it important to consider these factors. For drug delivery enhancement, surface modifications can slow the diffusion from the lens or hold drugs for prolonged delivery periods. Danion et al. attempted to slow down elution through the addition of layers of liposomes containing drugs on the surface of their CLs. Their results showed the release was broken into two periods; a smaller burst release of drug trapped in the surface liposomal layers can still occur if the lenses are soaked (Danion et al., 2007). More importantly almost half as much drug was retained leading to a much

longer diffusive release compared to no liposomal layers thus demonstrating that trapping drugs on the surface can be an effective method of increasing the drug delivery period (Danion et al., 2007). Hu et al. attached β-cyclodextrins to the surface of pHEMA hydrogels in order to enhance drug delivery through steric hindrance on the surface with a polymer containing hydrophobic domains which entrap drug and slow diffusion (Hu et al., 2016). Hu et al. noted that their system did increase the release period and reduced the amount of drug loss during the burst release, but only for lenses with low drug concentrations.

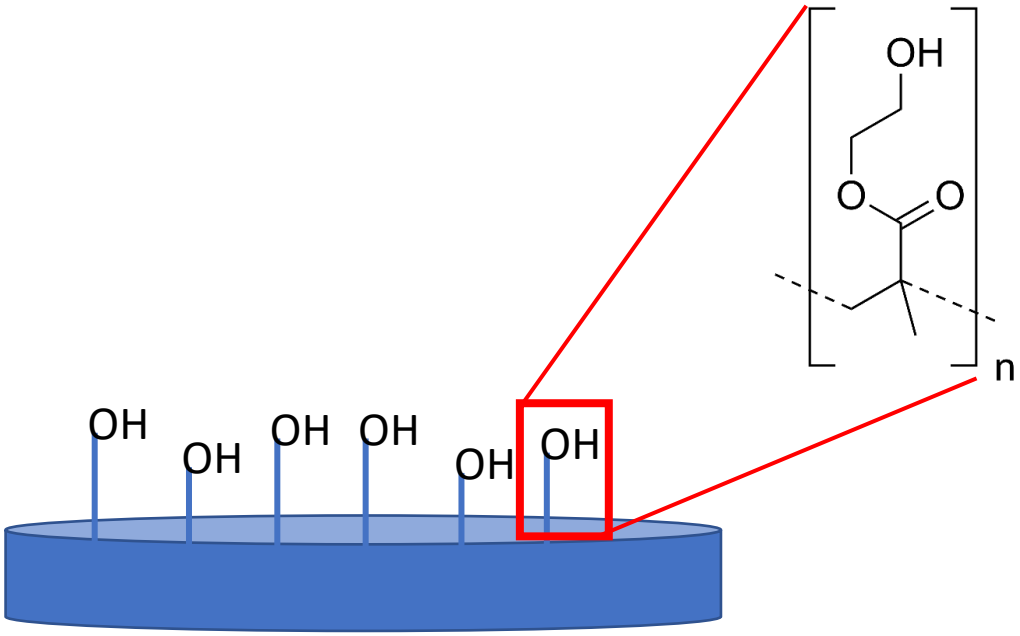

Figure 2: *Surface of pHEMA hydrogel model contact lenses. The surface has the tail end of the monomers pointing out with hydroxyl groups available for activation. The linkage of monomers occurs at the branches at the bottom of the monomer seen here.*

Surface modification generally should consist of rapid steps to prevent major structural changes, have low toxicity, and be site selective on the surface to prevent alternative structures from forming to interact with tissue. One of the most prominent sites on the HEMA monomer is the -OH group which can be found sterically available on the surface of pHEMA hydrogels (Figure 2). One commonly used technique for the

modification of -OH groups is the activation of these primary alcohol groups surface using CDI. This acyl transfer agent can react with a low toxicity reaction with easy preparation for amides; it is commonly used reagent in the pharmaceutical industry for large scale applications (Lanzillotto et al., 2015). This technique utilizes the imidazole leaving group of CDI to act as a temporary placeholder available for the attack of nucleophiles like peptides and proteins.

2.5 DNA and RNA Aptamers

The concept of controlled drug release indicates any method which can be used to alter the drug release kinetics to allow for more prolonged or more time dependent drug. As noted above, surface modification has the potential to alter and control the release of drugs from CL and can offer selectivity in terms of drug interactions with the material. Affinity molecules, including antibodies and others have a great deal of potential in this area. One potential affinity molecule that may be useful in drug delivery applications is the aptamer. Aptamers are single-stranded chains of oligonucleotides, either DNA or RNA nucleic acids, with a specifically designed affinity for certain targets. They are designed *in vitro* by the systemic evolution of ligands by exponential enrichment (SELEX), a process first described in 1990 which sought to process a large pool of single stranded RNA for strands that specifically interacted with a known non-nucleic target (Ellington & Szostak, 1990; Stoltenburg et al., 2007). RNA and DNA strands can have very complex, yet highly specific three-dimensional structures which enable their ability to be target specific like antibodies. The SELEX process became a selection procedure to process these large pools of RNA and eventually DNA chains and continues to be enhanced for various targets. These targets can range from small

molecules to large proteins. Binding is typically through hydrogen bonding and/or van der Waals forces with an affinity that can be controlled (Mairal et al., 2008; Zhu et al., 2015). Part of their specificity also includes the ability to recognize specific chiral molecules from their counterparts making this a strong target tool (Stoltenburg et al., 2007). In more recent developments, aptamers can have different ranges of affinities for targets, they can be chemically modified to improve binding and stability including resistance to biological nucleases found *in vivo* and *in vitro*, and they can be modified for labelling purposes such as with the use of fluorophores (Esposito et al., 2014; Stoltenburg et al., 2007).

2.5.1 Drug Applications

Aptamers have also been used in drug applications for increasing specificity to target sites, predominantly in cancer research. Aptamers can bind target molecules through non-covalent binding or can be conjugated with drugs through covalent binding. Early work by Huang et al. and others has shown that drugs conjugated to aptamers that specifically target cancer cell markers or overexpressed proteins such as tyrosine kinase can be effective at increasing the effectiveness of these therapeutics or at the least, reduce systemic side effects (Huang et al., 2009). Since then, aptamer research has included studies of binding multiple drugs to single aptamers to increase the bioavailability of the therapeutic to using aptamers as a therapeutic (pegaptanib). This FDA-approved drug is a PEGylated anti-VEGF used for the treatment of age-related macular degeneration (AMD) and was a milestone in showcasing that aptamers could be modified to exhibit endonuclease resistance (Ng & Adamis, 2006; Zhu et al., 2015). These modifications have been noted as a necessary feature as these nucleic acid

aptamers have high degradation rates when exposed to *in vivo* nucleases, particularly RNA aptamers. Most common modifications include substituting the hydroxyl groups on RNA with 2'-O-methyl or 2'-fluoro on various nucleotides along the chain as well as terminal PEGylation (Kruspe et al., 2014; Zhu et al., 2015).

In hydrogel applications, aptamer-drug conjugates have been incorporated into the gel matrix to reduce release rates, primarily through binding of drugs within the matrix (Liu et al., 2012). The aptamers are modified to attach to the gel network and are generally well protected from enzymatic degradation within the gel. Particularly with growth factor treatments, porous hydrogels have been tested with aptamers with varying binding affinity for their target. Battig et al. showcased the difference affinity makes in terms of release noting a range between 14% and 90% of drug retention within the first 24 hours compared to gels without aptamers (Battig, Huang, Chen, & Wang, 2014). The varying binding affinity can prolong the release of the drug, however most aptamers do break down over time, especially if exposed to DNAse or RNAse, thus this must be considered for tailoring aptamers for specific treatments (Battig et al., 2014).

2.5.2 Anterior Ocular Applications

The application of aptamers within the ocular space is very limited and still a ground-breaking field in terms of drug delivery. Currently, one of the only known delivery applications involves an improvement in delivery efficiency of eye drop drug through the attachment of drug via aptamers on a DNA based micelle nanoparticle (Willem de Vries et al., 2018). As mentioned, a large portion of eye drops are rapidly cleared before penetrating the layers of the eye; this group developed a model using DNA amphiphiles

which adhere to the corneal tissue for extended periods of time. Aptamers were used in this delivery system to hold kanamycin B and neomycin to their DNA nanoparticles and demonstrated a retention of antibiotic activity (Willem de Vries et al., 2018). For applications like antibiotics such as kanamycin B, extended corneal surface retention is ideal for longer lasting therapeutic effects against bacterial infections in the eye. Willem de Vries et al were able to demonstrate this increased antibiotic activity using their aptamer-based system and correlated this increased activity to their increased corneal surface adherence via aptamers (Willem de Vries et al., 2018). It has also been demonstrated that for RNA aptamers, the presence of RNAse was required for active drug activity whereas DNAse was not needed when DNA aptamers were used. This is quite conditional on the aptamer affinity as mentioned previously, as some bind strongly to their target while others may not. Thus, while aptamers have not played a part in delivering drugs to the specific site, they were able to retain and hold drugs until the target site was reached.

2.6 Scope of Research

Therefore, the objective of this thesis was to examine the effects that aptamers could have on drug release from a model contact lens prepared using pHEMA hydrogels which can carry drugs straight to the lower layers of the tear film. More specifically, RNA aptamers designed to hold the antibiotic, kanamycin B, were attached to the model pHEMA lens to improve drug loading and slow the rate of drug release.

Contact lenses are generally worn daily and thus the use of aptamers to prolong delivery for a short period of time would be ideal. Therefore, RNA aptamers were chosen as they have been used in previous ocular drug attachment studies. These

aptamers can specifically hold drug in place on the surface of the lenses and thus create steric hinderance to reduce the rate of drug release from the lenses. It was hypothesized that with the presence of aptamers holding drugs on the surface, a smaller burst release would occur and more prolonged drug release would be observed based on the affinity of the aptamer for the drug and changing drug concentrations between the internal and external environment of the lenses.

3. MATERIALS AND METHODS

3.1 Materials

2-Hydroxyethyl methacrylate (HEMA) (99%, Sigma-Aldrich, Missouri, USA) and ethylene glycol dimethacrylate (EDGMA) (98% Sigma-Aldrich) were passed through a column of inhibitor remover (Sigma-Aldrich) for the removal of hydroquinone and monomethyl ether hydroquinone stabilizers. Irgacure® 184 photoinitiator (BASF) used for radical polymerization reaction was used as received. 1,1'-carbonyldiimidazole (CDI) (Sigma-Aldrich) was purchased and stored with Parafilm® M seal. CDI was sealed with nitrogen bubbles for prolonged storage. Phosphate buffered saline (PBS) was prepared based on the recipe presented in Appendix A. Aptamers were custom designed and obtained from Integrated DNA Technologies (IDT™, Iowa, USA) and Gene Link™ and were delivered as a lyophilized powder. They were stored before use at -30°C. All DNA and RNA were modified with a 5' amino modifier C12 spacer. Fluorescent DNA had a 3' modification with a 6-FAM (fluorescein) fluorophore. Kanamycin B RNA aptamer (K8 aptamer) was designed from the Aptagen library with previously mentioned modification. Nuclease-free (DNAse, RNAse free) UltraPure™ distilled water (Invitrogen) was used as received.

3.2 Synthesis of Poly-(2-hydroxyethyl methacrylate) Model Hydrogel Lenses

These hydrogels were designed to model a commercial conventional hydrogel contact lens with maximum aptamer surface reactivity. The methods were adopted from previously developed group models using pHEMA hydrogels cut into 4mm diameter discs for drug release (Van Beek et al., 2008). To a 20mL glass vial, 4000mg of HEMA monomer (95 wt%), 122.1mg of EDGMA crosslinker (3 wt%), and 4mg of photoinitiator Irgacure® were added. The vial was covered with aluminum foil for light protection and

the monomers mixed to complete dissolution for 20 minutes. The mixture was injected using a 20G needle into a custom mold. The mold, depicted in Figure 3, consisted of two acrylic plates, each covered with clear polyester sheets to hold the solution between the spacer, and finally screwed together with an appropriate (1mm) Teflon spacer in between. The initial mixture was injected into the mold and cured using 400W UV chamber at wavelength 365nm for 12 minutes (Cure Zone 2, Illinois, USA). After the polymerization, the polymer film remained in the mold for 24 hours and then was subsequently removed and placed into a glass container filled with 300 mL of Milli-Q water. This allowed for the removal of any unreacted monomer or remaining components. After soaking, the polymer film was punched into circular 4mm diameter discs, which were subsequently dried for 12 hours under vacuum conditions (Isotemp Vacuum Oven Model 280A, Fisher Scientific, Massachusetts, USA) at 50°C. Dried discs were stored in a 37°C oven until needed.

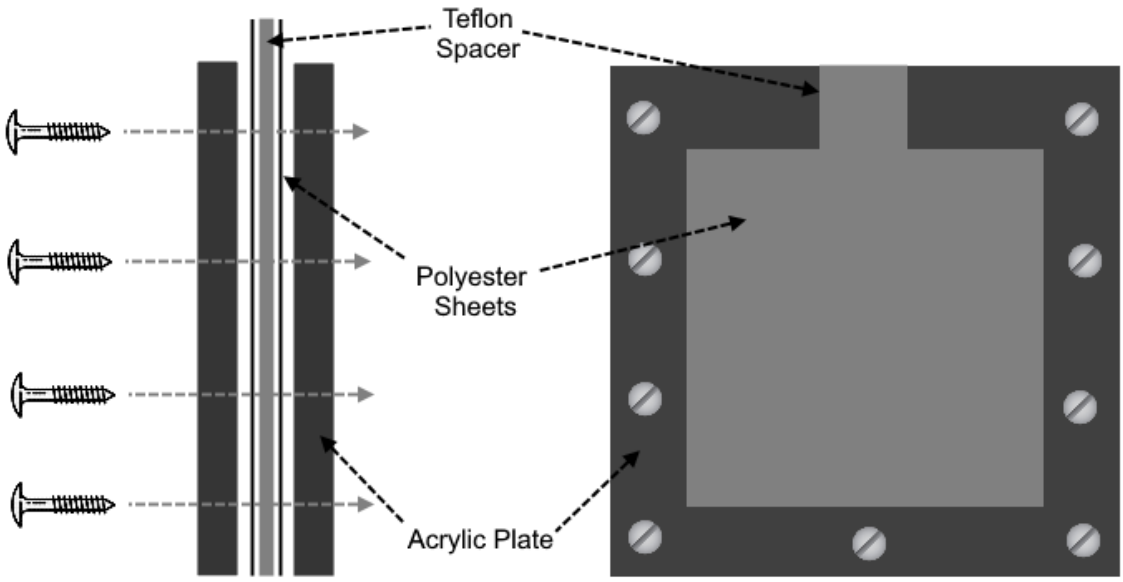

Figure 3: *Depiction of the hydrogel molds consisting of 2 acrylic plates, each covered with a clear polyester sheet to hold and shape the liquid mixture. Each half is screwed together with the appropriate size Teflon spacer in between. The gels used in this study were 1mm in thickness which correspond to the Telfon spacer used in these molds.*

3.3 Surface Activation of Hydroxyl Groups on the pHEMA Surface Through CDI Chemistry

The method of surface activation was adapted from a previous application described by Carvalho et al. (2010). CDI at a 10:1 ratio to available hydroxyl groups on the surface (12.5mg, 0.08mmol per 3 discs) was dissolved in 10mL of anhydrous 1,4-dioxane under a nitrogen atmosphere. The mixture was shaken for 10 minutes at room temperature to ensure complete dissolution. As previously described pHEMA discs were placed in previously dried 8mL glass vials (under vacuum at 50°C) and sealed under nitrogen. After shaking, 1mL of the CDI mixture was added dropwise through a stainless-steel needle injection under nitrogen to each vial containing a single pHEMA disc and the vials were gently shaken at 37°C for 3 hours. Upon completion, discs were then quickly removed to be washed, dabbed with a Kimwipe (Kimberly-Clark Professional, Georgia, USA) to remove excess surface dioxane solution and then placed in new vials filled with 1,4-dioxane. The wash phase consisted of 10-minute periods of shaking, followed by similar gentle tapping of both surfaces of the discs by Kimwipes and placed in a new vial filled with 1,4-dioxane, repeated 3 times to ensure all excess, unbound CDI was removed from the surface. Discs were then dried for thirty minutes under nitrogen atmosphere for full solvent evaporation and then either stored at 37°C until use or immediately used for aptamer attachment.

3.4 Kanamycin B (K8) Aptamer Attachment onto pHEMA-CI Activated Surfaces

The Kanamycin B (K8) aptamer was selected from the Aptagen Apta-Index™ (ID# 117) and was developed and shipped by Gene Link (Florida, USA) (Table 1). For preparation, the lyophilized aptamer was warmed to room temperature and briefly

centrifuged for 5 minutes at 1000rpm. The aptamer was then dissolved in a TE buffer (UltraPure™ distilled, DNAse, RNAse free water (Invitrogen, California, USA), 10mM Tris, 1mM EDTA, pH7.3) to the desired stock concentration through shaking for 30 minutes at room temperature. The aptamer solution was then heated to 90°C in an oil bath for 5 minutes for aptamer unfolding, then left to cool at room temperature for 30 minutes after which the solution was ready for use. 800uL of aptamer solution ($2.33x10^{-4}$ mol/L) and single pHEMA-CI discs were added to individual 8mL glass vials and then gently shaken at 37°C overnight (12 hours).

Table 1: *Sequences for the K8 RNA aptamer and DNA aptamer used in this study. Both contain a 12-carbon spacer with an amino attachment to form a peptide bond with the activated pHEMA surface. The DNA sequence contains a 3' end modification with a 6-FAM fluorescent probe.*

	5' End: Carbon Spacer	**Aptamer**	**3' End: 6-FAM**
K8 RNA Aptamer	H_2NC_{12}	/GGG AGC UCG GUA CCG AAU UCU CGC CCU AUA GGG GUG UUG AGG GAA AUG UGU GCG ACA AGG UGC GGU GGC CAG AAC UUU UCG UUC UCA UCA AAA GCU UUG CAG AGG AUC CUU/	
DNA Aptamer	H_2NC_{12}	/GC CGT TGC AAC GAC AAC GCA TCG CGC CGG CAT TGC GCA TTA AT/	$C_{21}H_{12}O_7$

3.5 Lens Characteristics

3.5.1 Surface Wettability

Contact angle was used to assess any changes in the surface wettability. Lens were soaked for 24 hours in UltraPure™ water at room temperature. Upon measuring, the surface of the lenses were removed of excess water by tapping with Kimwipes on both sides. The contact angle was measured on the high-speed contact angle OCA 35 (Future Digital Scientific, New York, USA) using 5uL of dispensed water droplets. Angles were measured 3 times for each side of the lens. A t-test was used to assess the significance of the results.

3.5.2 Aptamer Attachment Detection using Fluorescent Probes

Fluorescent probe testing was conducted on 2mm discs to do a validity check of the chemistry used for attachment. The DNA aptamer was modelled using RNAstructure© for a linear three-dimensional shape with an attached 6-FAM probe at the 3' end. Like the K8 aptamer protocols, this DNA was dissolved in the DNAse/RNAse free TE buffer and heated to unfold at aptamer changes. Discs were similarly exposed to the DNA aptamer while being placed in 8mL glass vials filled with 800µL of aptamer solution for 3 hours [0.75µmol]. Upon initial results, the method was amended to soak the gels for 24 hours first to reduce uptake of aptamer through swelling, as well as the exposure time was reduced to 2.5 hours.

3.6 Kanamycin Detection

3.6.1 Hydrophilic-Interaction Chromatography (HILIC) Columns

The Shodex VG-50 4D HILIC column was designed to retain hydrophilic molecules and release them under the appropriate gradient changes of the mobile phase. Mobile phase A consisted of 0.1% formic acid (FA) in water, while mobile phase B was acetonitrile (ACN). The flow rate was 200uL/min with an injection volume of 2uL. Table 2 shows the various gradients and timing that were tested for a quick and controlled separation. All gradients were linear and started 2 minutes after running the initial ratio of mobile phase to clear any remaining contaminants. The column was then reset to the initial gradient to end the single sample run and then washed for 2 minutes with 100% B before equilibrated with the initial mobile phase ratio for the next sample.

Table 2: *Various gradients were tested for quick and controlled separation of Kanamycin.*

	Mobile A → B Ratio Before Gradient	**Timing of Gradient**	**Mobile A → B Ratio Before Gradient**
Test 1	5:95	13 minutes	97:3
Test 2	20:80	13 minutes	95:5
Test 3	3:97	10 minutes	60:40

3.6.1.1 Mass Spectrometry Conditions

The mass spectrometry (MS) conditions were as follows: the polarity of the MS was run in positive ion mode. The ionization spray voltage was set at 4000, the desolvation temperature was set at 600°C, ion source gas 1 was set at 40, while ion source gas 2 was set at 50. Multiple reaction monitoring (MRM) was used for kanamycin quantification with molecular size 324 for quadrupole 1 (Q1) and 163 for Q3 filtering. Q2 is usually the chamber for further fragmentation where a collision energy of 20eV was applied. The standard calibration curve was created between 1.25ug and 50ug as 50ug was half of the total uptake of the gels. 1.25ug was the closest set point that could be reached for the calibration curve before approaching the LOD.

3.6.2 Reverse Phase (RP) Columns

The LUNA C18(2) (Phenomenex, California, USA) is a reverse phase (RP) column with C_{18} bound with ligands to create a strong hydrophobic stationary phase. Mobile phase A consisted of 0.1% FA in water while mobile phase B consisted of ACN. The second RP column tested was the Synergi polar-RP (Phenomenex, California, USA). The injection volume remained the same while the flow rates used were 200uL/min and 1000uL/min respectively. Gradients tested were like the HILIC columns as we wanted low hydrophilic mobile phases at the start to prevent early release (Table 2). After initial tests, a high hydrophilic mobile phase was tested with A starting at 95%

for the first 2 minutes, followed by a quick gradient to 50% A in 3 minutes. The column was then cleared with 95% solvent B for 2 minutes, before equilibrated again at 95% A for 2 minutes.

3.7 Drug Release of Kanamycin

3.7.1 Release Collection

Stock kanamycin solutions were created using 10mg mixed in 10mL of UltraPure™ water. For uptake of drug, three discs of K8 aptamer conjugated pHEMA and three regular pHEMA discs were placed in individual 1.5mL Eppendorf tubes filled with 1mL of kanamycin solution and gently shaken for 24 hours at room temperature. After the uptake period, the discs were transferred to individual 1.5mL Eppendorf tubes, filled with 1mL of UltraPure™ water and gently shaken at 37°C. Specified time intervals were determined for solution changes, where the discs were removed and placed in new Eppendorf tubes with 1mL of UltraPure™ water. Samples were changed hourly for the first three hours, then every two for the next six hours, overnights were 15-18 hour intervals, day 2 was every two hours of sampling (4x), and day 3 was four hour intervals (2x) (72 hour end point). Individual samples were stored in the 4°C fridge until analysis.

3.7.1.1 Hydrogel Drug Uptake Measurement

Uptake values were measured from the remaining kanamycin in solution. Samples of 1mg/mL were created from one stock solution and used for drug loading. After the hydrogels were transferred to release solutions, the loading solutions were immediately prepared for liquid chromatography-mass spectrometry (LC-MS) measurement to determine the remaining concentration of drug.

3.7.2 LC-MS Sample Preparation

Samples were lightly shaken for 10 minutes after removal from fridge storage. 120uL of sample solution was added to LC-MS AQ™ Brand inserts for vials (MicroSolv Technology Corp, North Carolina, USA). Samples were sealed in an LC-MS vial (Waters) and transferred to the machine where they were tapped prior to being scanned to release any air bubbles trapped at the bottom of the insert during the transfer. A calibration curve was set up and measured prior to each session of sample reading (1.56ug – 50ug). The limit of detection (LOD) and limit of quantification (LOQ) were measured using equations 1 and 2 (Evard et al., 2016).

$$1)\ LOD = \frac{3.3 \times S_y}{b}\ , S_y = Standard\ deviation\ of\ the\ responses, b = intercept$$

$$2)\ LOQ = \frac{10 \times S_y}{b}$$

4. Results and Discussion

4.1 Physical Characteristics of the Hydrogels

It is a challenge to control the release of highly hydrophilic drugs like aminoglycoside antibiotics from hydrogels in an aqueous environment. Thus the aim of this study was to examine if surface attached aptamers could create affinity for the drug at the surface of a model contact lens and/or could create a layer of interaction to reduce the initial burst of drug released from the hydrogel.

4.1.1 CDI Modification of the pHEMA Surface

CDI modification, as described in Section 3.3, was confirmed using Fourier-transform infrared spectroscopy (FTIR) (Figure 4). The addition of the CDI would replace the hydroxyl to form a pHEMA-imidazolyl carbamate. Thus on the unmodified surface, the presence of hydroxyls and no evidence of a carbonyl group is seen in Figure 4a (Carvalho et al., 2010; Lanzillotto et al., 2015). Figure 4b shows that, after the reaction, there is a significant decrease in the hydroxyl amounts marked by the peak at 3405 cm^{-1}. Additionally, a new peak at 1761 cm^{-1} appears which is indicative of the carbonyl found on a carbamate compound. This intermediate can be characterized as an alkoxycarbonyl imidazole which reacts with an amine nucleophile. The RNA and DNA aptamers used in these experiments contained 5'-amine modifications in order to attack the activated pHEMA surfaces (Guo et al., 2016).

4.1.2 Aptamer Attachment Confirmation

The aptamers used for drug testing were commercially purchased RNA aptamers specific for the drug Kanamycin B. Confirmation of the aptamer reacting with the surface was challenging due to the small size and the characteristics of RNA aptamers. These aptamers do not have unique chemical features that absorb light at a

specific wavelength and thus UV or FTIR were not ideal. Initial FTIR readings also identified remaining CDI on the surface meaning that not all the CDI was reacted and replaced with aptamer. Additionally, the RNA quantity was much smaller relative to the surface area of the pHEMA hydrogels and so elemental quantification was difficult.

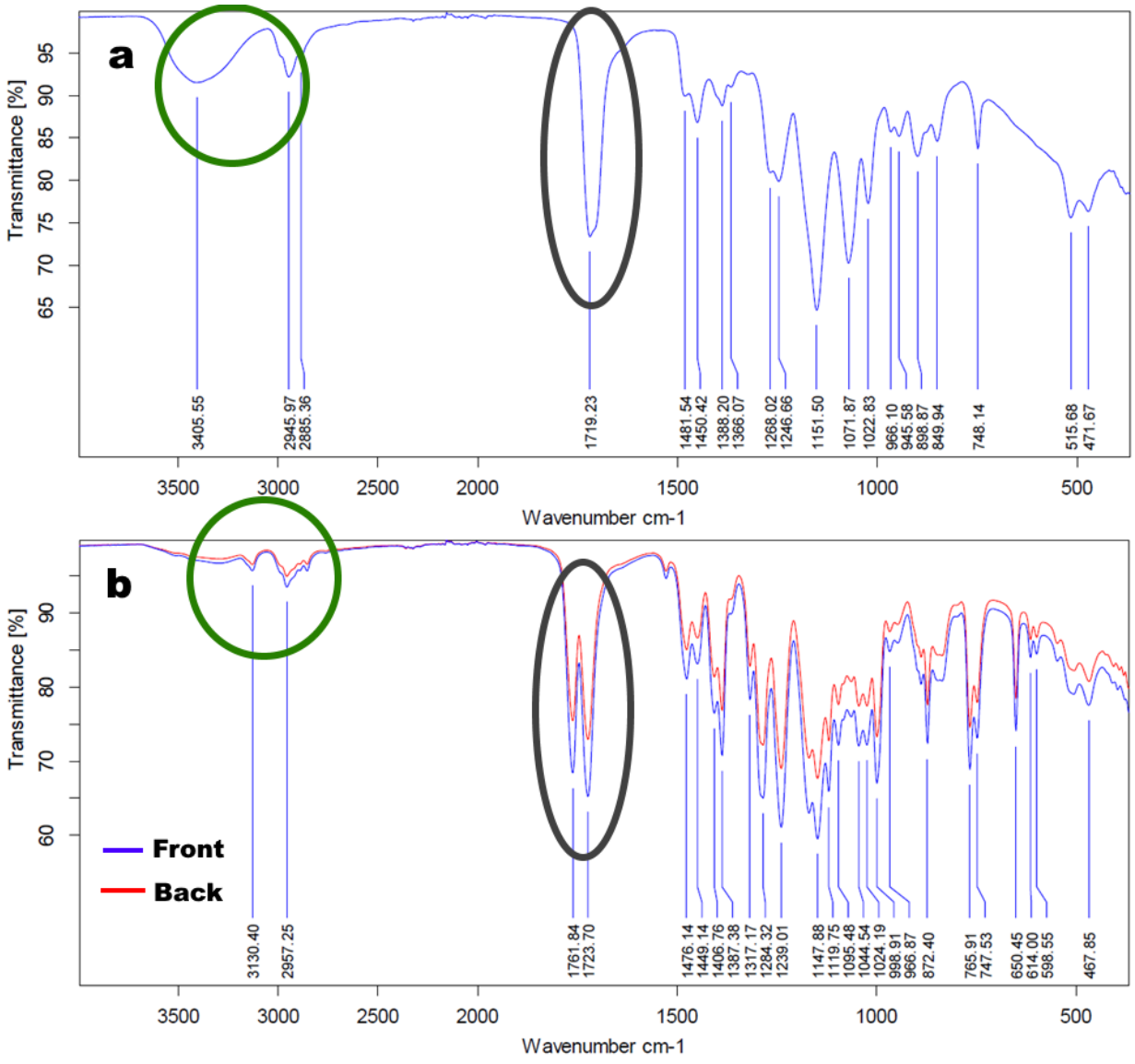

Figure 4: *FTIR output of CDI reacted pHEMA surfaces. A change at the 3405cm^{-1} and 1761cm^{-1} indicate the removal of hydroxyl groups and addition of imidazole respectively. The reaction took place in anhydrous 1,4-dioxane so any air exposure would have reduced the loss of hydroxyls. The 1,4-dioxane has no reactive species for this reaction and thus the only source of a carbonyl is the CDI.*

4.1.2.1 Fluorescent Probes

Aptamers with fluorescent probes were tested to confirm whether attachment occurred using the same chemistry. These DNA aptamers had similar 5 prime end amino group modifications like the RNA but also 6-carbofluorescein (6-FAM) probes attached to the 3-prime end. The 6-FAM probes are highly water soluble like the aptamers, thus they did not affect the solubility of the aptamers. DNA aptamers were used in these experiments as they are far more stable than RNA, especially unmodified single stranded RNA in a serum environment which degrade in seconds compared to DNA, which can maintain its structure for up to 60 minutes (Shaw et al., 1991; Takei et al., 2002; White et al., 2000). Even though this experiment was conducted in a nuclease free environment, DNA was used to reduce any loss between the attachment and scanning of the hydrogels. Furthermore, DNA were easily accessible and were available in larger quantities. Initial tests using the TECAN M1000 Fluorescent Plate Reader found no difference between hydrogels exposed to aptamer with and without CDI indicating these aptamers were small enough to be carried into the hydrogel through water absorption (Figure 5).

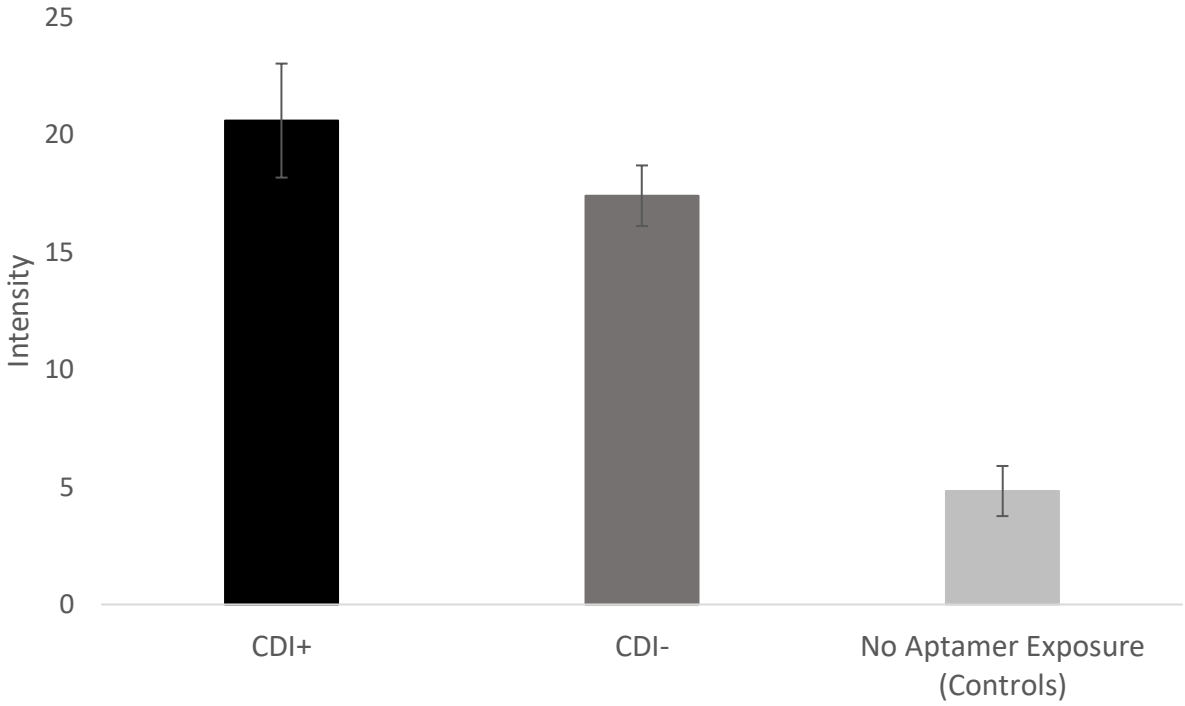

Figure 5: *TECAN readings of the pHEMA hydrogels from the initial batch of aptamer reacted experiment. CDI+ hydrogels contained CDI for aptamers to react and attach to the surface. The CDI+ group showed no significant difference from the CDI- and both showed a difference from the controls indicating diffusion of the aptamers are possible through the hydrogel pores.*

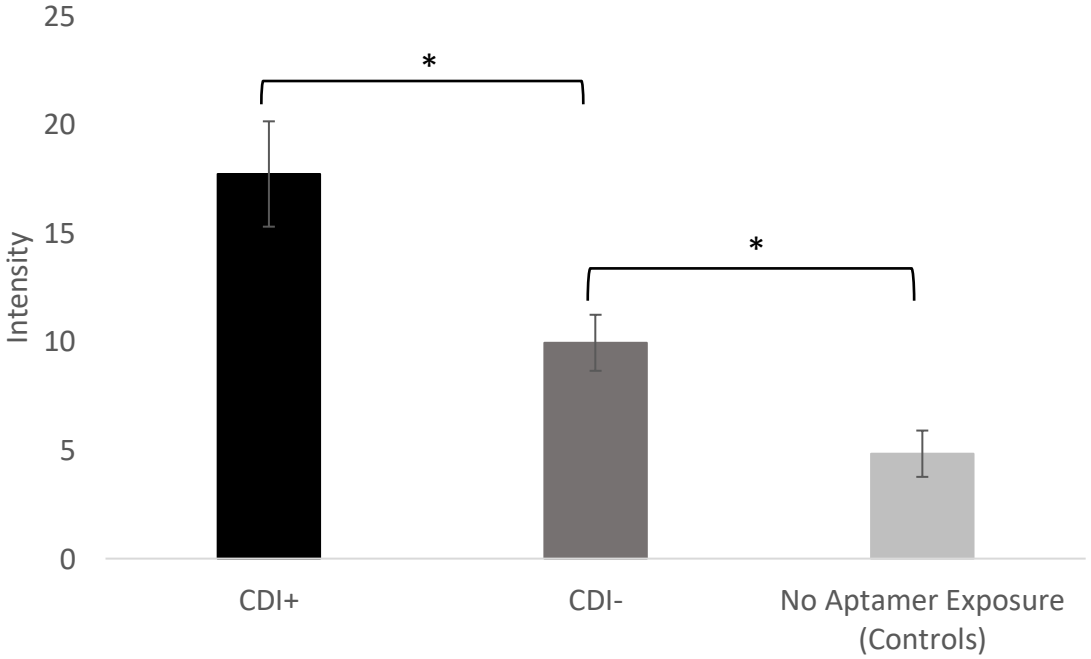

Figure 6: *TECAN readings of the pHEMA hydrogels following the amendments of the methodology (Section 3.5.2). CDI+ hydrogels contained CDI for aptamer to react and attach to the surface while CDI- did not. Both were exposed to the aptamers for the same period of time and a significant difference can be noted between all groups.*

Revisions to the protocol in order to reduce the amount of aptamer absorption included soaking the gels in water for 24 hours before being exposed to aptamer solutions as well as reducing the exposure time with aptamers. Fluorescence measurements under these conditions yielded a significant difference between the CDI+ and CDI- samples and controls (Figure 6). These results were confirmed with high quality imaging using the Amersham Typhoon 5 biomolecular imager (Cytiva, Malborough, MA, USA) and ImageJ for quantification where a more precise reading of the hydrogels showing the CDI+ were they only ones with present aptamer was observed (Figure 7,8). Figure 7 shows that the CDI- do in fact have some aptamer present as seen by the darker regions of the gels, indicative of absorption, but a significant portion was not absorbed due to the saturated nature of the gels. These results highlight the importance of swelling hydrogels prior to aptamer exposure to ensure greater surface exposure and overall depict how the CDI reaction is effective for attaching aptamers onto pHEMA discs which could further be applied to a commercial silicone-based contact lens.

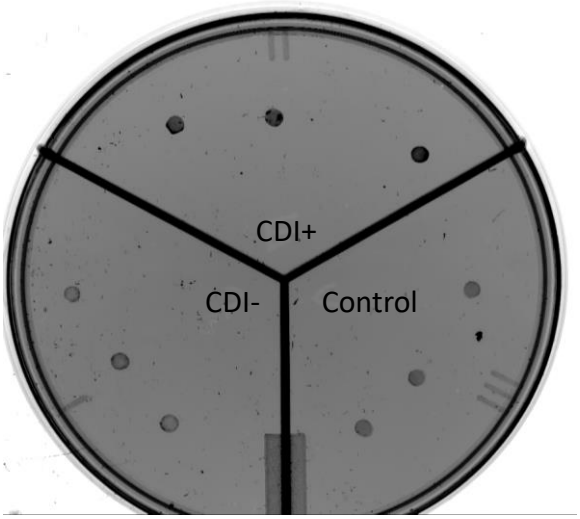

Figure 7: *Fluorescent imaging of a single batch using the Typhoon 5 Imager. Dark shades represent the 6-FAM fluorescent probes highlighted on the hydrogel. The CDI+ group clearly shows a higher intensity compared to the CDI- and controls. The CDI- still shows slight highlights indicating some did permeate into the gel.*

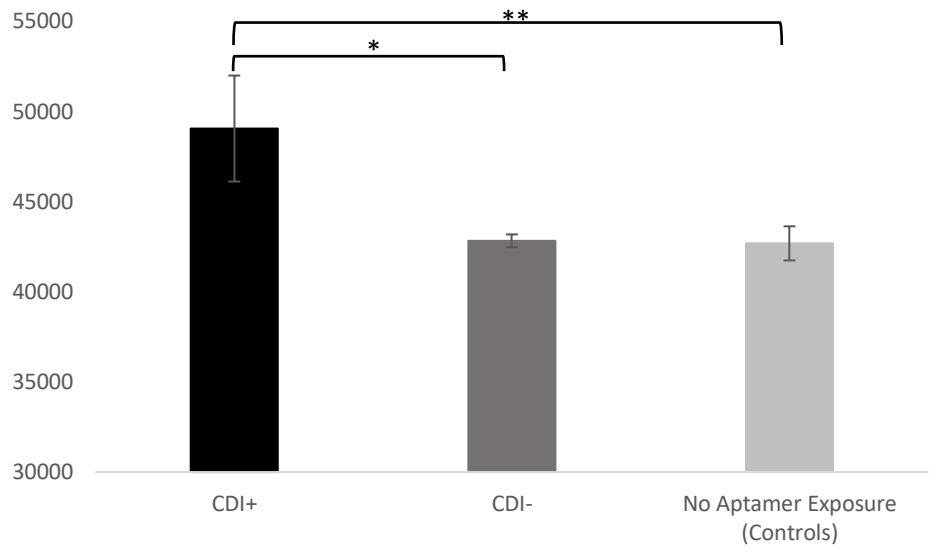

Figure 8: *Intensity of 6-FAM fluorescence measured from the analysis by ImageJ on the fluorescent image (Figure 4). A significant difference was found between the CD+ and CDI- groups indicating aptamer attachment was confirmed with the new methodology (* p=0.041).*

4.1.3 Contact Angle

The two-step process of attaching RNA aptamers to the surface utilized hydrophilic compounds and thus it was expected that there would be an increase in the hydrophilicity of the surface. RNA molecules are inherently hydrophilic in nature due to nucleotides which contain hydroxyl groups used for hydrogen bonding. The attached aptamers are single stranded RNA meaning that there are minimal hydrogen bonds making them open to attracting water molecules. Figure 9 shows how the addition of aptamer resulted in a decreased contact angle, maintaining the hydrophilicity of the surface. Additionally, CDI is also a water miscible compound and thus any remaining CDI on the surface could have contributed to this decrease.

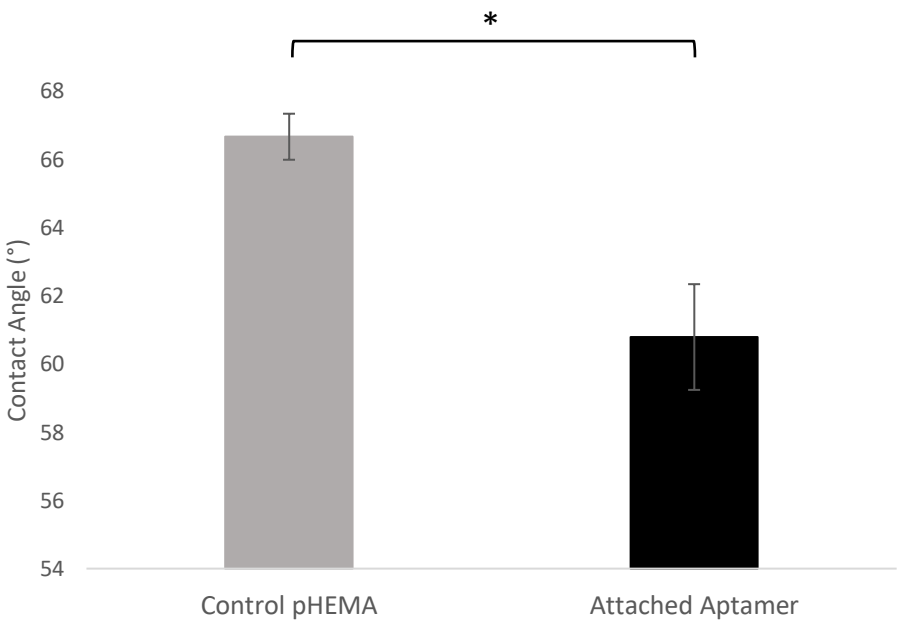

Figure 9: *Wettability test measured through contact angle. A significant difference was confirmed using a t-test (p=1.20E-8, n=4) which shows the aptamers do increase the hydrophilicity of the surface as expected.*

4.2 Kanamycin B Separation and Detection

4.2.1 Detecting Kanamycin B

Kanamycin B is an aminoglycoside with a very basic structure, and no unique chemical features when compared to the pHEMA surface and RNA making ultraviolet (UV) spectroscopy not an ideal detection method (Figure 10). Furthermore, any degraded RNA would contain nitrogen and thus any elemental differentiation was not ideal for this drug testing. Previous research has worked with HPLC separation and UV detection by modifying kanamycin amine groups in samples pre-column and post-column separation. These modified derivatives enabled kanamycin to be detected via UV or even fluorescence (Zhang et al., 2019). A more common method of detection is using the mass of kanamycin which is very distinguishable compared to any other compound in the release solution and thus liquid chromatography-mass spectrometry (LC-MS) was utilized for the detection.

4.2.1.1 Kanamycin Fragmentation

LC-MS utilizes a separation by chemical and physical nature of a compound and thus all kanamycin in solution was separated via column separation, followed by electrospray ionization which results in fragmentation of the drug. Mass spectrometry was used to first determine the fragmentation pattern of Kanamycin B which has a molecular weight of 483.51g/mol. This can be identified as the precursor ion in Figure 11. The drug fragments were confirmed through comparisons to blank solutions to be 324.2g, 205.0-205.3g, and 163.9g product ions (Acaroz et al., 2020).

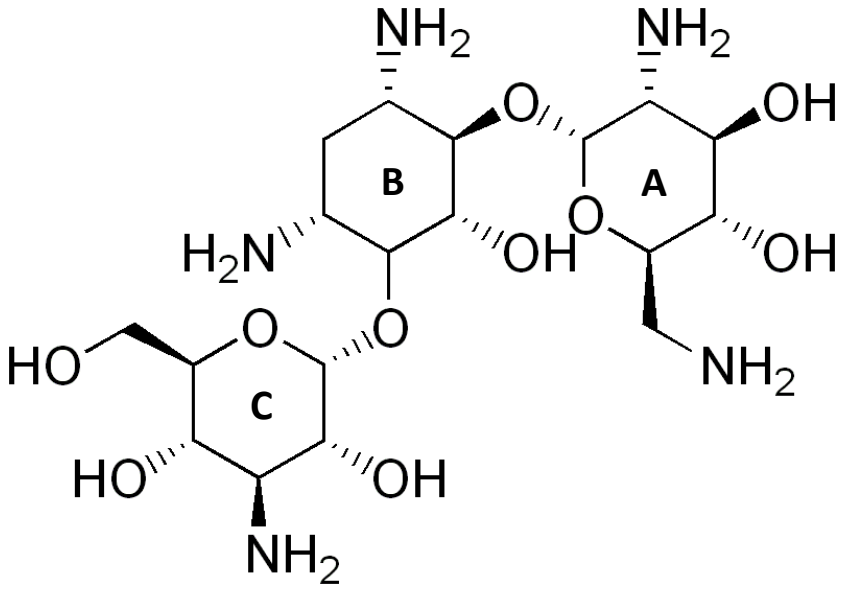

Figure 10: *Structure of Kanamycin B. A three-ring structure (labelled A, B, C) comprised of hydroxyl and amine groups.*

4.2.1.2 Column separation using Hydrophilic-Interaction Chromatography

Liquid chromatography separation was the first step in the detection of the drug released and this involves using an appropriate column. Like most liquid chromatography columns, there is a polarity difference between the stationary phase or

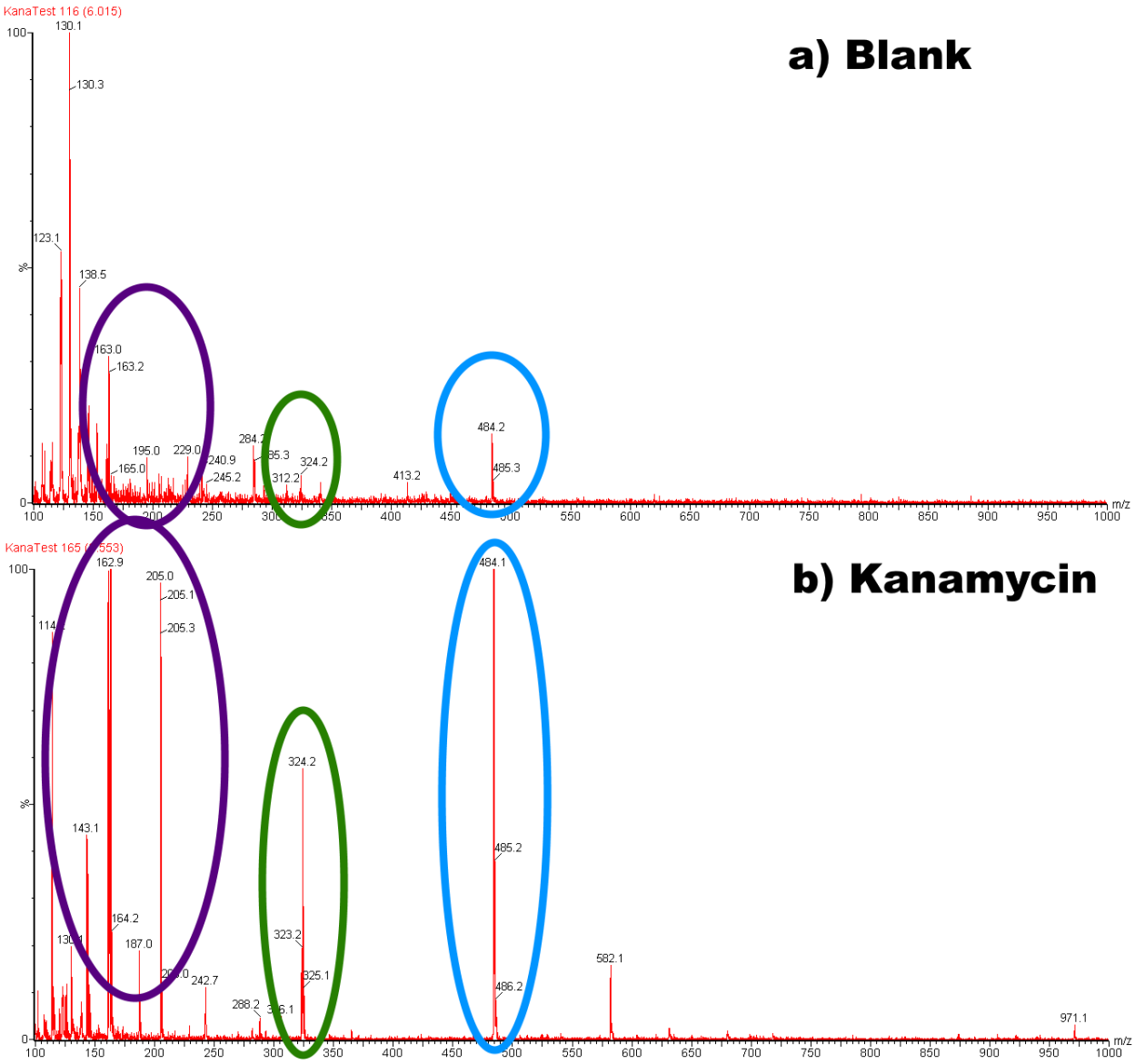

Figure 11: *Mass spectrometry reports of blank DNAse/RNAse water compared to Kanamycin (0.5mg) mixed solutions. Precursor ions (blue) were ionized and detected over various concentrations. The product ions determined that best represent Kanamycin presence were 324.2 and 162.9 (green and purple respectively).*

column packing compared to the mobile phase, which is regularly a solution passing through the column, containing the target compound. A polar stationary phase was first attempted in order to retain the highly hydrophilic Kanamycin B and release at a later stage after filtering other compounds. Kanamycin is a very polar compound and thus a regular normal phase with 100% organic mobile phase was not used. Hydrophilic-Interaction Chromatography (HILIC) is an alteration of normal phase which adds water

to an organic mobile phase in order to provide a competing compound against the drug for attachment to the polar stationary phase. With the use of a gradient, the mobile phase water content increases, thus building the polarity of the mobile phase and releasing the analyte attracted to the polar stationary phase (Acaroz et al., 2020; Zhang et al., 2019).

This was tested with various gradients, beginning at very low hydrophilic mobile phases and quickly changing to highly hydrophilic to elute the drug. Figure 12 depicts the common result of a gradient change from 5% to 97% hydrophilic mobile phase (0.1% FA) and what was noted was the high retention seen by the very broad spectrum.

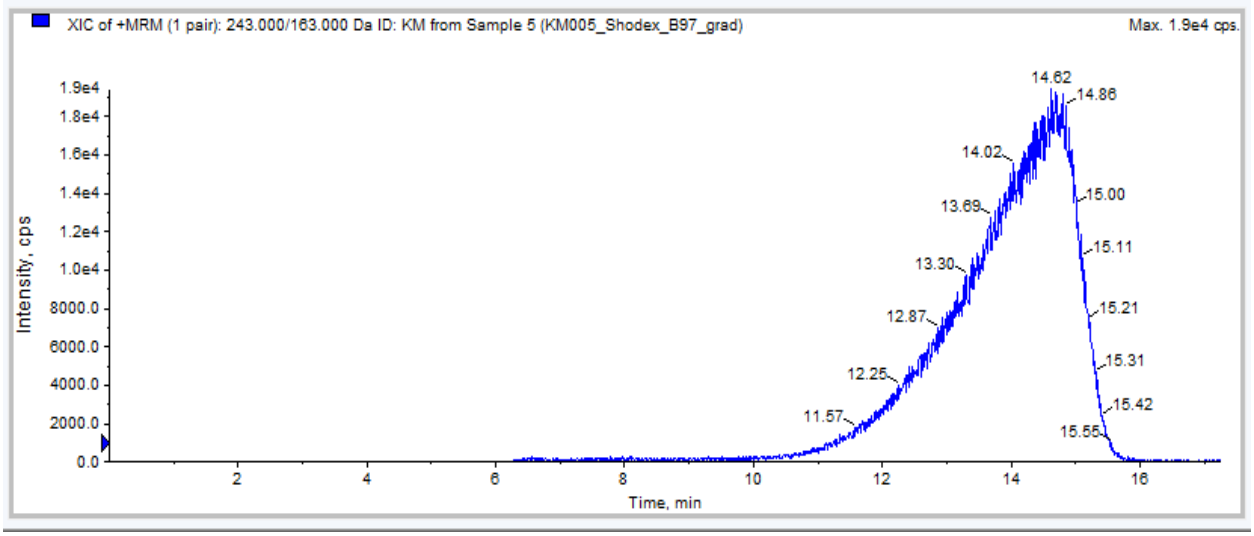

Figure 12: *Depiction of the Kanamycin detection starting with a 5:95 A to B solvent ratio (0.1% FA (solvent A), ACN (solvent B)). A gradient lasting from 2 minutes to 15 minutes resulted in a 97:3 solvent mixture however Kanamycin was very slow to release even by the end of the gradient. The drop off at 14.86 mins was indicative of the solvent mixture changing back to the initial low hydrophilic mixture. Fragments 243.0 and 163.0 were measured for Kanamycin detection.*

While this method enabled a late release well past any dead volume clearance, it was not clear if all the drug was released for an accurate measurement or if some were still retained on the column. This initial testing did exemplify that long gradients were not ideal for the detection of Kanamycin and thus quick gradient changes were used in all

subsequent studies. A narrower gradient from 20:80 to 95:5 solvent A yielded slightly greater success in release accuracy as it was noted that all precursor kanamycin signals were released before the end of the gradient at fifteen minutes (Figure 13). However, the broad spectrum was still present and thus the potential for fragments to have been retained on the column still existed using this basic HILIC setup. Previous research has shown that HILIC is able to separate from less polar solutions such as bovine milk in comparison to the ultrapure water in the present study. Acaroz et al. demonstrated that kanamycin could be extracted from bovine milk using a similar HILIC setup of 0.1% FA in water (solvent A) and ACN (solvent B) (Acaroz et al., 2020). Their HILIC setup was an isocratic system as all kanamycin was detected before any change in the mobile phase ratio. When using a gradient, a stronger mobile phase may be needed to ensure high resolution. HILIC systems using higher concentrations of FA in both mobile phases have been able to extract kanamycin at appropriate resolutions (Zhang et al., 2019). For future attempts, HILIC separation is a viable option but would require the use of 1% FA instead of 0.1% in both mobile phases to yield a sharper peak as increasing the acid content reduces the interactions between the target drug and the column stationary phase (Li et al., 2010). Highly concentrated (150mM) ammonium acetate could also be a viable mobile phase A to replace water and FA.

Lastly, Acaroz et al. had an immediate release with very little dead volume at the start of the chromatography which could contain potential contaminants in the present study as broken-down RNA or hydrogel could be present in the highly polar release solutions. Many studies that tested LCMS separation from bovine milk have early releases which suggests a HILIC release may work from a less polar sample solution,

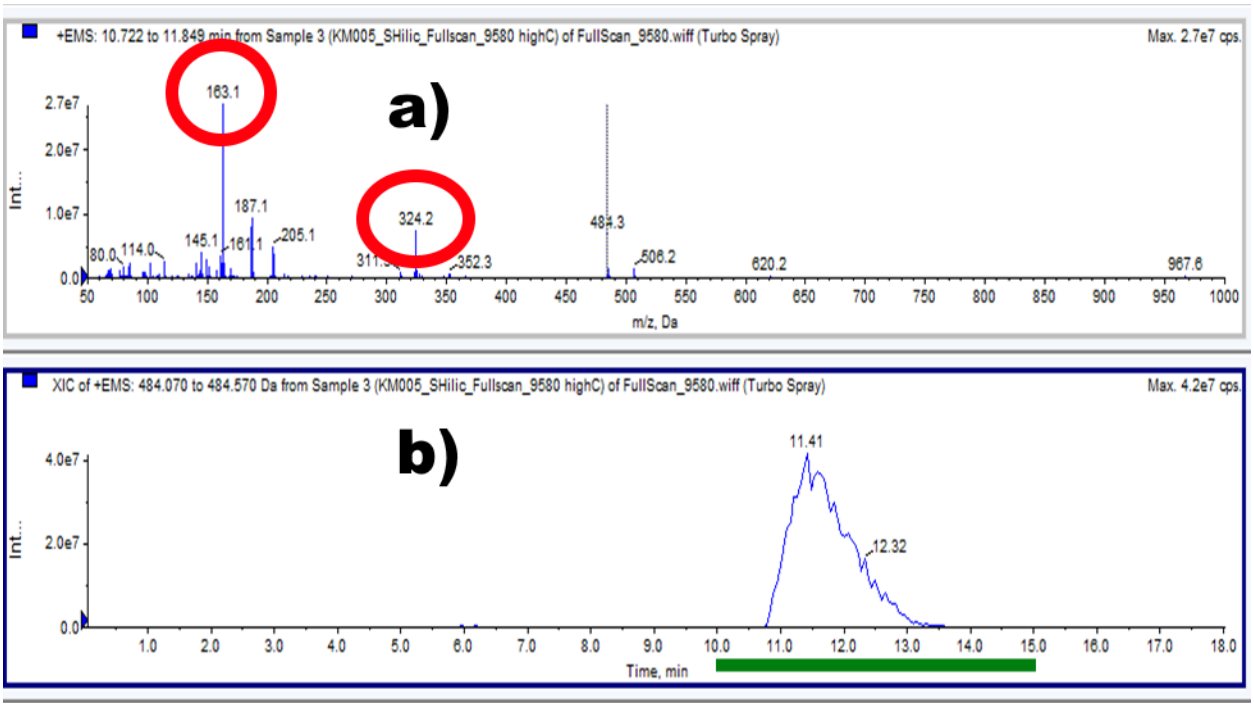

Figure 13: *20:80 to 95:5 gradient over the 13-minute period (2 - 15 minutes). Fragments were still detected over a wide range (a, red), however all precursor Kanamycin B (484) compounds were released before the end of the gradient (b, green bar).*

whereas the setup in the present study utilized highly filtered water with very little initial contaminants meaning kanamycin may have been much harder to separate from the solution and attach to the column (Acaroz et al., 2020; Goutalier et al., 2012). An isocratic method was not tested in the present study as we had aimed for a larger dead volume at the start of testing.

4.2.1.3 Column Separation using Reverse-Phase chromatography

With the high polarity of the sample solution and high retention found on polar stationary phases, the alternative option of RP was considered with the aim to reduce the retention. A gradient system was implemented in order to attempt to slow the release for as long as possible to enable a dead volume release. A basic carbon-18 bonded silica (C_{18}) column was initially tested (LUNA C18(2), Phenomenex) showing

very little retention of kanamycin which was to be expected with the high polarity of the drug (Figure 14) (Mugabo et al., 2015).

Figure 14: *Kanamycin separation using the LUNA C18(2). The precursor ion and product ions were able to be identified (red, b) while 324 was also detectable (c). The release was very early near the dead volume and had a broad tail end (d).*

A broad spectrum was still present; however, it was much smaller and repeatable using the RP separation. As seen in Figure 14, a slight taper at the end was present indicating some level of unstable retention or release. Previous research does show that this taper region persists in most releases, however it can be reduced to less than 30 seconds (Mugabo et al., 2015). A second C18 column was also tested with very similar results and thus it was clear an alteration on RP was needed in order to improve the accuracy.

The Synergi polar-RP column was selected as a C_{18} column with specific ether-linked phenyl groups to increase retention of highly polar target compounds. With the addition of 0.1% FA in both mobile phases, a clean separation and early release of the drug target kanamycin was achieved with this column (Figure 15). Precursor and the 242.8 product ion were present in the full scan indicating both could be detected. Previous results have confirmed that RP is a useful and accurate method to detect kanamycin from a variety of solutions and thus this column and method were chosen for drug release measurement (Zhang et al., 2019).

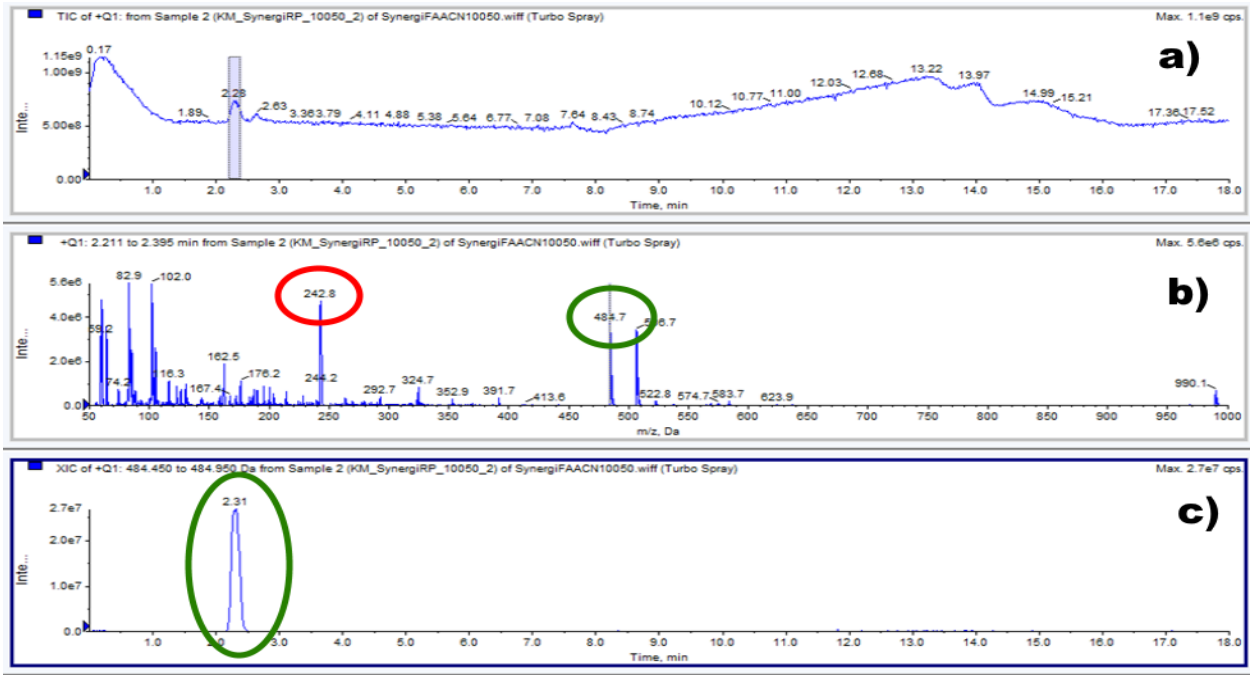

Figure 15: *A full scan of sample solution containing kanamycin B using the Synergi polar-RP column with the high hydrophilic mobile phase method described in 3.6.3. kanamycin was all detected early at the start of the gradient (a). The product ion (red) and precursor ion (green) are identified (b) and can be confirmed to only appear at this early release (c) depicting a clean and accurate release.*

4.3 Release of Kanamycin from pHEMA Hydrogels

The release of the antibiotic kanamycin B from the modified and unmodified pHEMA lenses was used to determine if aptamers could make a difference in drug release. Specifically, the burst release was examined followed by the change over 72

hours. The end point of 72 hours was selected as the readings had dropped within range of inaccuracy determined by the LOD (1.71ug/kg). The K8 aptamer is highly specific through hydrogen bonding of the -OH groups, particularly the ones in the A ring seen in Figure 10 (Kwon et al., 2001). These hydroxyl groups on the drug make for a tight, strong bond and thus giving the aptamer a strong hold on the drug at the surface until the RNA breakdown. Figure 16 depicts the release of kanamycin B from both the controls and aptamer positive hydrogels which was found to have no significant difference.

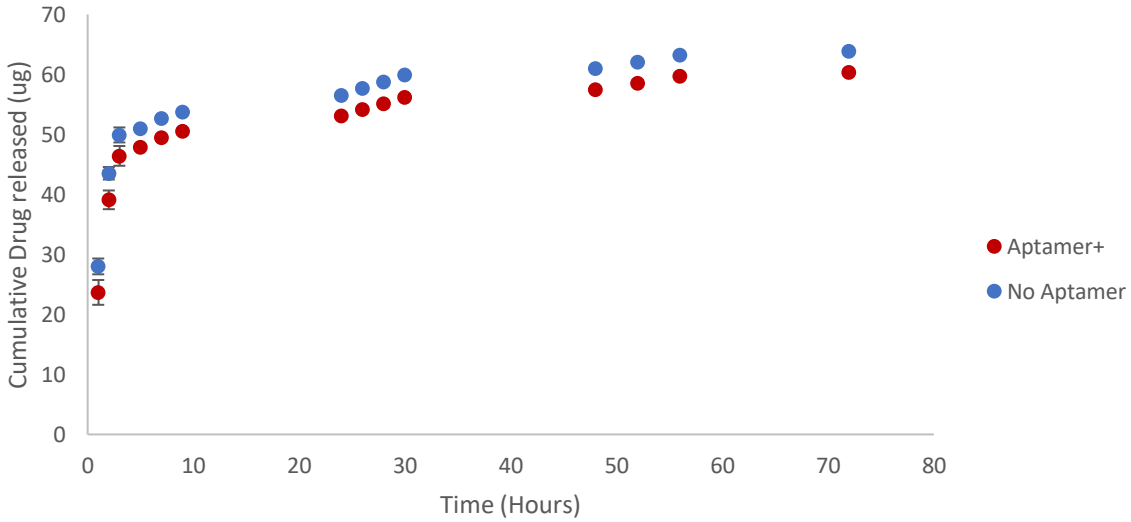

Figure 16: *Release profile of kanamycin B from aptamer positive hydrogels (red) and aptamer negative hydrogels (blue) detected through LC-MS quantification (n=3). The release was measured for 72 hours before the endpoint was decided as the change in drug release was within range of the LOD and deemed inaccurate. A t-test assuming homoscedastic variance confirmed an insignificant difference between the release profiles (p=0.8721).*

While the overall release trends did not have significant differences, the examination of the burst release also showed an insignificant difference, albeit much closer to a statistical difference compared to the rest of the release profile (p=0.0666). This may be due to several different factors.

4.3.1 Multiple Reaction Monitoring Setting Error

Multiple Reaction Monitoring (MRM) is a highly specific technique for filtering and quantifying mass targets, including their product ions following fragmentation. For most triple quadrupole MS instruments like the one used in this study, there are three segments compounds transition through. Q1 usually targets the most common fragment or the precursor ion itself. Once these pass through Q1, these are usually further fragmented in Q2 through a collision-induced dissociation involving neutral molecules in a gas state (You et al., 2013). The final fragments transition to Q3 where they are filtered to send a specific mass to the detector which outputs a single signal. With the current setup, an error was found where Q1 was filtering one of the product ions (324.7) instead of the precursor ion (484.2). The impact of this error can be quite substantial in that it indicates a significant amount of target was lost in the initial filtering at the first quadrupole. Figure 15 shows a full scan with little fragmentation of kanamycin solution; ions at 484 represent a significant portion of the scan, while the ions at 324.7 were only a small fraction. However, the MRM did use a higher entrance collision voltage compared to the enhanced mass scan (EMS) which was used for the full scan and thus higher fragmentation could have occurred at the initial ionization into Q1. Had the Q1 filter been appropriate at 484, the results may not have been impacted as it could have just simply increased the signal strength of both drug groups by a similar factor.

4.3.2 Lack of Surface Aptamers

The efficiency of aptamer attachment through CDI chemistry is not a well-documented reaction. A ratio of 20:1 CDI was exposed to the discs to ensure a saturated surface activation. However, the amount of aptamer used to follow this step

was about a tenth of the overall -OH concentration on the surface due to limitations in the amount of aptamer available. It was assumed that neither the CDI nor the aptamer would react to 100% completion and thus the surface was not expected to be saturated, however the results suggest that the amount of conjugated aptamer was smaller than anticipated. To provide further evidence of this, X-ray Photoelectron Spectroscopy (XPS) was used as a highly specific surface test in case the aptamers could be detected by surface element changes.

4.3.2.1 XPS Surface Analysis

XPS was performed on a batch with the fluorescent DNA probes to assess if the aptamers were on the surface. pHEMA gels have high concentrations of carbon and oxygen while the CDI on the surface has nitrogen and thus the only unique element DNA contains compared to these is the phosphorous present in the phosphate which make up the backbone of DNA. Table 3 displays the averaged results of XPS. Oxygen levels do rise with the aptamer presence which corresponds to the hydroxyl groups found on nucleotides of RNA. Nitrogen is found in both CDI and DNA, however DNA does contain more per moles reacted. Thus, the aptamer modified surfaces should have had significantly more nitrogen present. However, no significant difference was seen between the aptamer positive (Apt+) group and CDI only groups (CDI+).

Table 3: XPS values the control (apt-) discs, aptamer present (Apt+), and CDI+ without aptamer exposure (CDI+) discs. Values were determined by analysis of 3 different points on each disc and averaged. Nitrogen can be identified on the CDI+ discs including those reacted with aptamer with no significant difference (yellow). However, phosphorous was only seen on one disc indicating an unsuccessful detection of aptamer (red).

	C	O	N	P	
Control 1	79.3	16.3	0	0	
Control 2	76.7	20.6	0	0	
Control 3	74.1	22.1	0	0	
Apt+ 1	71	21.6	2.6	0	
Apt+ 2	62.9	27.7	3.5	0	Averaged From 0, 0, 2.1
Apt+ 3	51.1	28.1	5.6	0.7	
CDI+ 1	69.3	23.8	2.2	0	
CDI+ 2	74.2	21.1	1.8	0	
CDI+ 3	75.4	21	2.3	0	

In addition, the phosphorous was barely detectable, only being seen in one of the three discs. More specifically, phosphorous was only detected in one of the three specific readings per disc (0,0, 2.1). These XPS results suggest two concerns in the detection of aptamers on a surface. It appears that the use of CDI for aptamer attachment may not entirely be a uniform reaction. This non-uniformity is a concern in that the current XPS method used could not measure both sides of the disc due to a size limitation and thus if both sides of the discs were uneven in their aptamer distribution, the detection would be lower than expected. A possible identification of the problem was found in the aptamer reaction methodology. This step was done in a glass 8mL vial with a flat surface and the discs were placed flat at the bottom. During incubation, the vials were rotated in an upright position without any flipping or exchanging of sides of the discs.

Thus, one side of the discs were always against the glass. Changing this method to either flip the discs between the 12-hour exposure time or have the discs shaking more vigorously to ensure rotation could potentially solve the lack of uniform aptamer reaction. Nevertheless, within the context of future applications, only one side of these lenses need to be modified to release drug onto the surface of the eye. A future experiment could be done to modify one side by sealing one end with transparent sealing tape and place this face down in the vials. This would prevent activation of this surface with CDI and thus when exposed to aptamers, they will only react to one side of the disc.

The second explanation for these results is they could confirm there was not enough aptamer for a uniform spread. Therefore, future studies should focus on increasing the incubation time and speeding up the incubation by shaking with a saturated concentration of aptamer. However, previous research has also shown XPS is not commonly used to identify aptamer surface attachment and instead is more commonly used to identify surface modifications that enable attachment of aptamers to the surface (Metaferia et al., 2013).

4.3.3 RNA Degradation

Another plausible cause for the lack of change in the burst release or reduction in the prolonged release could be the degradation of RNA. While this experiment was conducted in a nuclease free, highly purified water environment, RNA is still much more unstable than DNA and contamination was possible with these protocols. In serum, RNA typically has a half-life of a few seconds, but they can be modified for higher stability and protection from nucleases (White et al., 2000). RNase contamination was

not impossible with the setup of changing discs from one solution to another at each time point, including the loading period, and thus it may be important to ensure modified RNA aptamers are used in future experiments to reduce any early degradation. The presence of the K8 aptamer after the drug release was not assessed in the current study; this study should be conducted with stability protecting modifications to assess if these aptamers can sustain being attached to the hydrogel surface for the period of an extended drug release.

4.3.4 Longer Release Period

The 72-hour period was quite a short release and given the values on the third day were too close to the LOD, any further results were deemed inaccurate. At this stage in the release, roughly 2/3 of the drug appeared to have been released, however as mentioned, the detection method had an error and thus there may be more drug remaining or released than it appears. The previous sections described the possible causes for the inaccuracy of the study and thus the possibility of a delayed release cannot be excluded until the previous errors are resolved. Drug release from hydrogels has been studied in the past and quite often are measured to at least 100 hours of release, if not more (Postic & Sheardown, 2019). With the assumption that the K8 aptamers were still present on the surface, their strong affinity to the drug may have delayed the release and thus a prolonged release could have been possible. The aim with these aptamers were to prolong release of drug through surface steric hinderance and drug holding through hydrogen bonding. In an *in vivo* setting, protected aptamers would be required to delay degradation on the ocular surface. Endonucleases are present in the tear film which would be the main source of DNA or RNA breakdown

(Yusifov et al., 2008). When the uptake values were examined, it was found that by the 72-hour mark, neither the controls nor experimental gels had released 100% of their drug. As previously mentioned, this missing drug could have been caused by inappropriate filtering of the MS quadrupole 1, but additionally some drug may remain associated with the gels. With the appropriate MRM settings, the signal strength may have been much higher and above the LOD borderline which would have enabled this study to pursue a longer release time.

5.0 Conclusions and Future Directions

The novel aptamer-based contact lenses developed in this work are one of the first applications of aptamers to control drug release from model contact lenses. Confirmed using fluorescent probes, it was demonstrated that these aptamers could be attached to the hydroxyl saturated surface. The use of CDI was appropriate and has been conducted in previous work to activate the surface of pHEMA. Aptamers were modified to have a free end amine group which could then form amide bonds with the pHEMA surface to create a strong bond for a longer lasting modified surface. The drug release found no significant difference with the use of aptamers, however given the number of variables that can be changed, the results of this study do not conclusively demonstrate that aptamers cannot be used to alter drug release from model contact lenses. The aim of these aptamer materials was to apply these to silicone hydrogels which have much longer wear time and thus could allow for drug release over longer periods for patients. Future studies should consider an amendment of the errors found during the drug release. Specifically, using 484 for Q1 and 163 for Q3 could yield results with higher signal strength and enabled longer release times to be measured. Additionally, a release profile with model lenses modified on one side should be examined to provide applicable results to a future use. Since a silicone-based application is expected for future use, attachment of the K8 aptamer to p(HEMA-co-TRIS) hydrogels will also produce a more realistic and applicable model.

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Appendix A - PBS buffer recipe

Created with 1L of MilliQ water, 2.47g sodium phosphate diabasic heptahydrate, and 0.345g sodium phosphate monobasic monohydrate, stirred for 30 minutes and placed in 4°C fridge until use.