POLYMER-PEPTIDE SYSTEMS FOR THE OCULAR SURFACE AND MEDICAL DEVICES

by
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Abstract

Hyaluronic acid (HA) wetting solutions effectively lubricate the ocular surface and are used clinically for corneal epithelial wound healing and to treat dry eye disorders resulting from Sjögren syndrome and keratoconjunctivitis sicca. However, HA has a low eye residence time of less than 10 min owing to limited adhesion, which necessitates frequent instillation of HA-based eye drops. Conversely, more viscous artificial tears are known to blur vision and interfere with blinking. We therefore developed a polymer-peptide system based on a heterobifunctional HA-binding peptide (HABpep) polymer-peptide system that binds and retains HA, from tear fluid or exogenous application, for longer periods of time at the surface of the eye. The key is to anchor HABpep polymer-peptide system on either the ocular surface or on the surface of a contact lens. To target the ocular surface, the anchoring peptide targeted sialic acid-containing glycosylated transmembrane mucins, the most peripheral extracellular ocular epithelium protecting and lubricating surface, or the ocular tissue matrix enriched in collagen type I. For contact lenses, the anchoring peptide targeted dimethylsiloxane units on the surface.

The first part of this thesis will demonstrate that a polymer-peptide system utilizing HABpep can enhance the biological and physical benefits of HA at the ocular surface and potentially be more effective in treating dry eye than a standard HA eye drop. The latter part will discuss how modifying contact lens surfaces with a polymer-peptide system can also reduce dry eye irritation in contact lens users.

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1. Introduction

Dry eye is a prevalent ophthalmic condition especially to elderly population, affecting over 15% of the US population (Ding and Sullivan 2012). Symptoms include discomfort, dryness or lack of hydration, visual disturbances and pain in the eye, which can hinder the performance of everyday activities (Abetz, Rajagopalan et al. 2011; Pouyeh, Viteri et al. 2012; Pili, Kastelan et al. 2014). Although dry eye is a multifactorial disease, it occurs mostly due to any alterations in the tear film composition and stability with potential damage to the ocular surface. There are three ocular tear film layers that have unique functions. The outermost lipid layer protects the eye from foreign particles, lubricates the inner eye-lid and exterior eye and slows down the water evaporation. The second, aqueous layer keeps the eye hydrated and contains water-soluble mucin, salts, proteins, and glucose for nutrition, while the transmembrane mucin layer provides ocular corneal protection and tear film adhesion (Figure 2.1). Tear film–related dry eye disease is further categorized into two major conditions: aqueous-deficient and evaporative. Aqueous-deficient dry eye is caused by lacrimal gland's inability to produce sufficient tear fluid, whereas evaporative dry eye is caused by increased tear film evaporation rate due to a compromised protective lipid layer (Horwath-Winter, Berghold et al. 2003; Sharma and Hindman 2014). To treat dry eye disorders, many different approaches have been employed (Young, Veys et al. 2002; Johnson, Murphy et al. 2006; Ali and Byrne 2009; Papas, Ciolino et al. 2013); however, these strategies are temporary, involving the application of a saline or an emulsion-based eye drop to reduce discomfort and increase water retention of the ocular surface (Young, Veys et al. 2002; Johnson, Murphy et al. 2006; Prabhasawat, Tesavibul et al. 2007; Ali

and Byrne 2009; Papas, Ciolino et al. 2013; Labetoulle 2015; Simmons, Carlisle-Wilcox et al. 2015). These artificial tears must be reapplied frequently owing to their short residence time in the eye (<10 min).

Currently, commercially available eye drops containing 0.1-0.2% hyaluronic acid (HA) solutions, such as Opticalm™, Scope™, and Oxyal™ are commonly used as an active ingredient in Europe to relieve dry eye symptoms, while in USA as an inactive ingredient of the eye drop formulation (e.g. in Blink™). HA is an anionic glycosaminoglycan that is present in many tissues and organs as a major component of the extracellular matrix, has been shown to ameliorate dry eye because it retains water through hydrogen bonding (Hargittai and Hargittai 2008), stabilizes the tear film and lubricates the surface (Tonge, Jones et al. 2001; Read, Morgan et al. 2009). HA has also been shown to slow tear removal and permit uninterrupted blinking (Tsubota and Yamada 1992; Nakamura, Hikida et al. 1993; Hamano, Horimoto et al. 1996). In addition, HA has several desirable therapeutic properties; for example, it encourages corneal wound healing by promoting epithelial cell migration (Stuart and Linn 1985; Shimmura, Ono et al. 1995; Gomes, Amankwah et al. 2004; Johnson, Murphy et al. 2006; Rah 2011), reduces inflammation (Pauloin, Dutot et al.) and protects cells from free-radical damage (Presti and Scott). Recent studies (Bray, J. Theor. Biol, 2001; Georgiev et al., Soft Matter, 2013; Cerretani et al., Adv. Colloid Interface Sci. 2013) further indicate that polyanionic polysaccharide moieties, including HA can enhance the spreading of the tear film lipid layer, which may be an added clinical benefit of HA for ocular application. HA eye drop solutions effectively wet and lubricate the contact lens and ocular surfaces, but similar to saline, poly(vinyl alcohol)/PVA, methyl cellulose (MC) and hydroxypropyl

methylcellulose (HPMC)-based eye drops, they suffer from low ocular residence time (<10 min) due to limited adherence to ocular surface (Snibson, Greaves et al. 1992; Mochizuki, Yamada et al. 2008; Papas, Ciolino et al. 2013); conversely, highly concentrated HA-based eye drops are too viscous and interfere with vision and blinking (Marner, Mooller et al. 1996; Reddy, Grad et al. 2004; Ali and Byrne 2009; Papas, Tilia et al. 2014). Therefore, an HA binding eye drop technology that prolongs HA retention and delivery on ocular surfaces would capitalize on the biological and physical benefits of HA.

We anticipate that this eye-drop and contact lens technology can further be designed to target dry eye disease helping millions of people who need long-lasting therapeutic eye drop solutions or contact lenses that due not induce dry-eye.

2.1 Eye-Drop Techonlogy

2.1 Introduction

We aimed to enhance the binding of HA to the eye surface, which would retain a thin film of moisture to mimic the smooth, hydrated and lubricated ocular surface. We hypothesized that a previously designed HA binding peptide (HABpep) (Tolg, Hamilton et al. 2012; Amemiya, Nakatani et al. 2005) could localize HA to the ocular surface through anchoring sites on the ocular surface. The ocular epithelium is covered by a layer of transmembrane mucins known as the glycocalyx that are abundant in sialic acid (Fig. 2.1). These cell surface mucins have a large O-glycosylated extracellular domain, and mucin-1 is the most abundantly expressed one on both corneal and conjunctival epithelia (Gipson et al. 2004). Therefore, we harnessed sialic acid–containing mucin-1 as one anchoring site for immobilizing HA using HABpep. Moreover, in case of severe dry eye or corneal abrasion, the ocular surface epithelium is damaged and mucin-1 on the ocular surface decreases significantly (Corrales, Narayanan et al. 2011). Therefore, we also selected collagen as an alternative anchoring site, as stromal collagen will be exposed when the epithelium is absent. We tested sialic acid binding peptide (SABpep) or collagen binding peptide (ColBpep) for their abilities to bind ocular tissues and further developed HABpep containing heterobifunctional poly(ethylene glycol)/PEG-peptide systems. Specifically, we present here in detail the efficacy of SABpep-PEG-HABpep eye drop solutions that prolonged HA delivery by 3 folds *ex vivo* and *in vivo* in rodents compared with HA-only solutions. We further assess the lubricating ability of eye drop solutions with *ex vivo* rabbit ocular tissues, as lubrication is critical for smooth surface

movement and uninterrupted blinking. Lack of enough lubrication many traumatize epithelium and develop a clinical condition known as lid wiper epitheliopathy or LWE, a frequent symptom of dry eye (Korb, Herman et al. 2005)

We anticipate that this eye-drop and contact lens technology can further be designed to target dry eye disease helping millions of people who need long-lasting therapeutic eye drop solutions.

2.2 Materials and Methods

2.2.1 Materials

Six different binding peptide candidates that could potentially bind ocular tissues were custom synthesized (NeoBioLab, MA, USA) (Table 1) (36-41). The peptides were biotinylated with a linker, *N*-hydroxysuccinimde (NHS)-PEG-biotin (Life Technologies, NY) with a standard method as recommended by the manufacturer's protocol. HA, HA-biotin and HA-fluorescein (HA-FL) (each with Mw 800 kDa) with a degree of substitution ~ 5 mol %, were purchased from Creative PEGWorks (Chapel Hill, NC). Heterofunctional poly(ethylene glycol) (PEG, Mw 1000 Da) with SABpep (GGSPYGRC) and HABpep (STMMSRSHKTRSHHVGC) (Amemiya, Nakatani et al. 2005; Tolg, Hamilton et al. 2012) were custom synthesized from SynPeptide (China).

2.2.2 Screening of ocular surface-binding peptides

Conjunctival tissue from the ocular surface was targeted to test the binding affinity of the multiple peptides. Conjunctiva was harvested from rabbit eyes (Pel-Freez Biologicals, Rogers, AR) with the underlying tenon's capsule removed, and then cut into 6 mm pieces

with a biopsy punch. The conjunctival epithelium was kept intact for testing SABpep, while the epithelium was scrapped off with a cell scraper for testing type I collagen-binding peptide (ColBpep). The tissue pieces were merged in the biotin-conjugated peptide solutions (1.0 mg/mL in PBS, pH 7.4; Life Technologies, NY) for 20 minutes and then washed three times with PBS to remove any unbound peptides. A HABA (4'-hydroxyazobenzene-2-carboxylic acid) assay (Life Technologies, NY) was used to measure the amount of bound peptide (biotin), per manufacturer's instructions. An aqueous solution of biotin (1.0 mg/mL) without peptides was used as a negative control. Peptides with the highest binding to conjunctiva corresponding to their fluorescence values were chosen for further experiments.

2.2.3 Immunohistochemistry with binding peptides

To visualize binding of SABpep and ColBpep to the ocular surface, we performed immunohistochemistry with biotin-conjugated peptides on tissue section of rabbit conjunctiva. Conjunctival tissue dissected from rabbit eyes were fixed with 4% paraformaldehyde (PFA) solution (Electron Microscopy Sciences, Hatfield, PA), embedded and sectioned (thickness~5 μm) in paraffin. The sections were first incubated with biotin-conjugated peptides (1.0 mg/ml in PBS pH 7.4) for 1 h at room temperature, washed thrice with PBS and then stained with streptavidin-Texas Red conjugate (Vector Laboratories, CA) in accordance to manufacturer's protocol. The samples were then washed again three times, mounted with DAPI mounting medium (Vector Laboratories) and imaged with Zeiss Axio Imager 2 microscope (Carl Zeiss, Jena, Germany). Sections stained with only secondary antibody served as negative control. Mouse anti-mucin-1 primary antibody (Novus Biologicals, Littleton, CO) and mouse anti-collagen type I

antibody (Abcam, Cambridge, MA) were used as replacements to SABpep and ColBpep in the positive controls, respectively, and stained with goat anti-mouse IgG secondary antibody (Life Technologies, NY).

2.2.4 Quartz crystal microbalance with dissipation (QCM-D) binding studies

We conducted QCM-D studies using a Qsense E4 system and gold-coated sensor crystals QSX 301 (Biolin Scientific, Linthicum Heights, MD) to directly evaluate the binding polymer-peptide constructs of SABpep and HABpep to mucin and HA, respectively. At a flow rate of 25 µl/min in PBS (pH = 7.4), mucin from the bovine submaxillary glands (type I-S, Sigma-Aldrich, St. Louis, MO) or HA-thiol (Creative PEGWorks, Chapel Hill, NC) were applied on pristine surfaces at c_w = 0.1 mg/mL in PBS at 37 °C. After a PBS rinse, either SABpep (0.1 mg/mL) or SABPep-PEG-HABpep (Mw 3698.43 Da, 0.1 mg/mL) was applied to the respective substrates. Changes of frequency (Δf) and dissipation (ΔD) were recorded, and the mass per surface area (Δm) for immobilized SABpep and HABpep was calculated using the Sauerbrey relationship on the fifth overtone ($\Delta m/C = -\Delta f$, C = 17.7 ng/cm^2) and for HA-thiol and Mucin substrates using the visco-elastic Voigt model (Reviakine, Johannsmann et al. 2011; Majd, Kuijer et al. 2014).

2.2.5 Ex vivo studies for HA binding

SABpep-PEG-HABpep and ColBpep-PEG-HABpep (1.0 mg/mL) solutions were applied on the corneal surface of the rabbit eyes for 20 minutes. The epithelium was scrapped off with a cell scraper for testing ColBpep-PEG-HABpep. After washing twice with PBS, 10 µg/ml of HA-FL was applied to the same area for 20 minutes. Positive controls received

HA-FL with no peptides and negative controls were treated with only PBS. For imaging, the eyes were washed again with PBS and corneas were excised and imaged under Axio Imager 2 microscope (Carl Zeiss). ImageJ was used to process and quantify the amount fluorescence in each image. We further performed overtime HA release studies on SABpep-PEG-HABpep treated samples by measuring fluorescence values with full area scan periodically every 5 minutes for a total of 25 minutes and also 2, 4 and 16 hours after the initial fluorescence measurement. The value of fluorescence was compared to positive and negative controls. All groups were performed in triplicate.

2.2.6 Friction measurement

Ocular biomechanical friction tests were conducted by Tannin A. Schmidt's laboratory at the University of Calgary.

2.2.7 In vivo studies of SABpep-PEG-HABpep

In vivo studies were performed on mice treated with an HA eye drop solution containing SABpep-PEG-HABpep. SABpep eye drop solution (total volume 5 μl) consisting of SABpep-PEG-HABpep and HA-FL was prepared by combining a 1:1 volume ratio of 5 mg/ml SABpep-PEG-HABpep and 1 mg/ml HA-FL solution (corresponds to 0.1% HA eye drop solution). Five mg/ml HA-FL solution mixed with an equal volume of PBS served as a control eye drop. Mice (C57BL/6, Charles river) were anesthetized with isoflurane (Baxter, IL) and SABpep-PEG-HABpep/HA eye drop solution was applied to the right eye while the control eye drop was applied to the left one. Isoflurane gas was then removed and the mice were allowed to regain consciousness. Mice were sacrificed approximately 5, 10 or 15 minutes after regaining consciousness. Whole mice were

imaged using a fluorescent dissecting microscope (Nikon, Melville, NY), allowing us to take images of the eye without harvesting the tissues. However, we also harvested eyes and imaged with Zeiss Axio Imager 2 microscope to obtain magnified images.

2.2.8 Statistical Analysis

One-way ANOVA was performed among groups to determine any statistically significance in mean values of HA retention on *ex vivo* rabbit ocular tissues (statistically significant values with $p \leq 0.05$ was marked as *, p< 0.0001 marked as ****, p<0.002 marked as ***, and p<0.005 marked as **). The measured kinetic coefficient of friction values between the treatment groups for each repeat of the test were analyzed by Cohen's d effect size. Cohen's d measure is defined as the difference between two means divided by the pooled standard deviation for the data and classifies effect sizes as small ($d = 0.2$), medium ($d = 0.5$), and large ($d \geq 0.8$) (Cohen 1988). The effect size was calculated instead of significance as it allowed for the determination the magnitude of the difference between the two treatment groups independent from sample size (n=3).

2.3 Results

2.3.1 Screening and selection of a peptide that binds ocular tissues

We screened multiple peptides that are reported earlier to bind different tissue components (Table 1). Out of the six-screened peptides that could potentially bind conjunctiva tissue, GGSPYGRC and SYIRIADTNIT, hereafter respectively referred to as SABpep and ColBpep, were chosen for further experiments (Fig. 2.2A). The chosen SABpep and ColBpep exhibited little to no cytotoxicity to epithelial cells as well (Fig. 2.6).

2.3.2 SABpep and ColBpep binding on conjunctiva tissue

Rabbit conjunctival sections bound SABpep specifically on the epithelial lining of the conjunctiva (Fig. 2.2B), indicating the strong presence of sialic acid on mucin-1 as compared to mucin-1 antibody staining. A higher magnification confirmed that SABpep staining was localized to only on cell membranes (Fig. 2.2C). Conversely, as expected, ColBpep staining was localized on conjunctiva tissue more indiscriminately, which is similar to type I collagen antibody staining (Fig. 2.2B). The binding specificity of SABpep was further proven by sialidase digestion and pre-incubation with sialic acid. SABpep could not bind to conjunctival epithelium if the section was digested by sialiadase before staining. However, SABpep still stained the epithelium even after preincubation with sialic acid solution (Fig. 2.7).

2.3.3 Mucin and HA binding studies by Quartz Crystal Microbalance with Dissipation (QCM-D) monitoring

Surface binding of polymer-peptide conjugates to mucin and HA was directly measured using QCM-D. First, sialic acid bearing mucin was adsorbed on gold substrates to mimic the interface at the corneal epithelium (Fig. 2.3A), as native mucin from bovine submaxillary glands is abundant with O-linked oligosaccharide chains, and can be used as a source of sialic acids. A visco-elastic layer of highly hydrated mucin was formed as shown by a frequency change of Δf=-32 Hz and dissipation change of ΔD=7. Then SABpep-PEG-HABpep was applied to the mucin layer and specific binding was indicated with a frequency change of Δf=-5 Hz. In turn, SABpep-PEG-HABpep was immobilized onto HA substrates with a Δf=-3 Hz (Fig. 2.3B). The Voigt mass quantified the consistent formation of visco-elastic substrates Mucin and HA (Fig. 2.3C). In turn,

HApep binding showed a frequency but not a dissipation change. Considering the relatively small molecular weight of SABpep-PEG-HABpep (Mw = 3698.43 Da) we observed a considerable binding to mucin and HA with Sauerbrey mass of $\Delta m = 68 \pm 28$ and 54 ± 5 ng/cm^2. For example, the drop in frequency directly corresponds to binding of the polymer-peptide to mucin and can be attributed to sialic acid chains branching out from the proteoglycan (Gipson 2004) with binding sites available in a 3-dimensional network.

2.3.4 Ex vivo HA binding and release profile

To confirm the binding of HA on SABpep-PEG-HABpep and ColBpep-PEG-HABpep treated ocular tissue samples, we applied HA-FL solution and monitored the fluorescence overtime. The peptide treated samples fluoresced with higher intensity compared to both positive (only HA) and negative controls (PBS) (Fig. 2.4A). The positive control had minimal fluorescence, and it was significantly lower than the peptide-treated groups, indicating that the ocular surface is poor at retaining HA without an aid from the peptide constructs. Fluorescent HA release studies performed on SABpep-PEG-HABpep treated samples showed ~1.8-fold more bound HA in within the first 25 min (Fig. 2.4B), and ~1.3-fold more after incubating overnight compared with HA only–treated controls (Fig. 2.4C). Although a detailed stoichiometric and control study to compare the binding ability of SABpep *vs.* ColBpep is required for an accurate comparison, our initial experiments with conjunctiva (physically abraded to expose collagen) showed a greater relative fluorescence value (only as an estimate) for SABpep+HA treated samples than ColBpep +HA treated samples (Fig. 2.8). We further calculated Area under curve (AUC) for HA release overtime (Fig. 2.9) that showed the total released amount of HA on ocular

surface bound through SABpep was significantly higher than that for samples with only HA after 25 minutes and after 16 h.

2.3.5 Lubrication of ocular tissues with eye drop solutions

Rabbit ocular tissues treated with SABpep-PEG-HABpep and HA solution had reduced coefficient of friction values, and therefore improved tissue lubrication, compared to HA-only treated tissues as control. Kinetic coefficient of friction values of $<\mu_{kinetic}, N_{eq}>$, expressed as the mean ± standard error of the mean (SEM), decreased from 0.05±0.01, to 0.04±0.01 (d = 1.25), 0.06±0.01 to 0.04±0.01 (d = 2.13) and 0.07±0.00 to 0.05±0.01 (d = 2.10) during the first, second and third repeats of the HA treated tissues compared to the SABpep-PEG-HABpep followed by HA treated tissues, respectively. A strong effect size was observed in all three repeats, indicating that there is a strong magnitude of difference between the SABpep-PEG-HABpep+HA group compared to the HA control group. Kinetic friction coefficient values for SABpep-PEG-HABpep followed by HA treated tissues were also compared to tissues treated with a PEG non-HA-binding peptide (SABpep-PEG) solution, which acted as a polymer control. $<\mu_{kinetic}, N_{eq}>$ values during the first, second and third repeats of the PEG non-HA-binding peptide solution were 0.08±0.01, 0.07±0.01 and 0.07±0.01, exhibiting strong effect sizes (d = 2.02, 2.70 and 1.34) when compared to the kinetic friction coefficient values of the tissues treated with SABpep-PEG-HABpep+HA.

2.3.6 In vivo HA retention studies

An eye drop formulation with equal volume mixture of SABpep-PEG-HABpep and 0.1% HA (as in commercial HA eye drops) was applied *in vivo* to one of the mice eyes, while

the other eye was applied with only 0.1% HA, and monitored overtime till 30 min. Eyes treated with SABpep eye drops retained HA longer than eyes treated with regular HA eye drops (15 minutes versus 10 minutes, respectively) (Figs. 2.5A&B). The fluorescence from eyes treated with only HA was quickly vanishing and lasted till 10 min, while the fluorescence from peptide and HA treated eyes was still intense at 10 min and lasted even at 15 min (Fig. 2.5C). We further harvested mice eyes and imaged them at a higher magnification, which, as expected, showed more bound HA on ocular surface at epithelial cell level at 5 min and lasted till 15 min (Fig. 2.5B&C). Initial and over time fluorescence of standard HA-FL eye drop solution with and without peptide solutions remained approximately the same that ruled out the difference in fluorescence values due to the pipetting errors (Fig. 2.10).

2.4 Discussion

A current challenge in HA-based eye drop technology is to retain HA on the ocular surface for a prolonged time. Increased HA retention can benefit dry eye patients by increasing corneal wettability and reducing tear evaporation. Although several eye drop solutions are commonly used, HA has been shown to perform better than other common lubricants used in eye drops. For example, HA is more effective at protecting human corneal epithelial cells against dehydration and retaining water when compared to CMC and HPMC (Zheng, Goto et al. 2013). In addition, HA not only lubricates the ocular surface, but also enhances the spreading the tear film lipid layer (Georgiev, Norihiko et al. 2013). An eye-drop technology that can address the challenge of the faster clearance of HA from ocular surfaces (retention time ~10 min) may have a profound eye health benefit. Therefore, we developed a novel eye drop solution with a peptide, HABpep that

non-covalently binds HA, to concentrate and retain HA to the epithelia of the ocular surfaces (Stuart and Linn 1985; Shimmura, Ono et al. 1995; Gomes, Amankwah et al. 2004; Johnson, Murphy et al. 2006; Rah 2011).

Recently, we reported a simple methodology to immobilize HA using an HABpep by developing a heterobifunctional polymer-peptide system (Singh, Li et al. 2015). For eye drop applications, HABpep polymer-peptide was integrated into a bifunctional molecule, containing also either SABpep, to target transmembrane mucins, or ColBpep, to target collagens present throughout the ocular surface stroma. Thus, we can specifically target the ocular surface through non-covalent interactions, to mimic the water-retaining capabilities of the healthy ocular surface, and in turn treat severe dry eye or wounded epithelia. Although HA can be directly immobilized on the ocular surface by chemically modifying it with a conventional conjugation strategy, such as the aldehyde-NHS type reaction, the peptide-polymer strategy based on non-covalent interaction of HABpep with HA provides us unique design flexibilities to tune the HA binding and release by appropriately selecting an HABpep and its concentration, which is related to the binding affinity of the HABpep to HA. Furthermore, HABpep sites can be replenished each time an HA-containing eye-drop is applied, which may further compensate a loss of HA due to the normal transport phenomena of the tear fluid.

The ability of the ocular surface to attract, retain and release HA depends on the strength of the interactions between HA, ocular surface tissue components, and the corresponding binding peptides. Therefore, we investigated six peptide candidates capable of binding to the ocular surface through different mechanisms; the most effective binding-peptide candidates, SABpep and ColBepep, were selected based on *ex vivo*

studies (Fig. 2.2A). The polymer-peptide system with collagen-binding peptide would be most useful for treating conditions related to severe dry eye, including sight-threatening complications, such as persistent corneal epithelial defect or sterile stromal ulceration, which allows the corneal stroma to be exposed to the external environment (Pflugfelder 2004). Whereas in conditions where epithelium is still partially or fully present (Gipson 2004), the eye can be treated with the mucin-binding peptide containing polymer-peptide system. The binding sites of SABpep on the epithelium was localized on the cell membrane only (Fig. 2.2C), and specifically it only bound to the sialic acid segment in O-linked oligosaccharides on membrane bound mucins. SABpep bound to the epithelium, even when preincubated with sialic acid; however, SABpep did not bind sialidase treated epithelium (Fig. 2.7). It is possible that SABpep is binding to the oligosaccharide conformation of the sialic acid in transmembrane mucin more preferentially than free sialic acid (Heerze, Smith et al. 1995). Here, our aim was to enhance surface lubrication and HA retention on ocular surfaces through immobilized HA using SABpep as an anchor. Therefore, in the following studies, we investigated SABpep-PEG-HABpep for the amount and duration of HA bound on ocular surfaces and found that SABpep-PEG-HABpep bound HA that is immobilized to the ocular surfaces through the sialic acid of transmembrane mucins acting as anchoring sites prolongs HA retention in both *ex vivo* and *in vivo* animal models. In the *ex vivo* rabbit eye model, HA was bound 1.8 times more in the beginning and ~1.2 times more at 24 h through SABpep-PEG-HABpep compared to control. In addition, we assessed the lubricating ability of the eye drops which showed a strong magnitude of difference in kinetic coefficient friction values for rabbit ocular tissues treated with SABpep-PEG-HABpep and HA solution compared to

the tissues treated with HA-only solution (~20% in repeat 1 and ~30% in repeat 2,3). The kinetic coefficient of friction measurements suggest that SABpep-PEG-HABpep was effective in immobilizing HA onto the ocular surface, and the surface bound HA without binding peptide sheared off from the tissue to a greater extent, which is consistent with the longer retention time of HA bound with SABpep-PEG-HABpep. Kinetic friction coefficient results from the SABpep-PEG-HABpep and HA solution showed a strong magnitude of difference compared to the tissues treated with a non-HA-binding PEG control peptide. This suggests that the PEG present in the SABpep-PEG-HABpep solution does not play a significant role in the kinetic coefficient of friction measurements obtained. While these results were performed on a rabbit model and not on human tissue, the rabbit animal model has often been used in ophthalmic research due to their similarities to human ocular anatomy. Although rabbit ocular tissues have differences in tear film stability and mucin structures compared to human ocular tissue (Tsonis 2011), the rabbit model is a better alternative than testing against synthetic materials. Lastly, while increased *in vitro* friction of commercial contact lenses has been correlated with increased discomfort of wears, there are no 'clinically relevant changes in tear friction' to reference from the literature. As such, the reported friction values are simply an *in vitro* measurement yet still provide a valuable information related to lubricating function of the peptide studied here.

In an *in vivo* mouse model, HA immobilized through the peptide was observed even at 15 min compared to 5 min for the control HA that was not bound with the peptide. Although HA bound was not quantified *in vivo*, the amount of HA-FL decreased more dramatically over the course of 30 min. However, this difference between *in vivo* and *ex*

vivo retention times is expected because in a live animal the tear film is active and is constantly flushing fluid.

From a combination of *in vivo*, *ex vivo*, and *in vitro* studies we posit using animal models that peptide-binding agents included in HA eye-drops can prolong the physical and biological benefits of HA. Although glycan compositions on the ocular surface are different across species, human ocular mucins have a higher occurrence of sialic acid than other species such as rabbits and canine and we hereby anticipate that SABpep eye drop technology could be extrapolated to human tissue too (Royle, Matthews et al. 2008). However rabbit tear film is much more stable compared to human tear film (Wei, Markoulli et al. 2013), thus the molecular ratio of HA and peptides need to be optimized in future studies for clinical application. The advantages of this technology are that it is easy to prepare and does not require any modifications to the standard HA eye drop solution – the polymer-peptide can simply be added to a standard commercially available HA-eye drop solution. Future direction for this work includes developing a multi-arm polymer peptide system to tune the binding and further prolong the *in vivo* HA delivery that may be a limitation of the linear heterobifunctional peptide binding system. Although future clinical investigations will be needed, we expect that this next-generation HA-eye drop would decrease the frequency of eye-drop instillation and will alleviate dry eye symptoms more effectively by improving water retention and wettability.

Table 2.1: Peptides Screened		
Peptide Sequence	**Description**	**Reference**
K-K(Palm)-K-K-K-K(Palm)	Cell membrane binding peptide	(Matsuda, Hatanaka et al. 2014)
GWQPPRARI	Fibronectin derived sequence – epithelial binding	(Mooradian, McCarthy et al. 1993)
GGSPYGRC	Sialic acid binding	(Heerze, Chong et al. 1992)
GGPQEQITQHGSPYGRC	Sialic acid binding	(Heerze, Smith et al. 1995)
RRANAALKAGELYKSILYGC	Collagen I binding peptide derived from platelet rich plasma	(Paderi, Sistiabudi et al.)
SYIRIADTNIT	Collagen I binding peptide derived from decorin	(Kalamajski, Aspberg et al. 2007)

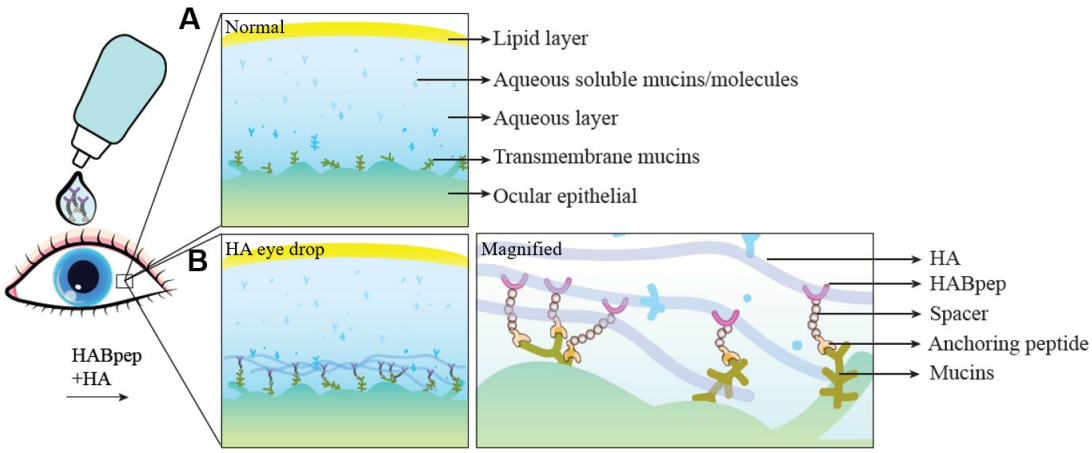

Figure 2.1 | Schematic of an HA binding eye drop technology. A) Exterior ocular surface has three layers: lipid, aqueous and mucin layers. **B)** On application of HA binding eye drop, HABpep binds HA through an anchoring peptide on the ocular surface. Specifically, sialic acid of transmembrane mucins is targeted (*magnified*); alternatively, collagen of the ocular tissue present in conjunctiva or cornea tissues can also be targeted to immobilize HA.

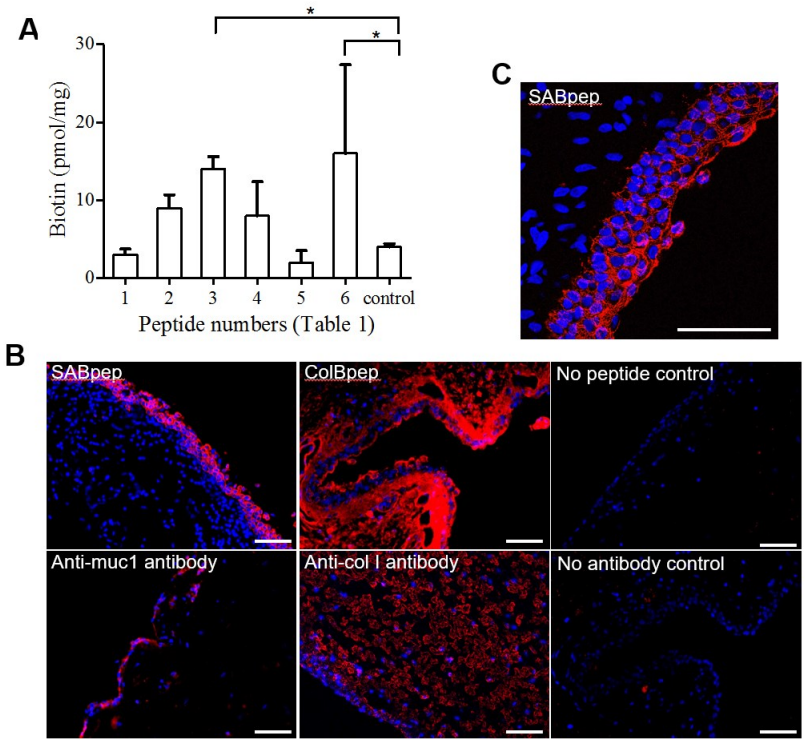

Figure 2.2 | Selecting ocular surface binding peptides: targeting sialic acid and collagen as sites for binding SABpep and ColBpep on the conjunctival tissue *ex vivo*. **A)** A HABA assay quantifying the amount of peptide bound to conjunctiva tissue (normalized to the dry weight) indicated that out of the six peptides tested (as listed in Table 1), peptide 3 (ColBpep) and peptide 6 (ColBpep) bound the most to conjunctiva tissue compared to control (p<0.5). **B)** Immunohistochemistry showed that both SABpep and anti-mucin 1 antibody stained positively for the transmembrane mucin-1 on the epithelial surface, while ColBpep binds on ocular matrices throughout cellular and non-cellular regions (scale bar = 100 μm). **C)** A magnified immunostaining image showing that SABpep specifically binds to the transmembrane mucin-1 along the cellular periphery (scale bar = 100 μm).

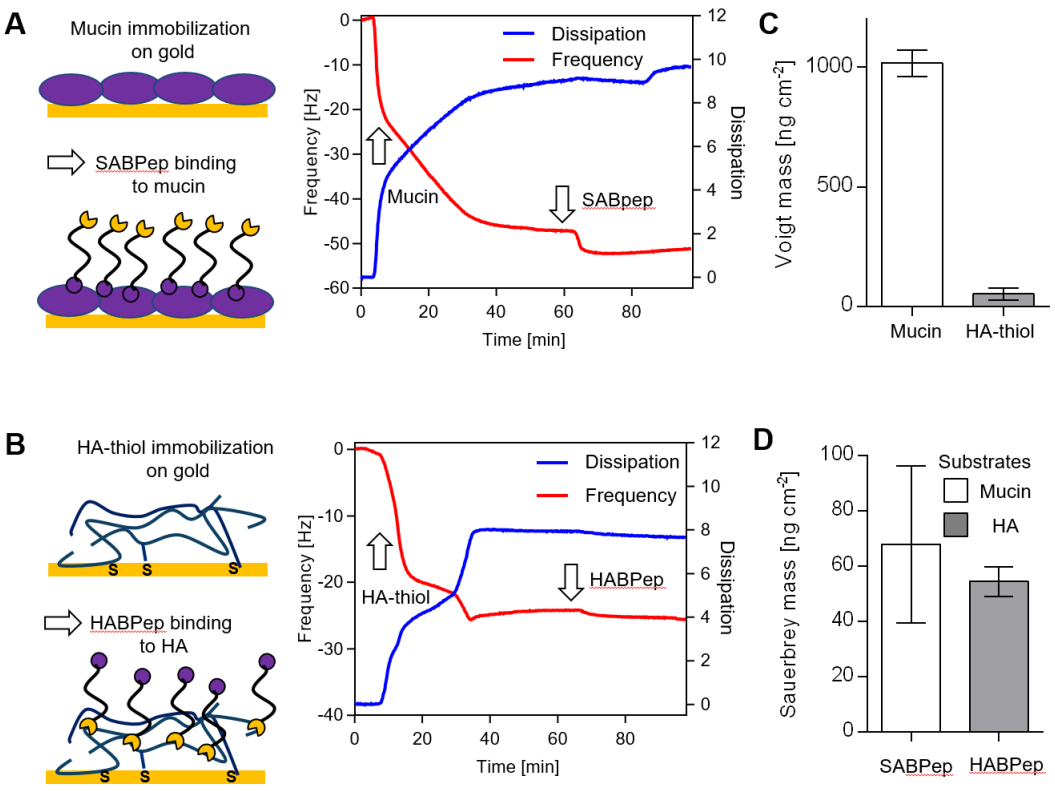

Figure 2.3 | QCM-D measurements of *in vitro* binding of SABpep and HABpep to Mucin and HA, respectively. QCM-D curves indicate successful deposition of substrates and binding peptides: **A)** Mucin adsorbed onto gold substrate followed by attachment of SABpep. **B)** Thiolated HA is covalently immobilized onto pristine gold, subsequent HABpep biding to HA. **C)** Deposition of the substrate layers of mucin and HA on pristine gold QCM-D surfaces quantified by the visco-elastic Voigt model, respectively. **D)** Attachment of binding peptides to mucin and HA substrates quantified by Sauerbrey relationship (overtone n = 5). Conditions: All concentrations c_w = 0.1 mg/ml, T = 37 °C, flow rate = 25 µL/min in PBS buffer (pH = 7.4) (n = 3).

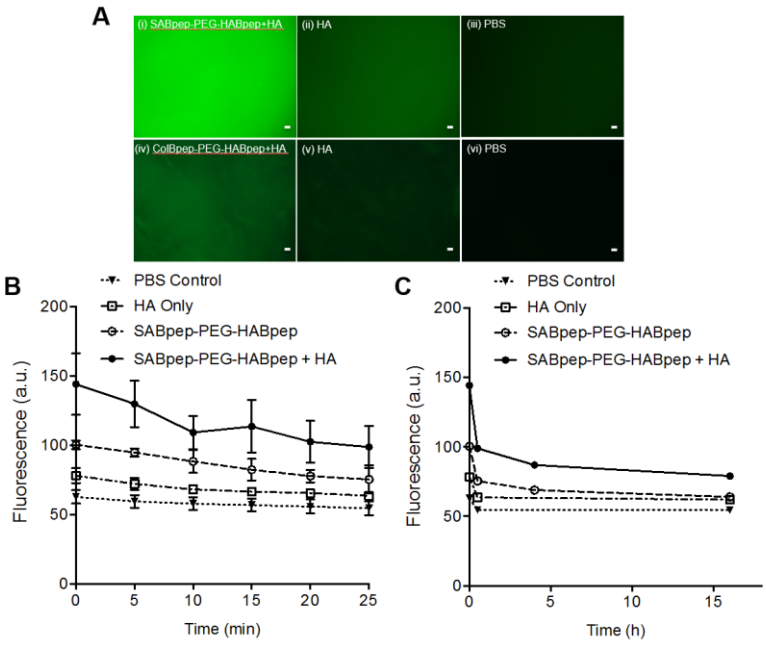

Figure 2.4 | HA binding through HABpep and overtime HA release profile. SABpep-PEG-HABpep treated eyes SABpep-PEG-HABpep and ColBpep-PEG-HABpep bind HA *ex vivo.* **A)** SABpep-PEG-HABpep treated rabbit eyes (corneas were dissected and imaged after treatments) *(i)* showed intense fluorescence compared to the eyes treated with only HA *(ii)* and PBS *(iii)*. Similarly, ColBpep-PEG-HABpep treated rabbit eyes *(iv)* showed a higher fluorescence compared to the eyes treated with only HA *(v)* and PBS *(vi)*. Scale bar 100 µm. **B)** Short-time HA release profile for SABpep-PEG-HABpep (-/+HA) treated eyes showed a higher amount of HA was releasing at each time point compared to HA without the peptide. SABpep-PEG-HABpep treated eyes bind to ~1.8 times more HA in the beginning as indicated by the greater initial fluorescence value and retain HA longer, showing signal significantly greater (~x1.6) than the control after 25 minutes. **C)** Long-term HA release profile for SABpep-PEG-HABpep (+/-HA) treated eyes showed that compared to the controls, a higher amount of HA was bound to the ocular surface even after 15 h.

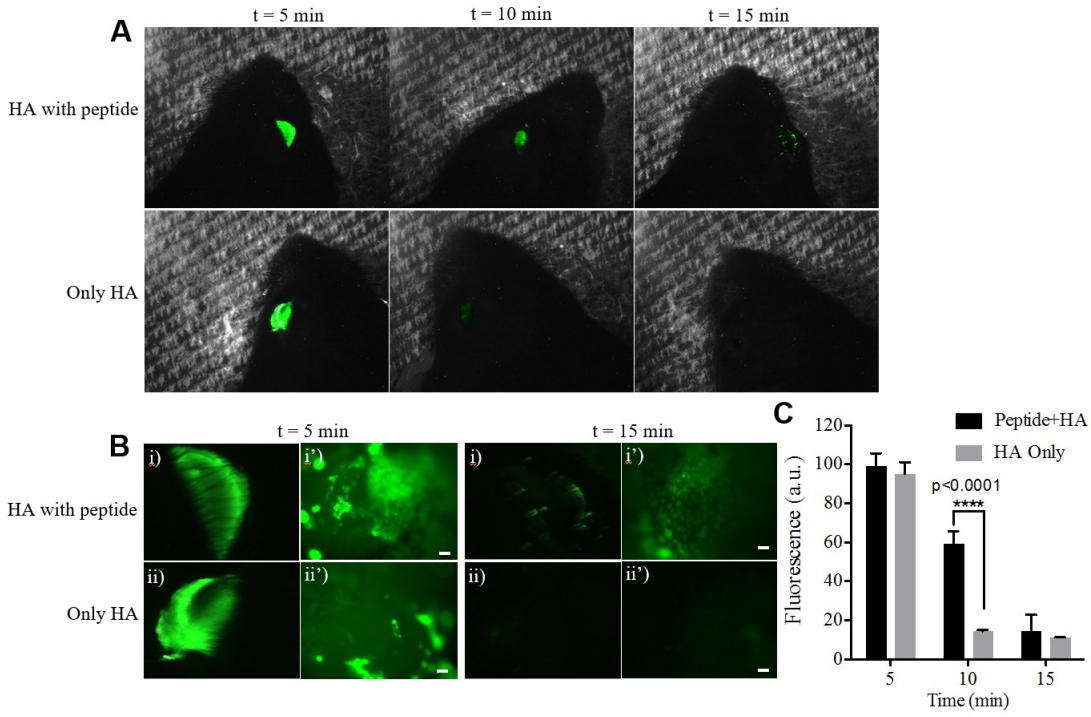

Figure 2.5 | HA retention on *in vivo* mice eyes. A) Harvested mice eyes applied with SABpep-PEG-HABpep+HA eye drops (top row) retained HA for at least three fold time period longer than those applied with control eye drops (only HA, bottom row) (n=2). Even after 15 minutes, HA was present on the samples treated with the binding peptide. **B)** Magnified images showed that both at beginning and after 15 min, SABpep-PEG-HABpep+HA (*i*- only eye, *i'*–magnified image of *i*) was bound homogenously to the ocular epithelial cells, while samples treated with only HA showed relatively less homogenous distribution. (Scale bar = 100 μm)

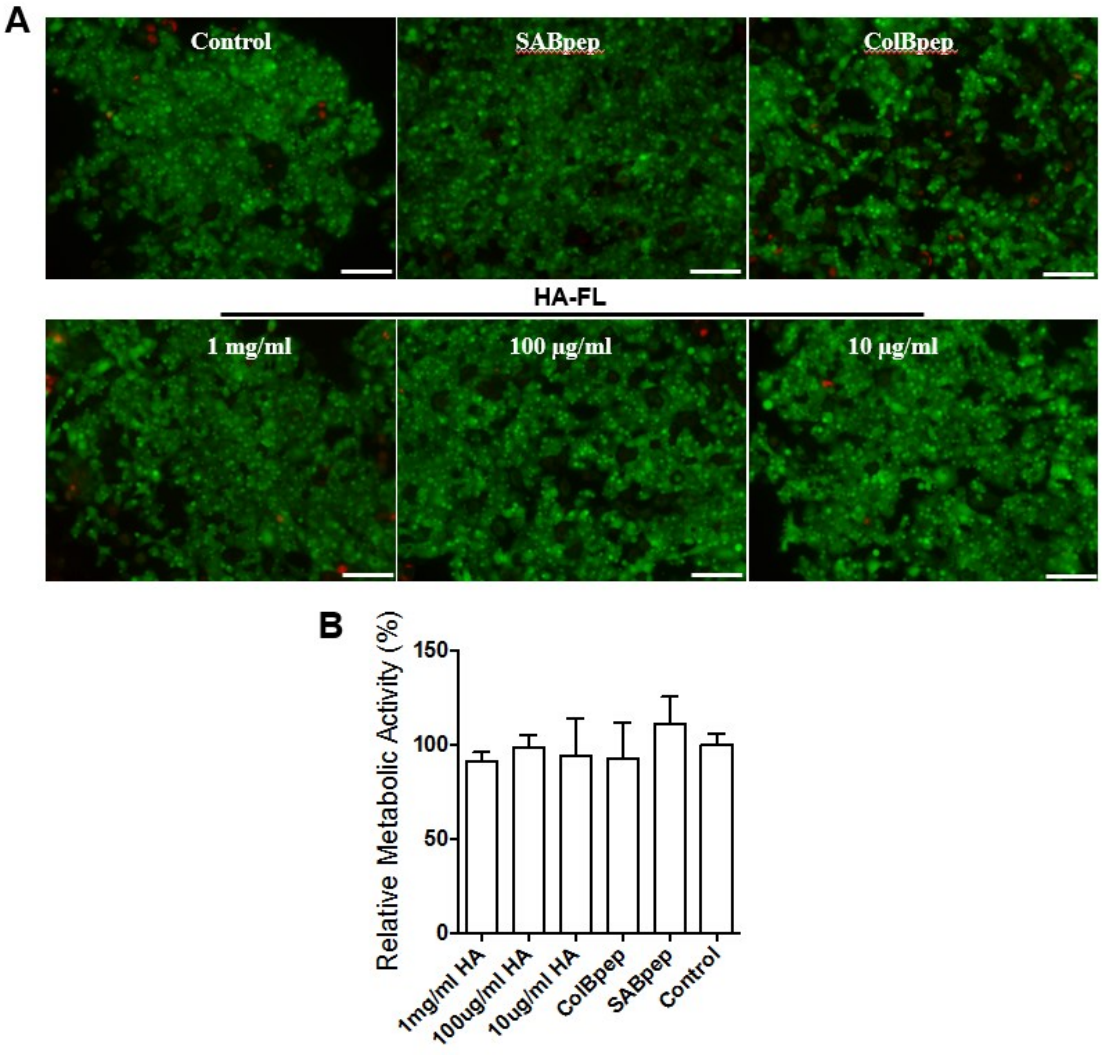

Figure 2.6 | Cytotoxicity of SABpep, ColBpep and HA-FL. Cultured conjunctiva epithelial cells were stained with the live/dead staining kit comprising calcein AM (green-live cells) and EthD-1 (red-dead cells) followed by a metabolic activity analysis by the AlamarBlue assay. Either binding peptide or HA-FL exhibited no cytotoxicity to cultured conjunctival epithelial cells. **A)** Live/dead staining with SABpep, ColBpep and different HA-FL concentrations (scale bar = 100 µm). **B)** AlamarBlue assay showed cell proliferation rate after exposure to HA-FL or binding peptides (normalized to control). No significant difference was found between groups

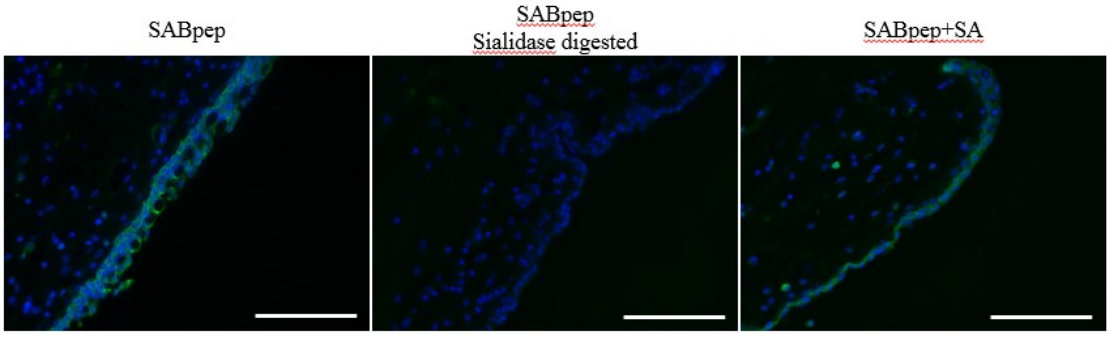

Figure 2.7 | Binding specificity of SABpep toward sialic acid of transmembrane mucin-1. Rabbits conjunctival sections with different treatments were examined for testing the binding specificity of SABpep. Streptavidin-FL (green) was applied to detect biotin conjugated SABpep. **A)** SABpep was added regularly and it resulted in a positive staining on the epithelium. **B)** No staining was observed when sections were treated with sialidase before adding SABpep. **C)** SABpep was incubated with sialic acid before being added to the sections and staining to the sections was not altered significantly.

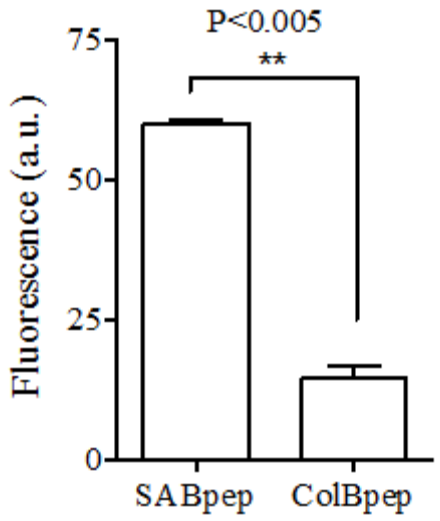

Figure 2.8 | HA binding on ocular surfaces through SABpep and ColBpep. Images from Figure 4A were analyzed using ImageJ to quantify the relative amount of fluorescence between SABpep+HA and ColBpep+HA treated samples. Background signal from the PBS controls were subtracted from the images.

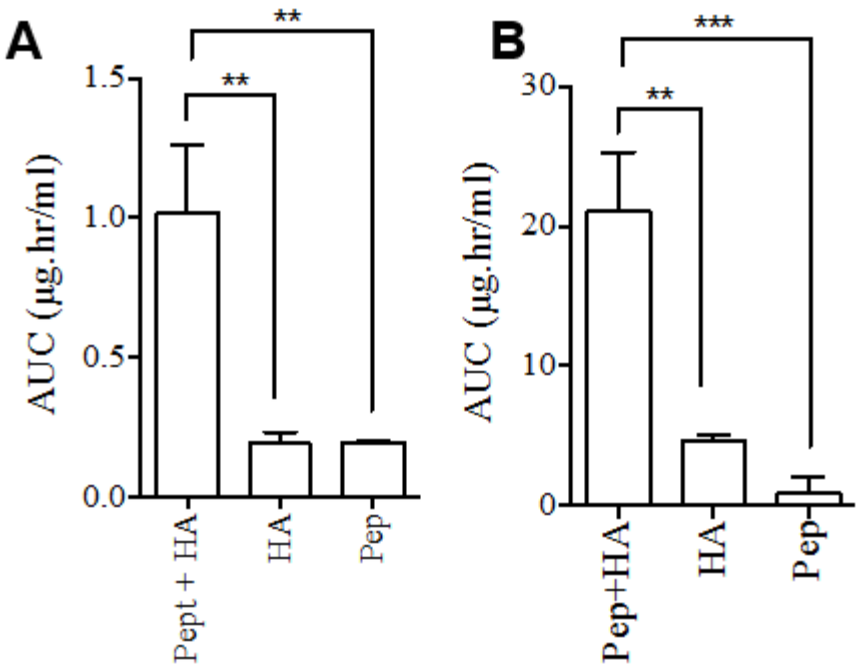

Figure 2.9 | Estimated HA release over time by area under curve calculation (AUC). Area under curve (AUC) was calculated for each experimental group. The background from PBS control was then subtracted from each AUC to quantify total amount of HA retained for each experimental condition. A standard curve of fluorescence versus HA was used to convert AUC values into HA (µg). AUC for release time of **A)** 25 minutes, and **B)** 16 h.

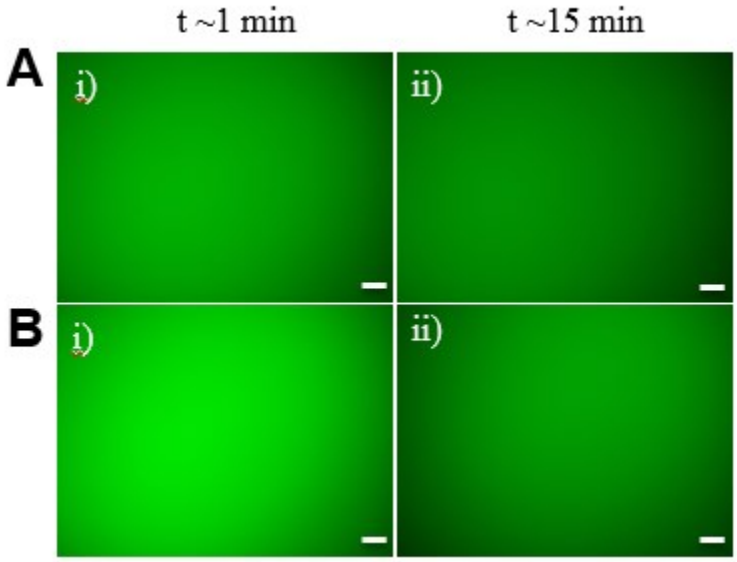

Figure 2.10 | Initial fluorescence of standard HA-FL eye drop solution with and without SABpep. Both eye drop solutions showed approximately the same amount of fluorescence over time implying that difference in fluorescence in figures 6 (*in vivo*) are not due to the difference in HA-FL concentration values of the corresponding solutions (due to pipetting errors) for **A)** HA-FL with SABpep **B)** HA-FL only at the beginning *(i)* and after 15 min *(ii)*. (Scale bar = 100 μm).

3. Surface Modified Contact Lenses

3.1 Introduction

We applied the same HA-binding peptide eye-drop technology to modify contact lenses that would help lubricate the ocular surface. The overall goal of our project was to design and develop a hyaluronic acid (HA)-binding contact lens that can bind and retain HA that is either present in the tear fluid or applied exogenously. To achieve this, we utilized siloxane-binding peptide (SiBpep) to develop a heterofunctional PEG-peptide (SiBpep-PEG-HABpep) that would non-covalently bind to dimethylsiloxane units of the lens at one end and bind HA at the other. Surface modification and HA-binding on the lenses were verified and HA-release and water retention profiles were quantified using different experimental techniques. Contact lenses were obtained from CooperVision® and two different brands of lenses were used – Stenfilcon A™ and Comfilcon B™. PDMS molds containing siloxane groups were also treated with peptide and were stained with dyes and imaged to further demonstrate the technology's efficacy.

3.2 Materials and Methods

3.2.1 Water Retention Studies

Contact lenses (n=3) (CooperVision, CA), either treated or untreated with peptide, were soaked in HA solution (5mg/ml, Mw 1000 kDa) for 1 hour. Negative control lenses were kept in PBS. Lenses were then washed twice in PBS (pH 7.4; Life Technologies, NY). After washing, lenses were sealed on a 1.5 mL microcentrifuge tube (inner diameter 1.0 cm and outside diameter 1.3 cm; USA Scientific) containing1 mL of PBS and glued to the rim with instant Krazy glue (Elmer's products). The leak-proof tube was positioned horizontally in such a way that the solution completely hydrated the lens and subsequently kept on an analytical balance. The weight of the tube was recorded every minute for 10 minutes.

3.2.2 HA Biotin Release

Lenses were soaked in 5 mg/mL HA-Biotin (~30% mol tagging) PBS solution for 1 hour. They were then briefly washed in PBS three times (quickly dipped to remove extra HA on the surface). Lenses were then periodically transferred to a well containing fresh PBS every five minutes in a 24-well plate. The absorbance of each wash was measured at 535 nm using a Quant*Tag Biotin Quantitation Kit assay (Vector Laboratories, CA) and a Synergy 2 microwell plate reader (BioTek, Winooski, VT). The values were compared to a standard curve to quantify the amount of HA-biotin in the wash at each time point and were used to back-calculate the initial amount of bound HA.

3.2.3 PDMA peptide treatment

For all experiments PDMS molds (12 mm diameter) were treated with peptide solution (0.5 mg/ml) overnight. Control samples were kept in PBS. For all experiments, n=3.

3.2.4 PDMS Staining with Alcian and Toluidine Blue

Peptide treated and untreated PDMS samples were placed in HA solution (1 mg/mL) for one hour. Negative control samples were placed in PBS. Samples were then washed twice with PBS and were either placed in 1% w/v Alcian Blue in 3% v/v acetic acid or 0.1% w/v Toluidine Blue (Sigma-Aldrich, St. Louis, MO) solution for an hour. After washing twice with PBS, the absorbance of the samples were measured using a Synergy 2 microwell plate reader. Absorbance was measured periodically every five minutes, with a PBS wash between each measurement. Photos were taken after 1 hour of washing.

3.2.5 Confocal Imaging

PDMS samples were treated with HA-fluorescein (HA-FL) solution (10 ug/mL) for an hour and then washed with PBS for 45 minutes. Images obtained with z-stacking were taken on a confocal microscope (Carl Zeiss, Jena, Germany).

3.2.6 HA Release Profile with HA-FL

Lenses were first washed in PBS to remove any solution in the packaging. Modified lenses were treated with 0.5mg/ml of peptide overnight, while control lenses were kept in PBS. Peptide treated lenses and untreated control lenses (n=3) were soaked in 10ug/ml HA-FL solution for an hour. Lenses were then briefly washed and transferred to a 24 well plate in PBS. The amount of fluorescence from bound HA-FL was measured using

a Synergy 2 plate reader (485/528nm excitation/emission). To obtain the release profile, the lenses were periodically transferred to fresh PBS every 5 minutes, with the fluorescence of the lens being measured each time. This resulted in a direct measurement of bound HA versus time without having to back-calculate using values from the wash.

3.2.7 HA Reloading Release Profile with HA-FL

To remove most of the bound HA from the contact lenses, lenses were placed in 1.5 M NaCl solution (in PBS). Every 5 minutes, lenses were transferred to a fresh sample of 1.5 M NaCl solution, and fluorescence was recorded for each time point. Once fluorescence values began to plateau to a minimal value, indicating that the salt had removed most of the bound HA from the lens, the lenses were reloaded with 10ug/ml HA-FL solution. The HA release profile for reloaded lenses was obtained using the protocol previously described above. Afterwards, the lenses were treated with 1.5 M NaCl and were reloaded a second time to examine whether the modified lenses would still have a different release profile from the control. Once this release profile was obtained, the lenses were fluorescently imaged with a Zeiss Axio Imager 2 microscope (Carl Zeiss, Jena, Germany)

3.2.8 Statistical Analysis

One-way ANOVA was performed among groups to determine any statistically significant differences in values of means. $P \leq 0.05$ was considered statistically significant (marked as *).

3.3 Results

3.3.1 Water Evaporation

Both Stenfilcon and Comfilcon lenses treated with both peptide and HA had slower evaporation rates than untreated lenses in HA and PBS (Figure 3.1a&b). Stenfilcon lenses had a 49% and 24% reduction in evaporation rate compared to untreated lenses in PBS and HA, respectively. Comfilcon lenses had a 55% and 30% reduction (Table 3.1).

3.3.2 HA Biotin Release Profiles

Modified lenses demonstrated approximately a 300-400% increase in initial bound HA. (Figure 3.2). After 30 minutes, almost all bound HA from control lenses was released, while treated Stenfilcon retained 50% and Comfilcon retained 67% (Figure 3.2). Even after 90 min of washing, Comfilcon lenses still retained about 50% of bound HA.

3.3.3 PDMS Staining with Alcian and Toluidine Blue

For the Toluidine Blue assay, peptide treated PDMS bound to approximately 35% more HA than untreated PDMS when both groups were normalized to the negative control. After 45 minutes, bound HA on treated PDMS almost reached the same level as the untreated sample (Figure 3.3). Alcian Blue stains achieved even better normalized results – about 50% more HA was initially bound in the treated group and after 90 minutes of washing about 40% of bound HA was retained for the treated group while the untreated group retained almost no HA (Figure 3.4). Images taken after washing show that there is visibly more Alcian blue staining on treated PDMS samples (Figure 3.5). Peptide treated PDMS Toluidine Blue samples also showed more staining than the control groups, but to a lesser extent.

3.3.4 Confocal Imaging

3D-box plots and 2D scans showed that PDMS treated with both peptide and HA bound more HA than samples treated with only HA and the negative control (Figure 3.6a). Comfilcon lenses treated with both peptide and HA also bound more HA than untreated lenses, but HA was primarily binding only at the edge of the lenses (Figure 3.6b).

3.3.5 HA Reloading Release Profile with HA-FL

Bound HA values plateaued after 75 min for untreated lenses with only HA, while peptide treated lenses with HA took about 4h to reach the same value (Figure 3.7&3.8). After salting out and reloading HA twice, the peptide treated lenses still bound more HA for both reloads (Figure 3.8). Fluorescent images taken after second reload showed significantly more bound HA on the peptide treated lenses (Figure 3.9).

3.4 Discussion

Contact lenses treated with SiBpep-PEG-HABpep were able to retain water for a longer period of time and bind more HA compared to control lenses. This demonstrates that the polymer-peptide system can bind to the lens and simultaneously bind to HA to wet and lubricate the lens surface.

Toluidine and alcian blue assays with PDMS containing siloxane groups also demonstrated that presence of the peptide led to more bound HA. These assays were applied on PDMS containing siloxane groups instead of the contact lenses, because the dyes would stain the lenses regardless of whether they were treated or untreated. To show a qualitative comparison using lenses instead of PDMS, we approached this issue by using an assay with HA-FL. Confocal imaging showed that the presence of peptide increases bound HA, although the binding occurs primarily at the edges. This may be due to inhomogeneous distribution of dimethylsiloxane units on the surface of the lens.

The peptide-polymer system was still bound on the surface on the lens after multiple washes, which was demonstrated by salting out and reloading the lenses with HA. Even after the second HA reload, more HA was bound on the peptide treated lenses, indicating that system is stable after multiple washes. Therefore, we have shown that modifying the surface of contact lenses with a polymer-peptide system can increase bound HA while slowing water evaporation. In addition, our surface modification is relatively stable since it can continue to bind HA after multiple washes.

1. Water evaporation rate (uL/min)		
	Stenfilcon A	Comfilcon A
Control	0.75	0.82
HA (passively adsorbed)	0.5	0.5
PEG-HABPep+HA (bound)	-	0.34
SiBpep-PEG-HABpep+HA (bound)	0.38	0.35
2. (% reduction) Normalized to control [% reduction in water evaporation due to (peptide+HA)]		
Control	-	-
HA (passively adsorbed)	33%	39%
PEG-HABPep+HA (bound)	-	58%
SiBpep-PEG-HABpep+HA (bound)	49%	55%
3. (% reduction) Normalized to HA [% reduction in water evaporation due to binding HA though peptide]		
Control	-	-
HA (passively adsorbed)	-	-
PEG-HABPep+HA (bound)	-	32%
SiBpep-PEG-HABpep+HA (bound)	24%	30%

Table 3.1 | Water Evaporation for Contact Lens. Both Stenfilcon and Comfilcon lenses had a reduction in evaporation rate compared to untreated lenses in PBS and HA, respectively.

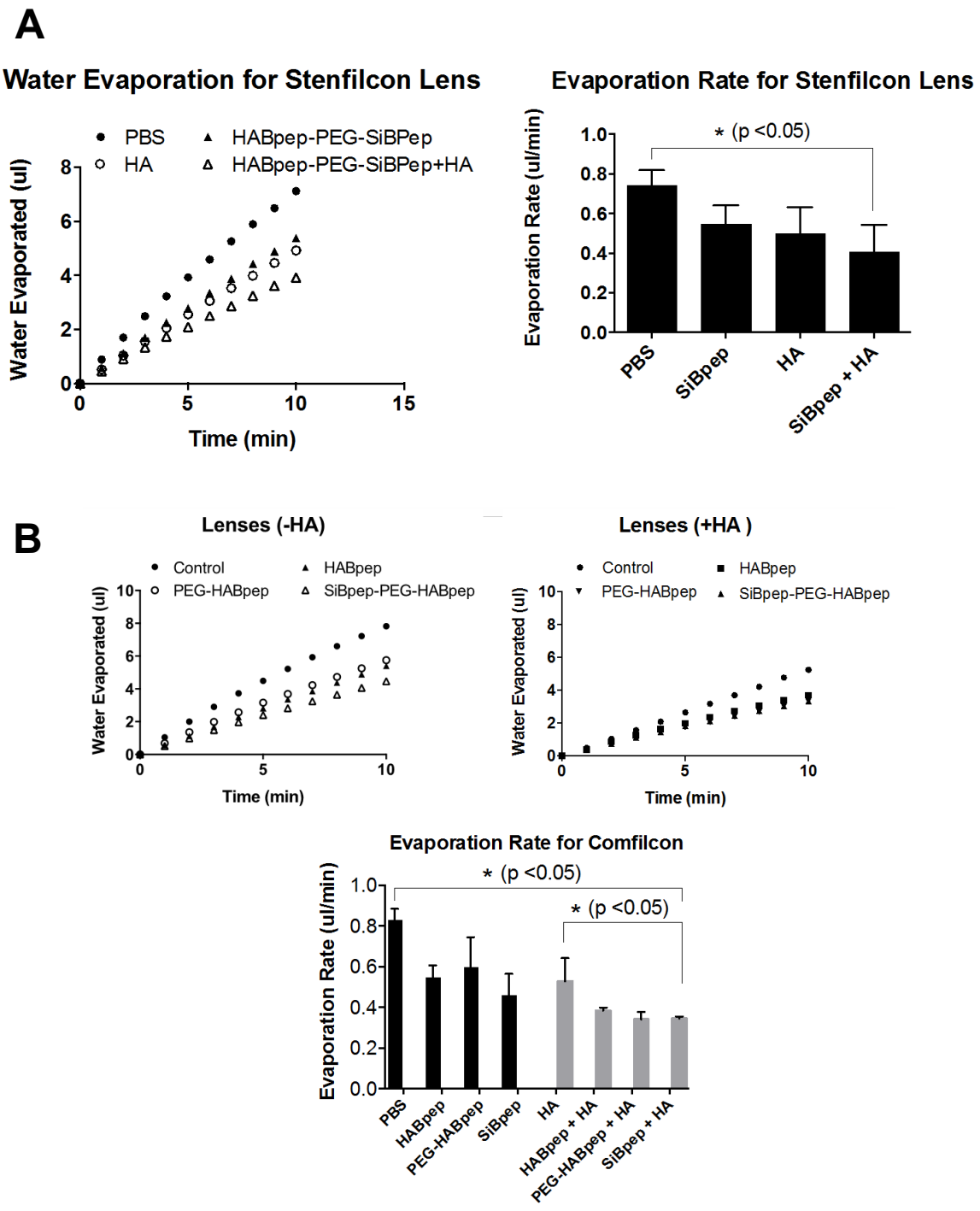

Figure 3.1 | Water Evaporation for Contact Lenses. Both Stenfilcon (Fig. A) Comfilcon (Fig. B) lenses treated with both HABpep-PEG-SiBpep and HA retain water longer and have a slower evaporation rate than lenses treated with only HA or only peptide.

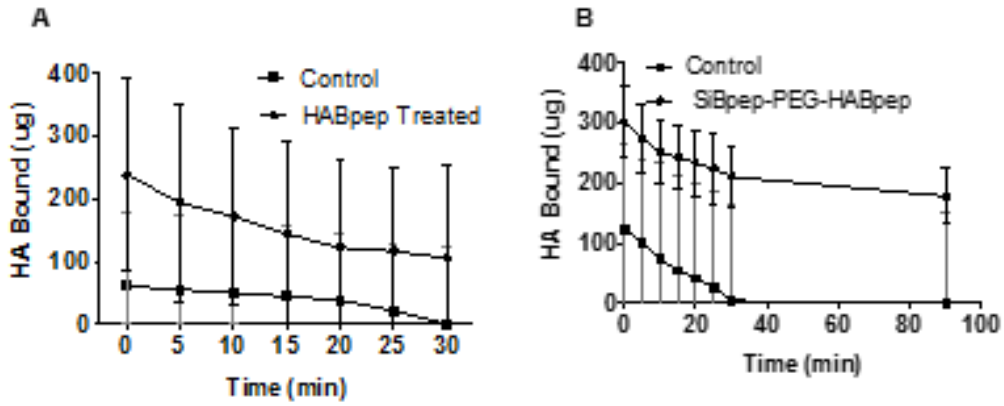

Figure 2: Overtime bound HA on A) Stenfilcon A and B) Comfilcon A.

Figure 3.2 | HA Release Profile. Modified lenses demonstrated approximately a 300-400% increase in initial bound HA (Fig. 2). After 30 minutes, almost all bound HA from control lenses was released, while treated Stenfilcon A retained 50% and Comfilcon retained 67% (Fig. 2). Even after 90 min of washing, Comfilcon lenses still retained about 50% of bound HA.

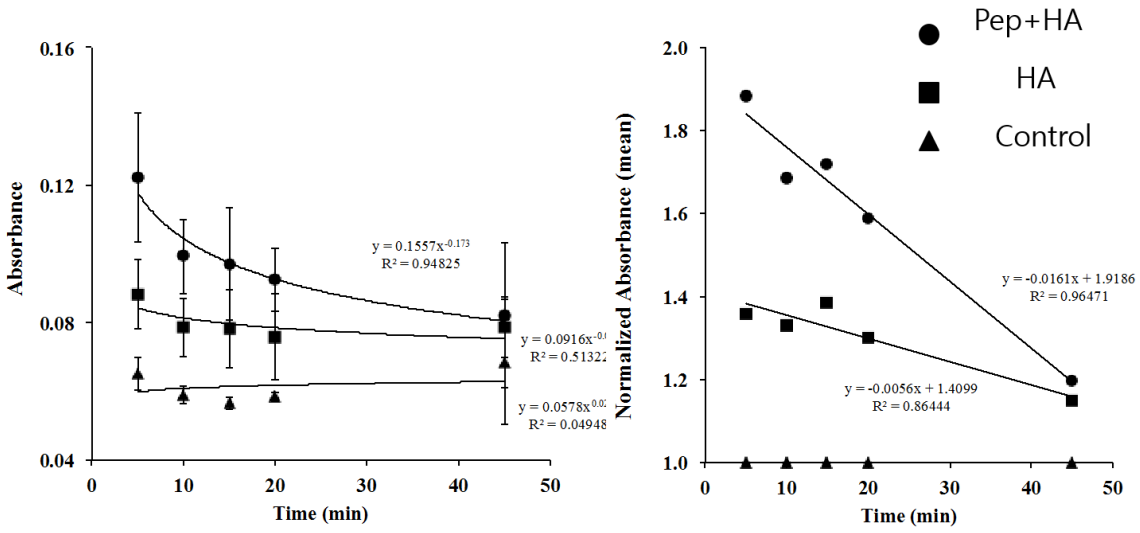

Figure 3.3 | Toluidine Blue Assay on PDMS. PDMS treated with both HABpep-PEG-SiBpep and HA retains HA longer than PDMS treated with only HA.

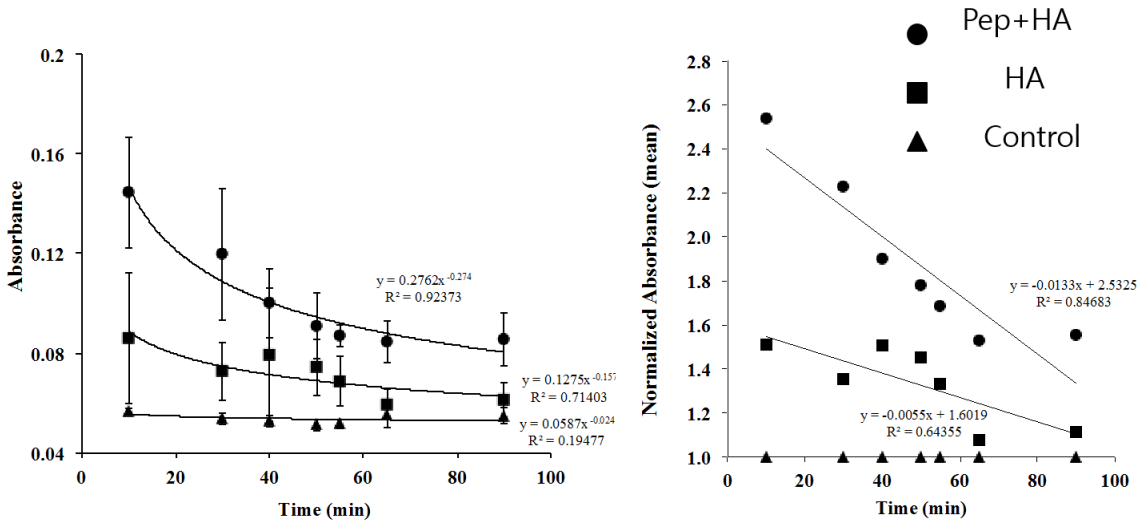

Figure 3.4 | Alcian Blue Assay on PDMS. PDMS treated with both HABpep-PEG-SiBpep and HA retains HA longer than PDMS treated with only HA.

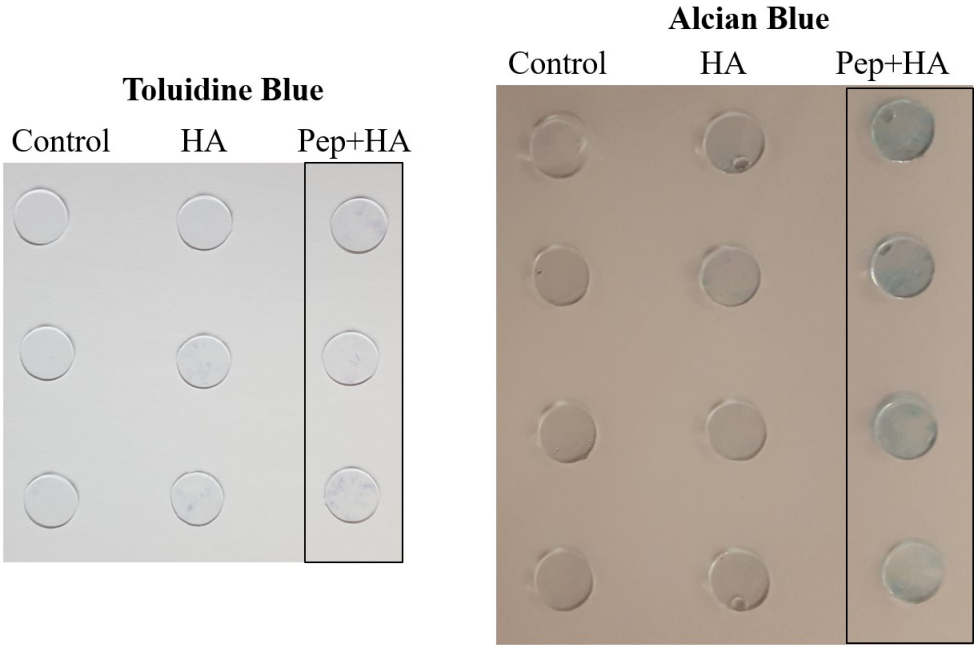

Figure 3.5 | Qualitative Comparison of Peptide Treated and Untreated PDMS. For both toluidine blue and alcian blue assays, presence of the peptide led to more bound HA. This can be observed by the higher amounts of blue staining in the peptide+HA group relative to the control groups.

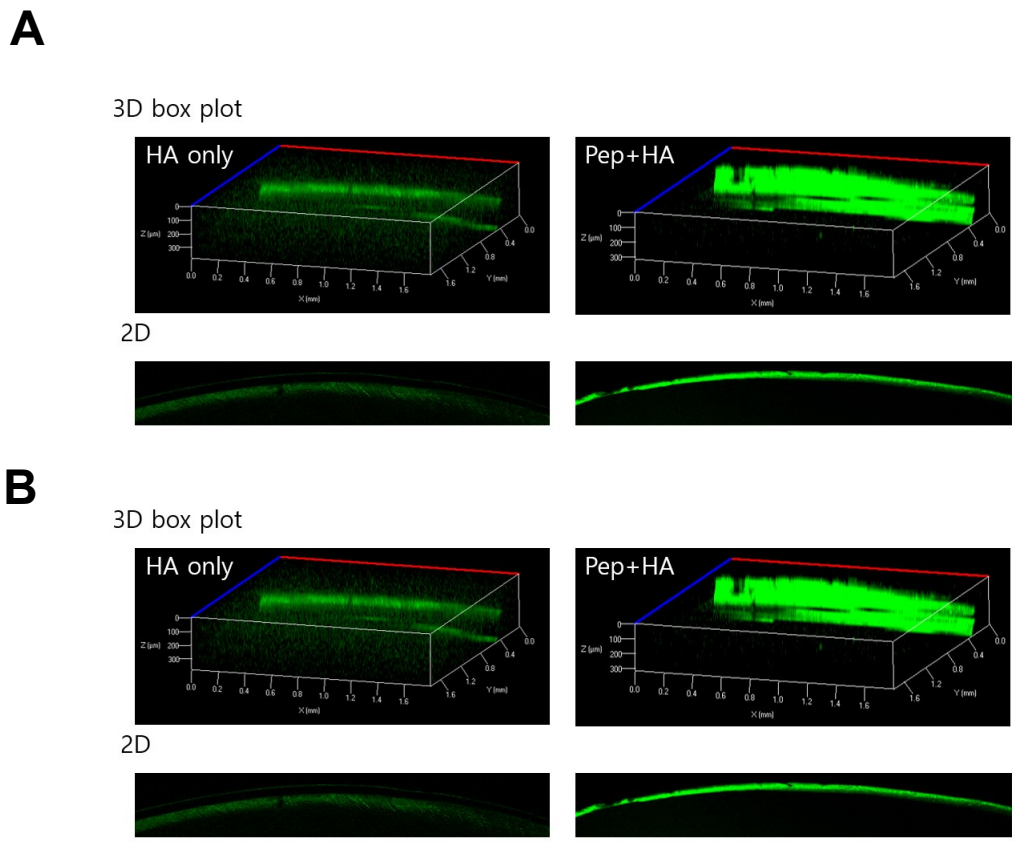

Figure 3.6 | Confocal Imaging of Contact Lenses. 3D box plots and 2D confocal images for Stenfilcon (A) and Comfilcon (B) lenses. Addition of peptide increases bound HA-FL. Binding primarily occurs at the edge of the lens.

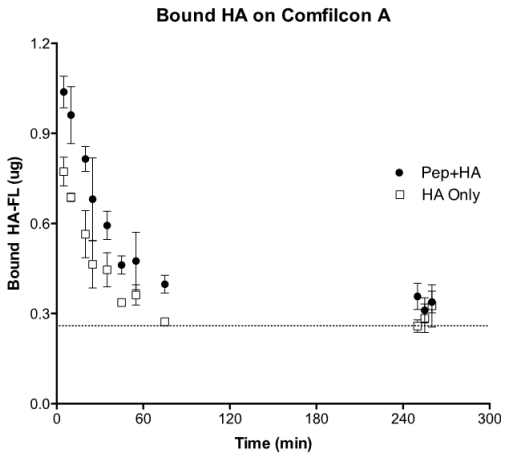

Figure 3.7 | HA-FL Release Profile for Comfilcon Lenses. Untreated lenses with only HA (no peptide) showed bound HA till 75 min (values plateaued after 75 min) while peptide treated lenses bound HA till about 4h (plateaus after 4 h, to reach the same value as with only HA).

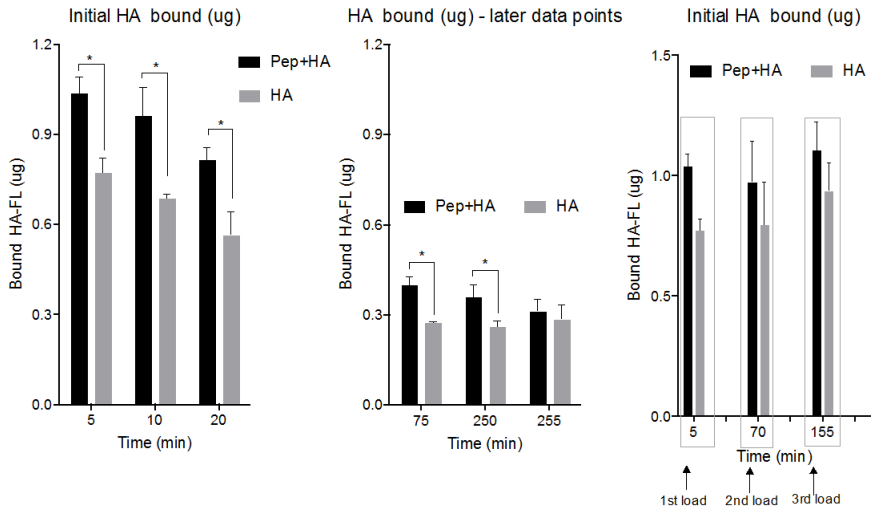

Figure 3.8 | HA-FL Reloading for Comfilcon Lenses. HA was salted out and reloaded twice. The peptide treated lenses still bound more HA for both reloads.

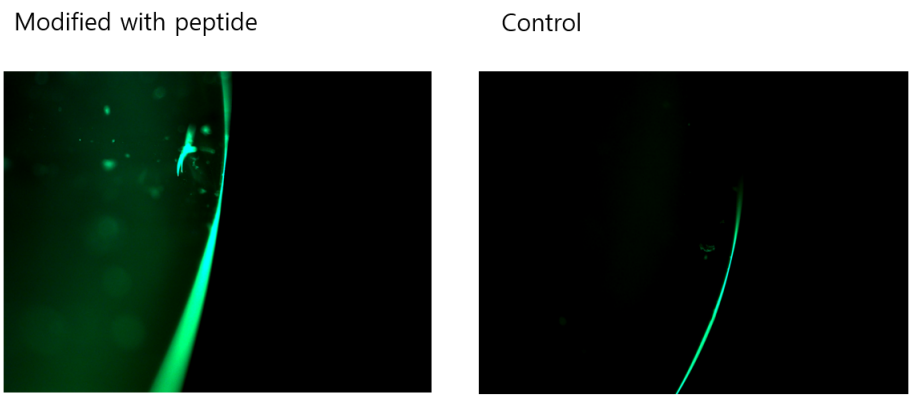

Figure 3.9 | Fluorescent images of bound HA-FL with and without peptide on Comfilcon A after second HA reload. Even after reloading the lenses with HA twice, modified lens showed more binding to HA, indicating that the polymer-peptide system is still bound to the lens after multiple washes.

4. Conclusion

Dry eye syndrome remains a very prevalent medical issue today and hyaluronic acid based treatments are not fully effective due to the short residence time of HA on the ocular surface. We have shown that polymer-peptide systems that utilize HA-binding peptide can be utilized in multiple ways to create new technologies for treating dry eye. This polymer-peptide system allows HABpep to be anchored on either the ocular surface or on the surface of contact lenses. This provides increased binding of free or exogenously applied HA, which causes improved water retention and lubrication on the eye.

Future direction of this work includes further improving HA retention for both eye-drop and modified contact lenses. This could possibly achieved by directly binding HA to the anchoring peptide without using HABpep to bind HA to the system. In addition, it is possible to utilize this polymer-peptide system on different kinds of surfaces. This can include both surfaces in the body, such as joints, and surfaces on different medical devices that require wetting and lubrication prior to implantation in the body.

5. References

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CIRRICULUM VITAE

EDUCATION

B.S. Biomedical Engineering, May 2014
with General and Departmental Honors
Johns Hopkins University, Baltimore, MD

M.S. Biomedical Engineering, May 2016
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RESEARCH

Elisseeff Lab
Fall 2014-Spring 2016
Developed eye drops and modified contact lenses that utilize HA-binding peptide to treat dry eye.

Bivalacqua & Singh Lab
Fall 2014-Spring 2016
Designed urinary conduits using collagen tubes and trachea scaffolds.

Green Lab
Fall 2013-Spring 2014
Used layer-by-layer assembly to construct a gold-based nanoparticle with temporal control over DNA release.

Yarema Lab
Spring 2013-Fall 2014
Synthesized sugar analogs to examine their effect on stem cell differentiation and sialic acid metabolic flux in cancer cells.

Boeke Lab
Fall 2011/Fall 2013
Synthesized a synthetic yeast genome by using PCR and yeast/E.Coli. transformation.

PUBLICATIONS

Lee D, Lu Q, Sommerfeld SD, Chan A, Schmidt TA, Elisseeff J, Singh A. Sialic Acid and Hyaluronan Binding Polymer Peptide System for Targeted Delivery to Ocular Surface Mucins. Under Review

Bishop CJ, Liu AL, Lee DS, Murdock RJ, Green JJ. Layer-by-Layer Inorganic/Polymeric Nanoparticles for Kinetically Controlled Multi-gene Delivery. J Biomed Mater Res A. 2015 Oct 31. doi: 10.1002/jbm.a.35610.