PRELIMINARY STUDIES ON CHITOSAN-HYALURONIC ACID SILOXY-AMINO MODIFIED CONTACT LENSES FOR OCULAR DRUG DELIVERY

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Abstract

Contact lenses are widely utilized for cosmetic and vision purposes. However, many health problems are caused by surface properties of contact lenses such as poor wettability and protein deposition. This work utilized chitosan (CS) and hyaluronic acid (HA) multilayers by immersive layer-by-layer (LbL) assembly on siloxy-amino functionalized generation 1 and 3 silicone hydrogel contact lenses. The water retention, protein deposition, and bacterial growth of the modified contact lenses were compared to unmodified ones. The ophthalmic drugs, timolol and norfloxacin, were trapped via the LbL assembly process and their retention times tested. The antimicrobial activity of the norfloxacin treated lenses were also examined.

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Introduction

This work examines the surface functionalization of generation 1 and 3 silicone hydrogel contact lenses to modify their charge (to fine-tune their adhesion and avoid folding), increase their moisture-retention properties (to address eye dryness), and impart antibacterial properties (by attaching chitosan to the surface). The lenses utilize chitosan-hyaluronic acid coatings prepared by electrostatic layer-by-layer deposition on the surface-modified contact lens material. The layer-by-layer (LbL) process will provide a surface to tether or encapsulate other medicinal compounds. Timolol (lowers pressure in the eye to treat glaucoma)[1] and norfloxacin (fluoroquinolone antibiotic)[2] are common ophthalmic drugs that were trapped by the LbL method and their releasing performances were tested.

Dry eyes is a condition that affects 10% of the population between the ages of 30-60; 15% of people, 65 years of age or older, will suffer from this disorder.[3] This condition is caused by a loss of moisture to the eyes either through evaporation, decreased production of tears or decreased clearance of tears.[4] Dry eyes can lead to an ocular surface disease, keratoconjunctivitis sicca (KCS). This disease affects the production of mucus caused by abnormal growth and differentiation of the ocular surface epithelium.[5] KCS can cause "blurred and fluctuating vision and increases the risk of sight-threatening corneal infection and ulceration".[6] The mechanism which causes KCS is unknown, but there is strong evidence that inflammation plays an important role in this process.[7] Thus people who suffer from conditions that can cause dry eye syndrome are not encouraged to wear contact lenses. This work will attempt to design contact lenses with multiple layers of chitosan and hyaluronic acid that will increase moisture retention for their users, but first a functional group needs to be placed on the silicone hydrogel lenses.

Hyaluronic acid can be found throughout the body (synovial fluid, cartilage, skin) and is an important extracellular component for the eye. It has been widely used in biomaterials because of its ability to absorb moisture and unique viscoelastic properties.[8,9,10,11] Chitosan is also anti-bacterial and a known tool for intraocular drug-delivery; it is also nontoxic, biodegradable (degradation products are amino sugars) and acts as a mucoadhesive.[4] Several biomedical applications of chitosan have been published to include: artificial kidney membrane, absorbable sutures, treatment of periodontics, supports for immobilized enzymes, blood anticoagulants, artificial skin, antimicrobial applications, hypocholesterolemia activity and some limited success as a method to accelerate wound healing.[12] Chitosan derivatives have been utilized in the field of controlled drug delivery systems in the past.[4] Since hyaluronic acid is a polyanion in water under physiological conditions and chitosan is a polycation, they can be alternatively layered on the surface of the amino-functionalized contact lens through electrostatic deposition.

Hu published a route for biocompatible hydrogels that were fabricated using methyl acrylic anhydride modified β-cyclodextrin and copolymerized with hydroxyethyl methacrylate to form a hydrogel that could potentially be used for contact lenses.[13] An alkynyl functionalized hyaluronic acid and an azide-functionalized chitosan were covalently bonded to the hydrogel via click chemistry. Unfortunately, the residing alkynyl functionality may not be suitable for use in contact lenses. The same group also used layer by layer self-assembly to modify a hydrogel with low oxygen permeability with a chitosan and hyaluronic acid to increase the water retention.[14] Another group has examined the surface wettability of silicone hydrogel polymers with five layers of chitosan and hyaluronic acid.[15] These findings demonstrate that the biopolymers, chitosan and hyaluronic acid, increased the water retention in the hydrogels and would do so for our modified lenses.

Generation 1 silicone hydrogel lenses (balafilcon A, PureVision2, B&L) are prepared from the co-monomers N-vinyl pyrrolidone (NVP) and 3-[tris(trimethylsiloxy)silyl]propyl methacrylate (TRIS) and then undergo a plasma treatment. This yields free hydroxy groups on the surface to increase the wettability of the lens. Generation 3 (enifilcon A, Avaira, CooperVision) use unique long-

chain siloxane macromers combined with other components.[16] Anderson discovered that silane groups grafted onto the surface of poly(2-hydroxymethyacrylate)/(methacrylic acid) PHEMA/MAA contact lenses absorbed less lysozyme and increased the wettability of the contact lenses.[17] It was decided to combine the effects of silyl moieties with the LbL depositions of chitosan and hyaluronic acid. The surface of the contact lenses was first modified by tetra-ethoxysilane (TEOS) and then further reacted with 3-amino propyltriethoxysilane (APTES) to provide primary amine functionality.[18] These amino groups provide a basis for either covalently linking the hyaluronic acid (carbodiimide coupling) or as a cation for layer-by-layer deposition as shown in Scheme 1.

Amino groups detected on the modified generation 1 and 3 lenses were used to determine which generation would work best with our procedure. Then different concentrations of the TEOS/APTES methods were examined to find the optimum process. These lenses were then tested for their water retention, protein deposition, and antimicrobial effects. Finally, the retentions of two common ophthalmic drugs, timolol and norfloxacin (incorporated during the LbL process), were examined in saline solutions over a timed period.

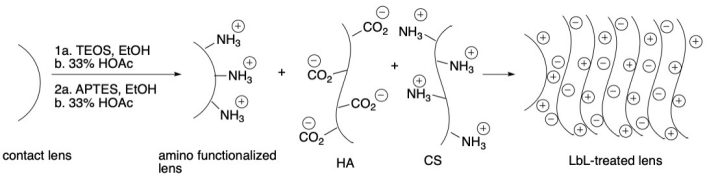
Experimental

TEOS treatment

A contact lens was rinsed with deionized (DI) water and dried by dabbing it with a Kimwipe and lightly blowing air on the lenses. The lens was placed in a clean 10 mL round bottom flask with a small stir bar in it. 2.0 mL of ethanol was added to the flask as well as 1.0 mL of TEOS. The flask and its contents were heated in an oil bath under a condenser at 50ºC. After a timed treatment, the flask was allowed to cool. Times tested were 18, 7, and 6 hours. The contents of the flask were poured into a 25 mL beaker. The contact lens was removed and rinsed with DI water. It was then dried completely by blotting it with a Kimwipe and lightly blowing air on it. Then the lens was placed in 33% m/v acetic acid and shaken on a Hybritech Incorporated Orbital Shaker. After 20 minutes, the lens was rinsed with DI water, dried, and placed on a piece of parchment paper on a labeled watch glass to dry overnight.

APTES treatment

Following a TEOS treatment, the contact lens was placed in a new, clean 10 mL round bottom flask with a small stir bar in it. 2.0 mL of ethanol was added along with 1.0 mL of APTES. The solution was heated in an oil bath under a condenser at 50ºC. After half of the treatment time of the TEOS treatment, the flask was removed from the oil bath and set aside to cool. Once cool, the contents of the flask were poured into a beaker. The contact lens was removed from the solution and rinsed with DI water. It was then dried completely. After it was dried, the lens was placed in 33% m/v acetic acid and shaken for 20 minutes. After 20 minutes, the lens was rinsed with DI water, dried, and placed on a piece of parchment paper on a labelled watch glass to dry overnight.



Scheme 1. Illustration of the electrostatic layer-by-layer deposition of hyaluronic acid (HA) and chitosan (CS) on amino-functionalized lenses.

Fluorescamine treatment

0.0050 g (1.797×10^{-5} mol) of fluorescamine was placed in a 20 mL beaker. 5.0 mL of ethanol was added to the beaker along with a small stir bar. The beaker with its contents were placed on a stirrer and stirred for 20 minutes. After the 20 minutes, 5.0 mL of DI water was added to the beaker and stirred for another 20 minutes. This solution was prepared under a hood which had tin foil covering the window of the hood to block the light from coming in. There was also a tin foil covered 400 mL beaker covering the 20 mL beaker. 5.0 mL of this solution was added to a vial. A contact lens that had been previously treated with TEOS/APTES was placed in the vial as well. The vial was capped and placed on a shaker overnight. The vial was covered with a tin foil wrapped 400 mL beaker.

The next morning, the lens was removed from the fluorescamine solution and rinsed with DI water. The contact lens was dried and placed on a piece of parchment paper on a labeled watch glass to dry for 3 hours. A mass was obtained of this contact lens. The lens was then placed in DI water for 3 hours. After the contact lens was thoroughly wet, a UV-Vis spectrum was obtained. The contact lens was placed back in the DI water and covered with a tin foil wrapped beaker when not in use. The lens was then dried, placed on a piece of parchment, covered and left to dry overnight. All procedures were performed with the lights off and the hood sash covered with aluminum foil.

Fmoc/Piperidine treatment

A lens treated by the 7 hour-TEOS:3.5 hour-APTES method was massed and placed in a vial. 0.04375 g (4.95×10^{-4} mol) of fluorenylmethyloxycarbonyl amino hexanoic acid (Fmoc-Ahx-OH), 1.0mL of dimethylform-amide (DMF), 0.01685 g (5.00×10^{-4} mol) hydroxybenzotriazole monohydrate (HOBt), and 20.0 µL (5.11×10^{-4} mol) N,N'-diisopropylcarbodiimide (DIC) were added to this vial. The vial was capped and placed on a shaker for 22 hours. The vial was covered with a beaker wrapped in tin foil to make sure no light would reach the lens.

The following morning, the contact lens was removed from the Fmoc solution and rinsed with DMF, then diethyl ether, followed by methanol, and lastly, DI water. The lens was then blotted dry with a Kimwipe and placed on the parchment paper on the watch glass to dry overnight. It is then massed again.

The contact lens that had been treated with Fmoc was placed in DI water for about three hours to thoroughly wet the lens. The lens was then analyzed via a Shimadzu UV-2450 UV-Vis spectrophotometer. Then the lens was placed in a vial of piperidine/DMF (1:2 v/v) solution. The piperidine/DMF solution was made by mixing 8 mL of piperidine with 16 mL of DMF and stirring it for 20 minutes. 3 mL of this solution and the contact lens was capped and covered with a tin foil wrapped beaker and shook for 16 hours. The following morning, the vial was removed from the shaker and the lens removed. The lens was treated with 2 mL rinses of CH_2Cl_2, then DMF, and finally isopropanol for two minutes each. This procedure was then repeated. These extractions were combined

with the DMF/pyridine filtrate and the solvent was removed under pressure. The resulting solid was dissolved in 25 mL volumetric flask with methylene chloride. 1.0 mL of this solution was used to make a 10 mL solution, which was then analyzed by UV-Vis.

Layer-by-Layer technique

Two solutions were prepared prior to treating a contact. In one solution, 0.050 g of chitosan was dissolved in 50 mL of 1% HOAc solution in 0.15 M NaCl. In the other, 0.050 g of hyaluronic acid was dissolved in 50 mL of 0.15M NaCl. A contact lens treated with the 7 hour-TEOS:3.5 hour-APTES method was weighed and placed in 0.15 M NaCl. The contact lens was lightly dabbed on a Kimwipe and placed in the hyaluronic acid (HA) solution for 20 minutes. After 20 minutes, the contact lens was rinsed in 0.15 M NaCl. It was then dabbed lightly with a Kimwipe and placed in the chitosan (CS) solution for 20 minutes. After 20 minutes, it was rinsed with 0.15 M NaCl. This cycle was repeated until the lens had 20 layers. (Each solution counts as a layer.) Then the lens was placed on a watch glass on parchment paper to dry overnight under ambient conditions.

Wettability Study

A contact lens was treated with the 7 hour-TEOS:3.5 hour-APTES method and then layered (20 layers), and then placed in DI water for 5 hours. The gently blotted lens was then put on a scale and the mass was recorded every 5 minutes for 3 hours. This data demonstrated the water retention properties of the layered lenses as an equilibrium swell ratio. The ratio was calculated using the following equation:

$$EQR = \frac{M_w - M_d}{M_w} * 100\%.$$

M_w is the mass of the wet contact lens and M_d is the last recorded weight once the mass was not changing further.

Protein Deposition

A standard curve was created following the instructions provided in Fisher's Pierce Bovine Serum Albumin (BSA) Assay kit. Using the same set of instructions, a 1mg/mL solution of BSA was created. 750 mL of this solution was poured into two contact lenses holders. A lens that had been layered twenty times was placed in one of the containers and an untreated lens was placed in the other. These lenses soaked in these solutions for 24 hours. A UV-Vis spectrum was taken before soaking in the BSA solution and after the 24 hours treatment.

A second test was run to compare the protein buildup on treated lenses versus untreated lenses. A 10 mg/mL solution of BSA Alexa-488 in Phosphate-Buffered Saline (PBS) was prepared.[21-22] A treated lens layered with 20 layers and an untreated lens were cut up. A small piece was taken from the approximately the same location on the lenses were placed in the BSA solution. The contact lenses were observed under a confocal microscope after 3 hours.

Bacterial adherence to lenses

Approximately 3x5 mm pieces of lenses were exposed to *Pseudomonas aeruginosa* by placing a portion of a lens in a bacterial suspension at Abs_{600} of 0.5, which equates to around $5x10^8$ cells/mL. The lenses were then incubated in the suspension for 30 minutes at 37°C and then washed with 0.8% saline solution to remove non-adherent bacteria. Lenses were then stained with LIVE/DEAD™ *Bac*Light™ Bacterial Viability Kit (Molecular Probes) per the directions. The lenses treated with *P. aeruginosa* were examined using confocal microscopy to quantify the amount of fluorescence and therefore the number of living and dead bacteria.

LbL with ocular medications

Lenses were layered following the same procedures stated under the "Layer-by-Layer Technique" section. The chitosan layer was made by dissolving 0.05 g of chitosan in 50 mL of 1% acetic acid solution containing 0.15 M NaCl. Then, 0.150 g of a drug (norfloxacin or timolol) was dissolved in this solution. This solution was used as the cationic layer for the LbL process.

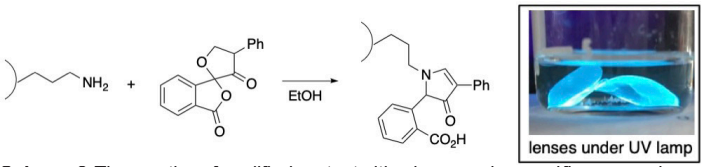
Drug Elution

A 7 hour-TEOS:3.5 hour-APTES treated lens was layered with hyaluronic acid and norfloxacin infused chitosan (20 layers). Another similarly treated lens with the same prescription was layered with hyaluronic acid and timolol infused chitosan (20 layers). These lenses were then placed in separate dishes containing 4 mL of 0.9% m/v NaCl solution for 5 minutes. After 5 minutes, the lenses were removed from this solution and again placed in separate dishes containing the NaCl solution for 5 minutes. This cycle continued for 60 minutes. Once this was done, each of the solutions were analyzed via UV-Vis to determine what quantity of the drug was released from the contact lenses (norfloxacin solution analyzed at 270 nm and 295 nm for the solutions containing timolol.)[18]

Results

Initially two different lenses were tested to find the best option for placing amino groups onto the contact. Generation 1 (Pure Vision 2, balaficon A) and generation 3 (enfilcon A) with similar base curves (BC:8.6,8.5) and strength of the prescriptions (PWR:-2.75, -2.75) were examined to try to keep the lenses as similar as possible. The presence of the added primary amino groups was confirmed by the reaction of the amine and fluorescamine (which is not fluorescent) as shown in Scheme 2 to produce fluorescent lenses.[19] The reaction of the fluorescamine with an enfilcon A lens gave a dark, discolored product with a lower absorbance of light (1.16) measured at maximum absorbance for the lens at 396 nm. The balafilcon A product was clear, light green and highly fluorescent and displayed a higher absorbance of light (1.53) at 424 nm. Balafilcon A lenses were then utilized for the remainder of this work.

The optimum concentrations for TEOS and APTES were determined by finding the maximum concentration of a fluorene released upon reaction of the modified contact lenses with an Fmoc derivative via UV-Vis analysis. Fluorenylmethyloxycarbonyl amino hexanoic acid (Fmoc-Ahx-OH), hydroxybenzo-triazole monohydrate (HOBt), and N,N'-diisopropylcarbodiimide (DIC) were reacted in DMF to produce an intermediate carbamate which was



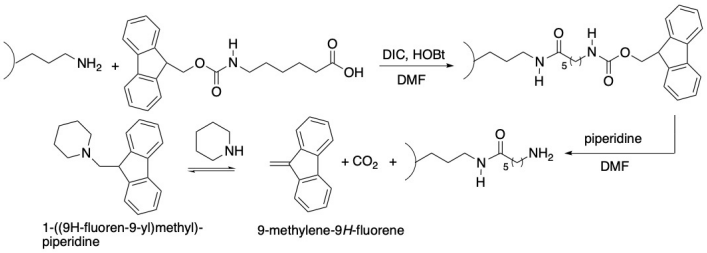
Scheme 2. The reaction of modified contact with primary amines and fluorescamine to produce fluorescent lenses, as shown in the photo to the right of scheme.

then reacted with piperidine to release 9-methylene-9H-fluorene, carbon dioxide, and the contact lens with free amino groups. The fluorene reacts with dimethylformamide in an equilibrium reaction to make the dibenzofulvene-piperidine product. The concentration of the Fmoc groups that reacted could be approximated by the absorbance of light at 301 nm (ε = 8021 $Lmol^{-1}cm^{-1}$) by the fluorene moiety emitted per gram of contact lens.[20] The reactions to produce the piperidine adduct are displayed in Scheme 3. This shows that the moles of primary amines have a one-to-one ratio with the moles of the Fmoc-derivative detected.

Contacts were prepared using three different times for the TEOS/APTES reactions: 6hr:3hr; 7hr:3.5hr, 18hr:9hr. 25 mL solutions of the overnight piperidine treatment filtrate's precipitate were diluted by a factor of ten and then analyzed for concentrations using Beer's law. Not surprisingly, the concentration per gram of contact lens increased as the reaction time increased as seen in Table 1. The 7hr:3.5hr time-period was determined to be the most efficient ratio. The moles of amine only increased by about 3% when the silyl reaction times were doubled. The 7:3.5 vs 18:9 ratios also meant the difference between two-day vs three-day reaction periods, as the lenses need to rest overnight between the TEOS and APTES treatments.

The presence of amines on the contacts confirms that the TEOS/APTES reagents reacted effectively with the generation 1 lenses. TEOS hydrolyzes on the hydroxy-groups of the lens polymer, forming the anchor C-O-Si-OH sites necessary for the introduction of amino-groups by the condensation with APTES.

Lenses made by the 7hour-TEOS:3.5hour-APTES method were reacted with fluorescamine to test the penetration of the amino groups into the contact. Slices of the contacts were imaged under a confocal microscope which showed insertion of the dye throughout the thickness of the lens and across the surface of the lens as shown in Figure 1. The lenses were treated with fluorescamine and viewed under confocal microscopy at a wavelength of 402 on the Olympus 1200 confocal scanning laser microscope (CSLM), 55 steps were taken each at 1.5 µm to show the total penetration of the lens.



Scheme 3. Production of 9-methylene-9H-fluorene from DIC coupling reaction of modified contact lens with Fmoc-Ahx-OH followed by formation of piperidine adduct

Table 1. Data/Results of different reaction times for silanization reactions to determine the moles of amine per gram of contact lens.

TEOS:APTES	mass of lens	absorbance	molarity/gram	mole/gram
6 hr:3 hr	0.0164 g	0.089	$6.79x10^{-3}$ M/g	$1.70x10^{-4}$ mol/g
7 hr:3.5 hr	0.0155 g	0.353	$2.82x10^{-2}$ M/g	$7.10x10^{-4}$ mol/g
18 hr:9 hr	0.0154 g	0.362	$2.93x10^{-2}$ M/g	$7.32x10^{-4}$ mol/g

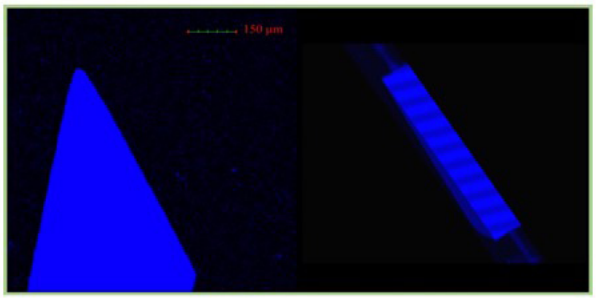


Figure 1. A slice of fluorescamine-labeled contact lens as viewed under confocal microscopy (left). The same slice turned to look through the lens on the Z axis to confirm the dye permeated through the lens (right).

Malachite green (MG) was used to confirm the layer-by-layer process. This dye was added to the hyaluronic acid solution and the absorbance of the lens was measured after each treatment during the LbL procedure. Figure 2 demonstrates that there is more MG chromophore present after the HA treatment and this decreases after a layer with the chitosan. In general, there is a trend for an increase in absorption of light as the number of layers increases.

The water retention of the lenses modified by the complete LbL process was tested by examining the change in the weight as the saturated lenses were left to dry under ambient conditions. Layered lenses retained water for longer periods of time than untreated lenses as can be seen in Figure 3. The layered lenses had an equilibrium swell ratio of 46.60%, whereas untreated lenses had a ratio of 39.11%.

Pseudomonas aeruginosa is a gram-negative bacterium that is hard to treat due to its extreme versality and resistance to known antibiotics.[21] Norfloxacin-modified lenses, as well as precursor

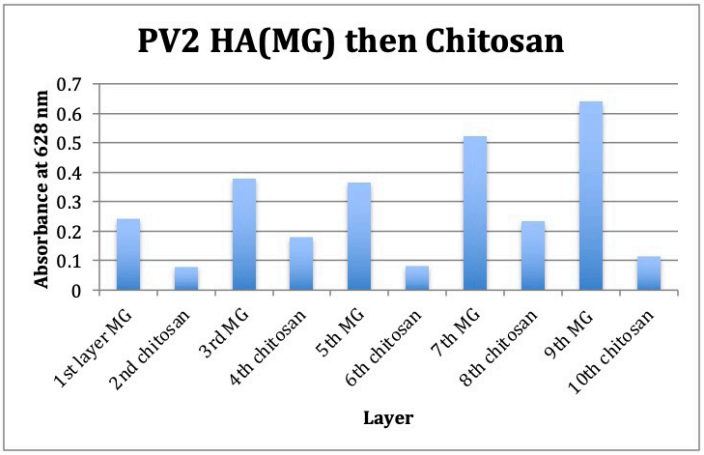


Figure 2. Absorbance of treated lens after LbL method with malachite green added to the hyaluronic acid layers.

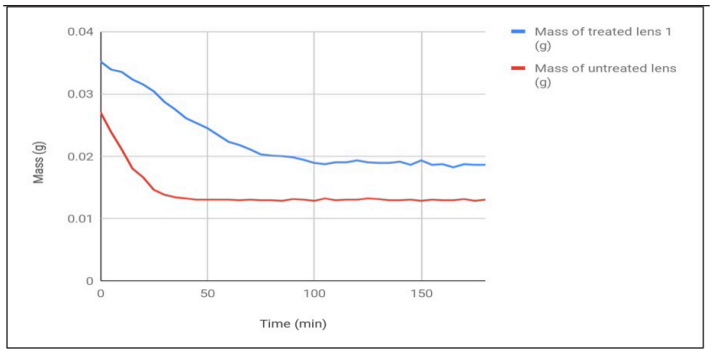


Figure 3. Water retention study of a layered PV2 lens and an untreated lens

lenses that were modified with TEOS/APTES and TEOS/APTES LbL were exposed to *P. aeruginosa* to test the ability of the treated lenses to control bacterial growth. Vitality of adherent *P. aeruginosa* was assayed using Live/Dead stain (Figure 4). The percent of bound bacteria that were alive was lowest on the norfloxacin-treated lens, as compared to the untreated lens and those with the precursor modifications. There was no statistical difference in the total number of live and dead cells on the four lenses types (data not shown). Therefore, the treated lens does not reduce the bacterial adherence to the surface, but reduces the viability of the attached microbes.

The LbL lenses were also tested for protein deposition. Lenses that had been layered had less protein build-up on the surface of the lens than the untreated lens when treated with the Fisher's Pierce BSA Assay kit. The concentration of BSA on the treated lens was 198.33 µg/mL, whereas the concentration of BSA on the untreated lens was 814.04 µg/mL. However, when the lenses were soaked in the 10 mg/mL solution of BSA Alexa-488 in PBS, no observable protein was built up on the untreated lens, whereas there was clearly immense protein build-up on the layered lens.

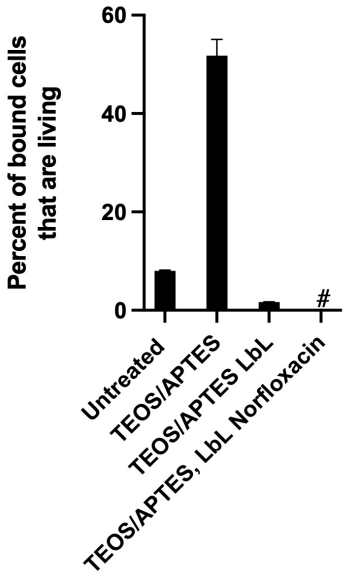


Figure 4. Effect of contact lens treatments on *P. aeruginosa* vitality. Error bars show standard deviations of 0.147, 3.28, 0.046 and 0.003 respectively. The percent of bacteria that bound to the lenses treated with TEOS/APTES, LbL, Norfloxacin (0.06%) was not visible on this scale.

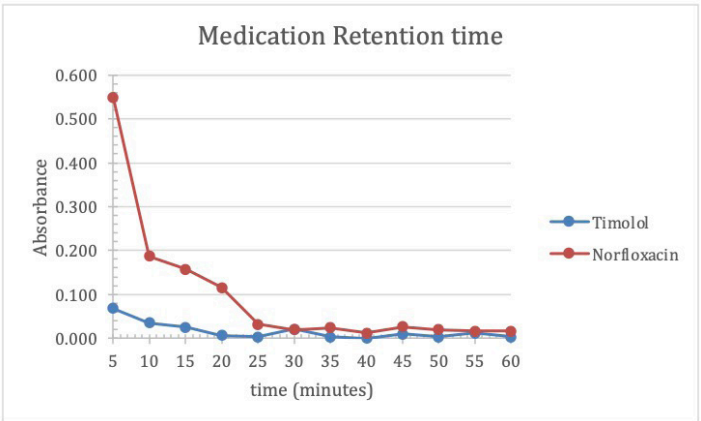


Figure 5. Absorbances of samples of 0.9% NaCl solutions with contact lenses layered with hyaluronic acid and timolol/norfloxacin infused chitosan for five-minute intervals.

The former results is not supported by previous reports on protein buildup for HA modified surfaces and needs to be reexamined in more detail. [11,15,22,23,24]

Lenses were then prepared by the LbL (20 layers) to incorporate either timolol or norfloxacin during the process to test the elution of these compounds in saline solution over time. Disappointingly, low timolol concentrations (294 nm) were detected in the examined saline solutions. Norfloxacin (275 nm) release could be detected in higher concentrations. Figure 5 shows that the detected timolol was very low even in the first five minutes and that a large amount of norfloxacin was lost during the first 10 minutes. Most of the norfloxacin was released after 30 minutes had passed.

Conclusion

This preliminary work examined lenses modified with siloxy amino groups and treated by a LbL process with hyaluronic acid (HA) and chitosan (CS) on generation 1 and 3 silicone hydrogel contact lenses. We are grateful for the donation of contact lenses and plan to purchase balafilcon A contacts with the same prescription strength to avoid any variables that might have contributed to the results of this research. The water retention, protein deposition, and bacterial growth of the modified contact lenses were compared to unmodified ones. Generation 1 (Pure Vision 2, balafilcon A) were found to react better than generation 3 (enfilcon A) under the TEOS/APTES treatment. The presence of the added primary amino groups was confirmed by the reaction of the amine and fluorescamine. Confocal microscopy illustrated that the amino groups were added throughout the lens and not just placed on the surface. It was found that the 7hr:3.5hr method was determined to be the most efficient ratio for the TEOS:APTES preparation. The dye, malachite green, confirmed the layer-by-layer process for the hyaluronic acid and chitosan solutions as it increased in concentration on the lenses during the process. Norfloxacin treated lenses exposed to *P. aeruginosa* demonstrated that the antibiotic was effective as only 0.06% of the detected microbes were alive. The drug, timolol, was not retained well on the layered lens and probably lost in the LbL process. Norfloxacin may be held better since its cyclic secondary amine has less steric hindrance than the t-butyl substituted secondary amine of the timolol. The bound norfloxacin was released after 30 minutes had passed. The authors intend to further investigate these amino modified LbL treated contact lenses' properties such as zeta potential and to find a better method to trap and then release ophthalmic medications

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