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Injectable drug depot engineered to release multiple ophthalmic therapeutic agents with precise time profiles for postoperative treatment following ocular surgery

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Abstract

A multi-drug delivery platform is developed to address current shortcomings of post-operative ocular drug delivery. The sustained biodegradable drug release system is composed of biodegradable polymeric microparticles (MPs) incorporated into a bulk biodegradable hydrogel made from triblock copolymers with poly(ethylene glycol) (PEG) center blocks and hydrophobic biodegradable polyester blocks such as poly(lactide-co-glycolide) (PLGA), Poly(lactic acid) (PLA), or Poly(lactide-co-caprolactone) (PLCL) blocks. This system is engineered to flow as a liquid solution at room temperature for facile injection into the eye and then quickly gel as it warms to physiological body temperatures (approximately 37 °C). The hydrogel acts as an ocular depot that can release three different drug molecules at programmed rates and times to provide optimal release of each species. In this manuscript, the hydrogel is configured to release a broad-spectrum antibiotic, a potent corticosteroid, and an ocular hypotensive, three ophthalmic therapeutic agents that are essential for post-operative management after ocular surgery, each drug released at its own timescale. The delivery platform is designed to mimic current topical

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application of postoperative ocular formulations, releasing the antibiotic for up to a week, and the corticosteroid and the ocular hypotensive agents for at least a month. Hydrophobic blocks, such as PLCL, were utilized to prolong the release duration of the biomolecules. This system also enables customization by being able to vary the initial drug loading to linearly tune the drug dose released, while maintaining a constant drug release profile over time. This minimally invasive biodegradable multi-drug delivery system is capable of replacing a complex ocular treatment regimen with a simple injection. Such a depot system has the potential to increase patient medication compliance and reduce both the immediate and late term complications following ophthalmic surgery.

Graphical Abstract

Keywords

Injectable; hydrogel; microparticle; controlled release; ophthalmology; postoperative

1. Introduction

More than 4 million ocular surgeries are performed every year in the US [1]. Careful management with topical medications is crucial for preventing post-operative complications including vision loss after ocular surgery [2]. Successful surgical outcome requires frequent applications of one or more ophthalmic drops over several days to several weeks and this process depends heavily on patient compliance. Similar to most other surgical procedures, incisional ophthalmic procedures carry a small risk of infection [3, 4] and a more common physiologic reaction of inflammation in the immediate post-operative period [5, 6]. Post-surgical inflammation as well as corticosteroid treatment may induce intraocular pressure (IOP) increase in many patients [2, 7–9]. Increase in IOP may require additional topical treatment with ocular hypotensives, adding to the overall burden of treatment and decreasing patient adherence. In the later stages of surgical recovery, secondary complications including rebound inflammation and elevated intraocular pressure may cause suboptimal vision, and on rare occasions may lead to vision loss, further surgery, and morbidity. Strict medication adherence in the post-operative period is necessary for preventing complications from inflammation, infection, and IOP elevation [10–12]. Numerous studies have shown that side effects of topically applied ocular agents and patient non-compliance are major contributing factors of therapeutic failure in ophthalmic diseases [13].

The vast majority of ophthalmic drugs are marketed as topical formulations. The delivery of topical drug formulations is greatly hindered by the ocular surface barrier and availability is limited by rapid clearance from the ocular surface by the tear flow [14, 15]. Despite these inherent shortcomings, a critical barrier for ophthalmic drug delivery continues to be poor patient compliance, limiting effective dosing and causing suboptimal therapy [13, 16, 17].

Patient adherence is affected by numerous factors, including decreased cognitive and motor function of geriatric patients, ocular surface irritation and allergic reaction with pain and discomfort caused by topical ophthalmic agents [13, 17–19]. Patient non-adherence is one of the main causes of post-operative complications after ocular surgery.

Alternative ocular drug delivery mechanisms have been developed to replace topical drop administration, improving drug dosing and reducing side effects. These mechanisms employ distinct approaches to the drug delivery problem and include contact lenses, implants and hydrogels [20–24]. Despite the extensive effort, there are only a handful of products that are marketed to date. A review of commercial implants can be found in [14]. These implants include Vitrasert® and Retisert® (Bausch & Lomb, Rochester, NY) as non-biodegradable implants to release biomolecules for prolonged periods. However, due to their non-biodegradable design, they require vitreoretinal surgery not only for implantation but also for implant removal [14, 25–29]. Ozurdex® (Allergan, Irvine, CA), a poly(lactide-co-glycolide) (PLGA) matrix that encapsulates dexamethasone. It is the first FDA-approved biodegradable formulation for ocular indications and useful to treat non-infectious posterior uveitis as well as macular edema secondary to diabetes and retinal vein occlusion [14, 30]. Iluvien® (Alimera Sciences, Alpharetta, GA) is a non-biodegradable intravitreal corticosteroid implant that unlike Vitrasert® and Retisert®, Iluvien® does not require surgical implantation and is delivered into the vitreous cavity via transscleral injection. Complications with Ozurdex® and Iluvien®, as depots of corticosteroid agents, include cataract formation and elevated IOP [14, 31–34]. In addition to implants, polymeric particles have been developed to sustain the release of drug molecules to the eye. Shmueli et al. synthesized nanoparticles by complexing a negatively charged peptide drug with a positively charged poly(beta-amino ester) [35]. The authors then loaded the resulting nanoparticles into PLGA MPs and were able to sustain the release of the single peptide drug for 200 days *in vitro* and ~100 days *in vivo* for the treatment of choroidal neovascularization [35]. For the treatment of retinal diseases, the goal is long-term release of many months following a single injection. In contrast, for postoperative treatment following ocular surgery, the goal is short to medium term release of small molecule drugs spanning from days to weeks.

The above-mentioned drug delivery systems are limited to single-agent strategies and there is a need to develop a multi-drug delivery system that can release disparate ocular drugs at different time scales. Thermoresponsive hydrogels are attractive options for long-term release of one or more therapeutics to the eye. In their optimal formulation, they are liquid solutions at room temperature to ensure injectability while they form a depot for drug molecules once they are warmed up to body temperature. A-B-A triblock copolymers are particularly desirable for ocular drug delivery due to their biocompatibility and biodegradability [36]. In these triblock copolymers, block A is a hydrophobic polymer such as PLGA, Poly(lactic acid) (PLA), Poly(lactide-co-caprolactone) (PLCL), or Poly(caprolactone) (PCL) while block B is a hydrophilic polymer such as poly(ethylene glycol) (PEG). Once in the solution, these triblock copolymers result in the creation of micelles, with a hydrophilic polymer creating the outer layer that is in contact with water. Simultaneously, the hydrophobic block forms the core of the micelle. The number of micelles, and the association between them is strengthened as the result of elevation of temperature, leading to the formation of a hydrogel network [37].

Gervais reported the use of cyclosporine A-loaded PLGA-PEG-PLGA thermogels for the treatment of posterior capsule opacification, one of the sequelae of cataract surgery. Their results showed a large burst release of drug followed by detectable release for up to a week [38]. Xie et al. loaded Avastin®, a large monoclonal antibody in PLGA-PEG-PLGA hydrogels for the treatment of vitreoretinal diseases and found the majority of it was released within 8 hours post incubation [39]. Thus, a central challenge is to enable long-term sustained release with such thermogel systems. Zhang et al. employed PLGA-PEG-PLGA hydrogels to release dexamethasone, which was loaded above the hydrophobic drug's solubility limit and shown to release for up to 18 days [40].

Hirani et al. synthesized PEG-PLGA nanoparticles loaded with triamcinolone acetonide and added the resulting nanoparticles to a PLGA-PEG-PLGA hydrogel network for the treatment of age-related macular degeneration (AMD) [41]. The authors found that the majority of the release occurred in the first 10 hours post incubation, but through the use of the nanoparticles they did not observe the large burst release evident in similar hydrogel studies [41]. Extended release of longer than a month was demonstrated by Duvvuri et al. who synthesized PLGA MPs (>200 µm) encapsulating ganciclovir and loaded these into PLGA-PEG-PLGA hydrogels [42]. While single-agent release may be helpful for certain indications, a multi-drug delivery platform that temporally regulates drug release of each agent would be ideal for the treatment of many ophthalmic conditions, including post-operative management requiring multiple agents for inflammation, infection, and elevated IOP. For other ocular diseases including age-related macular degeneration, studies have shown that combination therapy using multiple drug molecules can be more effective than single-drug therapy [14, 43]. Yet, a drug delivery system is still needed to provide increased flexibility to finely tune the drug release rate and duration of multiple ophthalmic agents that have differing drug properties.

This research is conducted under the hypothesis that an injectable depot can be engineered to allow for the simultaneous release of three ophthalmic therapeutics, each following its own precise release profile, through the tuning of biomaterial properties of a microparticle encapsulating hydrogel drug delivery system. To evaluate this hypothesis, a particular case of post-operative care following cataract surgery is considered where a multi-drug ocular delivery system might be helpful. Cataract surgery is the most commonly performed ocular surgical procedure in the US. Typically, during the postoperative recovery period, topical antibiotics are administered for 7 days to reduce the risk of infection and topical corticosteroids are applied for up to a month with decreasing frequency to reduce inflammation [10–12]. Corticosteroids are the first-line agents for the treatment of ocular inflammation. They are well tolerated with fewer side effects than other broad-spectrum immune suppressants [44]. Often an ocular hypotensive is also added to the post-operative treatment regimen to reduce IOP increase secondary to inflammation and/or corticosteroid use [2, 7–9]. Thus, a simple to administer, minimally-invasive ocular delivery system would be advantageous to the complex current standard of care and would improve patient compliance. If such a drug delivery system forms a localized depot in the eye that sits below the visual axis, it ensures no interferences with vision. The main motivation of this research was to develop the ability to bypass patient non-adherence to postoperative eye drops by developing an injectable localized depot for release of multiple drugs.

To mimic the current treatment paradigm, a broad-spectrum antibiotic, 4th generation fluoroquinolone moxifloxacin, is designed to be delivered over a period of 7 days by adding it directly to the hydrogel network (Fig. 1A). In addition, a potent corticosteroid, dexamethasone, is designed to be released from the drug delivery system for more than a month to suppress inflammation. Because increased IOP is seen commonly with intraocular steroid implants, an ocular hypotensive agent, beta-blocker levobunolol [45, 46], is also designed to be simultaneously delivered to control IOP. In this design, the anti-inflammatory and hypotensive drugs are encapsulated in PLGA MPs, which can sustain the release of the drugs. These particles can then be embedded into hydrogels to further regulate the drug release (Fig. 1A). With this design, the drug release rate of each agent (regardless of its hydrophobicity) can be individually tuned and then combined into a single easily injectable depot. By varying their hydrophobicity, the degradation rate of polymer microparticles, and consequently the drug release kinetics, can be finely tuned. Since this drug delivery platform is in liquid phase at room temperature (Fig. 1B) and forms a hydrogel network at body temperature (Fig. 1C), implantation is accomplished through an injection into the anterior chamber or vitreous cavity of the eye. Intraocular injections of drug delivery systems are done routinely during surgery in the clinic and have been tolerated by patients [2].

2. Materials and Methods

2.1. Chemicals

To make the hydrogel formulations, the following triblock copolymers were purchased from PolySciTech (West Lafayette, IN) and are called throughout this paper with their product number: AK12: PLGA-PEG-PLGA (with MWs of 1000:1000:1000 Da, ratio of lactic to glycolic acid (LA/GA): 1/1, viscosity at 5 °C: 0.37 Pa.s), Ak91: PLGA-PEG-PLGA (with MWs of 1500:1500:1500 Da, LA/GA: 6/1, viscosity at 5 °C: 0.25 Pa.s), AK100: PLA-PEG-PLA (with MWs of 1700:1500:1700 Da, viscosity at 5 °C: 0.098 Pa.s), AK108: PLCL-PEG-PLCL (with MWs of 1600:1500:1600 Da, caprolactone (CL)/LA: 3/1, viscosity at 5 °C: 0.073 Pa.s), and AK109: PLCL-PEG-PLCL (with MWs of 1700:1500:1700 Da, CL/LA: 3/2, viscosity at 5 °C: 0.091 Pa.s). Different types of PLGA were used to make drug loaded MPs. For levobunolol loaded MPs, multiple formulations of PLGA (with LA/GA of 60/40 (Product number: AP43), 75/25 (AP18), or 85/15 (AP87), all with number averaged molecular weight of 45–55kDa) were purchased from PolySciTech. For dexamethasone-loaded MPs, PLGA (Resomer 503H with LA/GA ratio of 50/50 and weight averaged molecular weight of 24–38 kDa) was purchased from Evonik Corporation (Essen, Germany). Dexamethasone (Product number: 46165) and moxifloxacin (Product number: PHR1542) were obtained from Sigma Aldrich (St. Louis, MO), while levobunolol hydrochloride (HCl) (Product number: 1359801) was purchased from United States Pharmacopeia (Rockville, MD). Poly(vinyl alcohol) (PVA, with molecular weight of 13–23 kDa, product number: 363170) was obtained from Sigma Aldrich. All other materials were obtained from Sigma Aldrich. Unless noted otherwise, the materials were used as received without further purification.

2.2. Microparticle Synthesis

One hundred mg PLGA (60/40, 75/25 or 85/15) was dissolved in dichloromethane (DCM) at a concentration of 61 mg/ml. This solution was added to 10.7 mg regular levobunolol HCl or deprotonated levobunolol dissolved in DMSO to 97 mg/ml. To remove the HCl salt from levobunolol HCl, 30 mg of the drug was dissolved in dimethyl sulfoxide (DMSO) at a concentration of 97 mg/ml. A one-to-one molar ratio of triethylamine (TEA) was added to that and the solution was constantly inverted at a speed of 11 rpm for 3 hrs to obtain deprotonated levobunolol. The resulting drug solution was kept at room temperature protected from light and was used to make the MPs the next day. The polymer-drug solutions were then mixed and sonicated with a bath sonicator for 45 seconds and split in half. Each half was transferred to a 40 mL aqueous solution of 0.75% PVA during homogenization at a speed of 15,000 rpm for 1 minute. The resulting MPs were transferred to a larger bath of PVA (80 mL at a concentration of 0.5%) while stirring at a speed of 990 rpm for 3.5 hours. The MPs were pelleted by centrifugation at a speed of 3300 RCF for 5 mins, and washed three times with milliQ water. The MPs were then lyophilized and stored at -20°C . To synthesize blank MPs, the same DMSO/TEA volume (115 μL) with no drug was mixed with PLGA solution in DCM. Dexamethasone-loaded MPs were made following the same protocol as described for levobunolol, except that PLGA (50/50) was dissolved in DCM at 68 mg/ml. In addition, 20 mg of dexamethasone was dissolved in DMSO at a concentration of 250 mg/mL and used as the drug solution.

2.3. Thermogel Preparation

To make the thermogels, the polymer solutions were initially dissolved at a higher concentration and adjusted to their final concentrations through the addition of drug molecules and diluent. Thus, the triblock copolymers with a final concentration of 20 % wt/vol were initially prepared in milliQ water at a concentration of 28.6 % wt/vol by shaking while cold ($2-8^{\circ}\text{C}$) for 3 days. The triblock copolymer solutions were then diluted to reach the intended polymer concentration by addition of excess water and 10X PBS. The volume of 10X PBS addition was chosen so that the final formulations were at 1X PBS concentration to minimize any osmotic pressure difference with the biological environment (isotonic concentration of 150 mM).

Three different hydrogel types were developed, namely, PLGA-PEG-PLGA, PLA-PEG-PLA and PLCL-PEG-PLCL hydrogels by blending different triblock copolymers. The ideal triblock copolymer solution is a solution at room temperature ($20-25^{\circ}\text{C}$) to ensure injectability and forms a hydrogel network at body temperature (37°C). To achieve this, different polymer solutions need to be blended so that the gelation temperature was approximately 37°C . The PLGA-PEG-PLGA was a 3/1 blend of AK91 and AK12 triblock copolymer solutions. The PLA-PEG-PLA hydrogel was made with AK100 triblock copolymer solution only. The PLCL-PEG-PLCL hydrogels were a 6/1 blend of AK108/ AK109 triblock copolymer solutions. By blending different amounts of triblock copolymers, the gelation temperature could be finely tuned.

2.4. Thermogel characterization

To characterize the hydrogels, two different schemes were implemented, a qualitative vial inversion test to determine the gelation temperature for each triblock copolymer solution and quantitative rheological measurements to determine the mechanical strength of the hydrogel at different temperatures. To determine the phase diagram, copolymer solutions at different concentrations of 10, 15, 20, and 25 % wt/vol were incubated at each set temperature point for 15 minutes. Subsequently, the vials were placed upside-down for 30 seconds and the triblock copolymer solutions were visually inspected. If a polymer solution was not able to flow during this time, it was considered a gel. Otherwise, it was a liquid, with or without a precipitate.

Rheological experiments were performed using an ARES-G2 rheometer (TA Instruments, New Castle, DE) with a stainless steel rheometer plate with a diameter of 40 mm. This rheometer plate with a large diameter was chosen to maximize the torque signal generated by the triblock copolymer solution and enhance the accuracy of the data. Rheological experiments were performed with a temperature step of 3 °C. At each specific temperature, a strain sweep experiment was done at a frequency of 0.1 Hz to determine the linear viscoelastic region of the material. Afterwards, a frequency sweep was performed in the linear viscoelastic region to determine the values of moduli at different frequencies. The elastic modulus (G') is a measure of elasticity of the material (solid like behavior), while the viscous modulus (G'') is a measure of viscosity of the material (viscous liquid like behavior).

2.5. Drug release studies

For the moxifloxacin, fast release of the drug within a week was desired, and as a result 500 µg of moxifloxacin was directly added to the hydrogel network without encapsulation into particles. To do so, moxifloxacin solution at 12.5 mg/mL was prepared and 40 µL was added to the triblock copolymer solution. A longer duration of release is desired for dexamethasone and levobunolol. In addition, for each of these two drug molecules a different release profile is required. For dexamethasone, the amount of drug release should be high initially and gradually decrease over time. However, levobunolol release should be low initially and increase later on to suppress any ocular pressure increase in the postoperative treatment period. To achieve this goal, these small drug molecules were encapsulated in MPs first and then the MPs were loaded in the hydrogel network. This provides two barriers against premature escape of the drug molecules. Unless noted otherwise, 7 mg of dexamethasone-loaded MPs and 17 mg of levobunolol-loaded MPs were loaded into the hydrogel network for drug release studies. As mentioned in the introduction, the mechanism behind hydrogel formation with triblock copolymers is the creation of associated micellar structures with hydrophobic cores (PLCL, PLA or PLGA) and hydrophilic shields (PEG). Having PLGA MPs in between micelles as external objects could decrease their ability to self-associate and create a hydrogel network. However, the amount of MPs added to the hydrogel network in this study was intentionally set low to not interfere with the gelation of the hydrogel network.

Hydrogels were made with 200 µL triblock copolymer solution and were kept in a 1.5 mL centrifuge tube. After incubation of hydrogel at 37 °C for half an hour, 1.2 mL of 37 °C

ionic buffer (PBS at normal isotonic concentration) was poured on it as the release media and the drug release was initiated. One mL of PBS was replaced with fresh PBS at each time point to simulate that the hydrogel was exposed to an infinite-volume bath to release the drugs and to determine the released amount over time. This was done to ensure that the obtained *in vitro* drug release results are close to *in vivo* outcomes. In addition, all drug-releasing samples were shaken in a 37 °C incubator to simulate mixing in the physiological environment.

To assess the effectiveness of hydrogels in sustaining the release of drugs, the drug release from the MP-loaded hydrogels was compared with that of MPs alone. To do so, drug release from MPs was studied by incubating the same amount of MPs (7 mg of dexamethasone-loaded MPs and 17 mg of levobunolol-loaded MPs) in 1.2 mL 1X PBS. At release timepoints, the tubes were centrifuged at 3000 RCF and 1 mL of PBS was replaced with fresh PBS.

In addition to drug-releasing hydrogels and MPs, three hydrogel samples representing PLGA-PEG-PLGA, PLA-PEG-PLA, and PLCL-PEG-PLCL hydrogels loaded with blank MPs as well as a blank MP sample without hydrogel were made and incubated to serve as the negative controls and any small background signal from them was subtracted from that of drug-releasing samples.

In the clinical application, the formulation is intended to be injected in the anterior chamber or vitreous cavity of the eye. Depending on the species, the temperature of different parts of the eye could range from 33.7 to 37.7 °C [47, 48]. The temperature at which the release experiments were performed (37 °C) was within the range of eye temperature. Small temperature variations within the biological temperature range are not expected to alter the obtained results significantly.

2.6. Drug loading and release characterization

To determine the loading of dexamethasone and levobunolol, PLGA encapsulating the drug molecules in MPs was degraded by incubation of particles in a basic environment (1M NaOH) for 15 minutes at 37 °C. The resulting solutions were neutralized by addition of acid (1M HCl) and lyophilized. Next, the dried drug molecules were dissolved in DMSO, diluted by addition of excess methanol (DMSO/methanol: 1/10 volumetric ratio), centrifuged at a speed of 4,000 RCF for 5 min and the supernatants were analyzed with high performance liquid chromatography (HPLC) in order to quantify the amount of drug loaded in the MPs. Loading was determined based on standard curves for each drug molecule with zero intercept. In this regard, dexamethasone was dissolved in water while levobunolol already in DMSO after deprotonation was diluted by addition of excess water. The drug solutions were turned into 1M basic environment by addition of the same base volume at 2M concentration. Subsequently, the drug solutions were incubated at 37 °C for 15 minutes (to simulate the steps taken to measure drug loading) and were subsequently neutralized by addition of acid (1M HCl). The samples were lyophilized, and reconstituted in DMSO/Methanol (1/10 volumetric ratio), and analyzed with HPLC to determine the standard curve for drug loading.

The release media taken from hydrogels or MP-containing samples were lyophilized and drug molecules were reconstituted in DMSO/methanol (at 1/10 volumetric ratio) to match the solvent system used to determine drug loading. The resulting solution was centrifuged at 21,000 RCF and supernatant was analyzed with HPLC to quantify the amount of released drug. To determine the standard curve for the analysis of release samples, moxifloxacin and dexamethasone were dissolved in PBS. Due to being in DMSO after deprotonation, a small volume of levobunolol solution was diluted with excess PBS. Next, the drug solutions in PBS at different concentrations were incubated at 37 °C for 24 hrs, lyophilized and reconstituted in DMSO/methanol (1/10 volumetric ratio) and analyzed with HPLC to determine the standard curve for drug release with zero intercept.

The mobile phase for HPLC was composed of a mixture of acetonitrile (20%) and water (80%) for the first 9.5 minutes to elute levobunolol and moxifloxacin. Then a linear compositional ramp was induced and the proportion of acetonitrile was raised to 34% in 1 minute. The acetonitrile was then kept at 34% for 6.5 minutes to elute dexamethasone, which was a more hydrophobic compound than the other drugs studied. Levobunolol, moxifloxacin and dexamethasone signals were read at 221, 295 and 240 nm, respectively. The drug content was always checked with its UV absorption spectra to distinguish drug content from baseline oscillations. The flow rate of solvent was set to 1 mL/min, and injection volume of the drug solution was 25 µL. To determine the percent of drug release, drug release at each time point is divided by the maximum detected drug release from the hydrogel.

2.7. Scanning electron microscopy

To determine the relative size of MPs and their surface morphology, a LEO/Zeiss scanning electron microscope (SEM) was utilized. MPs were deposited on a SEM mount, and were coated with a thin (<20 nm) layer of gold-palladium during 2 minutes deposition at a pressure of 200 mTorr. The operating voltage was set to 1kV to minimize sample damage during microscopy. At least four SEM micrographs from different parts of the samples at a magnification of 1000X were taken and the results shown are chosen to be representatives.

2.8. Statistical Methods

Error bars represent the standard deviations between replicate measurements. To compare groups across time points, Two-way ANOVA statistical tests were performed with Bonferroni's multiple-comparison post-tests and an alpha value of 0.05. In the case of p lower than 0.05, differences were considered to be statistically significant. (In this manuscript, *, **, and *** all are used as symbols that refer to $p < 0.05$)

3. Results and Discussion

3.1. Particle characterization

Figure 2 illustrates the SEM micrographs taken from the MPs loaded with levobunolol or dexamethasone in this research. This figure confirms the presence of spherical particles after loading different drug molecules and using different polymer types. The mean particle size and the standard distribution were calculated by analyzing different regions of each image

with ImageJ software with total of 150 particles analyzed in each image. Table 1 specifies the biophysical particle properties including number and volume based size as well as surface charge (zeta potential). In addition, histograms depicting size distribution of the particles are depicted in the Appendix A, Supplemental Information. The particle size distribution is somewhat polydisperse, which may have been helpful to enable the release profiles observed. Due to their higher surface area per mass, smaller particles lead to faster drug release [49]. Having a mixture of large and small particles leads to an average release behavior in between the two particle sizes. The particle sizes fabricated in this study enabled the multi-drug release behavior that met our design requirements. Optimal drug concentrations for encapsulation were chosen to be 68 mg/mL for dexamethasone and 61 for levobunolol to achieve high drug loading with acceptable particle morphology. Table 1 demonstrates that the PLGA MPs had a slightly negative zeta potential, presumably due to the carboxylic acid terminal groups of the polymer.

The final loading of dexamethasone in MPs was found to be 3.2 $\mu\text{g}/\text{mg}$ of MPs. In addition, the loadings of deprotonated levobunolol in MPs synthesized with 60/40, 75/25, and 85/15 PLGA were determined to be 6.9, 6.4, and 3.4 $\mu\text{g}/\text{mg}$ of MPs, respectively. With increasing hydrophobicity of PLGA, the encapsulation of levobunolol in MPs is decreased. The loading of levobunolol HCl in MPs made with 75/25 PLGA was less than with the deprotonated version and was found to be 2.5 $\mu\text{g}/\text{mg}$ of MPs. As levobunolol HCl is relatively hydrophilic (water solubility: 0.25 mg/mL [50]), it is not as easily encapsulated in hydrophobic PLGA as deprotonated levobunolol. Overall, the encapsulation efficiency of dexamethasone and levobunolol in MPs using this approach was low. This was mainly due to low solubility of the drug molecules in DCM and the use of DMSO to dissolve the drug molecules, allowing some escape of the drug molecules from the PLGA/DCM phase into the water phase.

3.2. Hydrogel characterization

Figure 3 illustrates the phase diagram for different triblock copolymers used in this study. All the formulations tested were at 1X PBS concentration.

For PLGA-PEG-PLGA and PLCL-PEG-PLCL, two different triblock copolymers with different molecular weights and ratios of LA/GA, or CL/LA (as discussed in the Materials section) were blended to make sure that the resulting polymer solution was a liquid at room temperature and a gel at body temperature. PLA-PEG-PLA polymer solutions already possessed this property without blending. Figure 3 demonstrates this and shows that at lower temperatures, all of the polymer solutions are liquid. However, they change into a gel form at approximately body temperature. Figure 3 also indicates that at very high temperatures, the polymers precipitate out of solution and can no longer form a gel network. According to Figure 3, for each triblock copolymer concentration, there is a gelation temperature window. When the polymer concentration is increased, this gelation temperature window becomes wider. All of the release studies were performed at 20% wt/vol concentration. At this high polymer concentration, small temperature variations do not impact the gelation of thermoresponsive hydrogels significantly.

At very high polymer concentrations, there are enough micelles associated with each other that the triblock copolymer solution could form a hydrogel network at low temperatures,

including room temperature. On the other hand, at lower polymer concentrations, the gelation window is too narrow to control a phase change. As a result, the polymer concentration for all of the hydrogels subsequently evaluated was set to 20% wt/vol. Interestingly, PLCL-PEG-PLCL polymer solutions have a much wider gelation window at each concentration than do PLGA-PEG-PLGA and PLA-PEG-PLA polymer solutions.

Figure 4 depicts the variation of storage and loss moduli for different triblock copolymers blended in this study. The polymer solution was formulated at 20% wt/vol in 1X PBS. This figure shows that the resulting blend of triblock copolymers had a low value for both the storage and loss moduli at room temperature, indicating liquid-like behavior. However, with increasing temperature, the mechanical properties of the gels shift as gelation occurs and the moduli are maximized near body temperature (37 °C). As temperature is further increased, the polymer precipitates and the desirable mechanical properties decrease. Quantitative rheological observations support the qualitative observations depicted in phase diagrams. Figure 5 depicts the variations of the moduli with frequency at different temperatures for the PLCL-PEG-PLCL triblock copolymer solution. According to this figure, regardless of oscillation frequency, the moduli are maximum at body temperature.

3.3. Effect of deprotonation on levobunolol loading and release

For ocular hypotensives, daily release on the order of 1 µg is required to ensure effectiveness of the molecule in reducing the ocular pressure [51, 52]. Thus, a high loading of levobunolol in MPs is required to ensure the proper effect. To enhance the loading, levobunolol was deprotonated in the presence of the base TEA leading to 2.6-fold increase in its loading relative to the levobunolol HCl. To determine the effect of deprotonation on the drug release profile, two samples were made: one with 17 mg levobunolol-loaded MPs and the other with 17 mg of levobunolol HCl-loaded MPs both made with 75/25 PLGA. Both samples had 7 mg of dexamethasone-loaded MPs made with 50/50 PLGA added to them to check whether the presence of levobunolol would impact the release of dexamethasone. Drug release from these samples is compared in Figure 6. The reason for selecting 75/25 PLGA is to ensure the MPs have high enough drug loading and can release the drug molecules for extended period of time (more than a month). Polymers with higher hydrophobicity degrade more slowly and can enhance the drug release duration. However, as mentioned previously, such polymers lead to lower drug loading. 75/25 PLGA achieved a balance between these two competing factors. As a result, this polymer is used for subsequent experiments.

The percentage of encapsulated drug released over time is approximately the same with levobunolol and levobunolol HCl. However, as the result of the higher loading of deprotonated levobunolol in MPs, its daily and cumulative release are more than 2-fold higher than the sample with levobunolol HCl (Figure 6). Thus, deprotonation enhanced the daily release of levobunolol from the MPs. Figure 6 also shows that daily release of dexamethasone from the two samples is the same, indicating that the presence of levobunolol MPs, whether protonated or unprotonated, and whether at 2.5 µg/mg or 6.4 µg/mg loading, does not impact the dexamethasone release.

3.4. Effect of hydrogel type on sustaining drug release

Next, the effect of the hydrogel network on sustaining the drug release from MPs was determined by comparing the results of MPs in hydrogel with those for MPs alone. To do so, four samples were made: one containing 17 mg of deprotonated levobunolol MPs with 75/25 PLGA and 7 mg of dexamethasone loaded MPs with 50/50 PLGA. In addition, three hydrogel samples composed of PLGA-PEG-PLGA or PLA-PEG-PLA or PLCL-PEG-PLCL hydrogel containing the same amount and type of microparticles plus 500 µg of moxifloxacin added directly to the hydrogel network.

Figure 7 compares the amount of moxifloxacin, levobunolol and dexamethasone released from each sample. Moxifloxacin is a hydrophilic drug molecule (water solubility: 24 mg/mL), and was added directly to the polymer solution, and upon hydrogel formation, it will not be held strongly within the hydrogel network. Its release profile as shown in Fig. 7A contains a high burst release followed by a decrease in daily drug release amount, with the majority of the drug being released within a week. This release profile is favorable for meeting clinical requirements to avoid infection [53]. Figure 7A also shows that the release profiles of moxifloxacin are generally similar between the different hydrogel types, although slightly less drug is released from PLA-PEG-PLA.

Release of levobunolol from MPs and different hydrogel types is shown in Fig. 7B. Comparing the MPs with PLCL-PEG-PLCL hydrogels one discerns that the levobunolol release is controlled by the MP formulation and the presence of the surrounding hydrogel does not influence the drug's release profile. On the other hand, as shown in Fig. 7C, release of dexamethasone from MPs is considerably slowed due to the surrounding PLCL-PEG-PLCL hydrogel compared to release from MPs alone (statistically significant for all time points). Evident in Fig. 7C is the large burst release of dexamethasone from the MPs with no hydrogel on day 1, which does not occur when the hydrogel is present, and this burst could lead to undesirable side effects. Drug molecules that are attached to or near the surface of MPs are presumably the main reason for the observed burst release [54]. Also, for MPs alone without hydrogel, dexamethasone is released within the first 5 days, without a prolonged sustained release. However, for the MPs loaded in the hydrogel, burst drug release is eliminated and daily release of dexamethasone is approximately constant for 10 days. Beyond this point, drug release is sustained with a gradual decrease in daily drug released over time (which would be generally favorable for the clinical application). Dexamethasone release from MPs alone resulted in minimal release past day 8, while for MPs in the hydrogel, the drug release was sustained for 30 days. The hydrogel without any MPs cannot sustain the release of drug molecules for an extended period of time. Drug release is directly affected by diffusion of drug molecules and due to large pore size of the hydrogel (~50–100 µm [39]) and small size of the drug molecules (~1 nm), the hydrogel is unable to ensure long-term sustained release of drug molecules on its own. Sequestering the drug molecules within MPs is necessary to sustain their release for an extended period of time. PLGA, PLA or PLCL polymers used in the hydrogel could entangle at the hydrogel/particle interface, which may further delay the release of drug molecules. Polymeric microparticles together within the hydrogel can also avoid premature drug leakage.

Throughout the 30 days, daily release of dexamethasone from the MP-hydrogel is in a range that could have a therapeutically significant effect in accordance with literature. Notably, the amount of dexamethasone released to the vitreous cavity by implants should be between 0.2–1.2 µg daily initially, and the amount of drug should decrease gradually over time for postoperative management following cataract surgeries [55].

The sustained dexamethasone release from the hydrogel is due in part to the chemical interactions between the drug, surrounding water, and the hydrogel. Dexamethasone contains a fluorine group, three hydroxyl groups, and two double bonded oxygen groups that could form hydrogen bonds and it is also quite hydrophobic, increasing its interactions with the hydrogel. On the other hand, levobunolol, with reduced hydrophobicity, hydrogen bonding groups, and molecular mass, can more easily diffuse out of the hydrogel network.

According to Figures 7B (levobunolol) and 7C (dexamethasone), drug release from PLGA-PEG-PLGA hydrogels is faster than from PLA-PEG-PLA or PLCL-PEG-PLCL for the first three weeks. This trend is more noticeable for levobunolol (statistically significant from 5 t 25 days for levobunolol compared to 5 t 15 days for dexamethasone). Compared with release from microparticles alone, it seems that the PLGA-PEG-PLGA hydrogels containing the same MPs have an accelerated release of levobunolol (Fig. 7B). This observation may be due to hydrolysis and cleavage of ester bonds of PLGA in PLGA-PEG-PLGA hydrogels, generating autocatalytic acidic degradation products that can make the microenvironment acidic, leading to faster degradation of microparticles that are embedded in the hydrogel network [56]. Acidic degradation products are also formed by the degradation of PLA-PEG-PLA and PLCL-PEG-PLCL hydrogels, but due to their higher hydrophobicity and slower kinetic degradation rate, those hydrogels are less efficient at creating an acidic microenvironment and thus their impact on acceleration of drug release is less pronounced.

3.5. Use of polymer type to fine-tune the drug release profile

Depending on the specific disease model and its progression, ophthalmologists might be interested in having different release durations of a specific drug molecule. To do so, the drug delivery platform should have the ability to finely tune the drug release profile. One way to achieve this goal is to vary the type of polymer encapsulating the drug molecule. To demonstrate this, deprotonated levobunolol loaded MPs were fabricated with PLGA with differing chemical compositions through the variation of LA/GA molar ratios to be 60/40, 75/25, and 85/15. A higher ratio of lactic to glycolic acid leads to greater hydrophobicity and slower degradation rate. Three hydrogels were prepared each containing 17 mg levobunolol-loaded MPs with different PLGA polymer types mentioned above, plus 7 mg dexamethasone-loaded MPs and 500 µg moxifloxacin. PLCL-PEG-PLCL hydrogels were used for this experiment. Figure 8 depicts the levobunolol release profile from these hydrogels. Evident in this figure is the ability of the drug delivery platform to finely tune the drug release profile by simple variation of the polymer chemical composition used to encapsulate the drug molecule. PLGA with a LA/GA ratio of 60/40 degrades faster and thus enables more rapid and approximately linear drug release kinetics. For this polymer, almost all levobunolol release happens within 25 days. On the other hand, 85/15 PLGA is the most hydrophobic polymer and releases the drug molecule more slowly and sustains the drug

release for 60 days. This polymer demonstrates a non-linear release rate with an increase in the drug release rate on the fourth week of drug release. Drug release from PLGA MPs is governed by the diffusion of water into the hydrophobic polymer matrix, the chemical kinetics of the ester linkages breaking (which can be autocatalyzed by its own degradation), and the diffusion of the drug out of the polymer. Drug release typically starts with burst release of drugs that are attached to the surface, and is followed by the intermediate stage which is governed by degradation of polymer and an increase in pore size, and lastly the drug release is finished by the loss of majority of polymer due to erosion [54]. Degradation and erosion enhance the network pore size and increase the diffusion of drug molecule and its release. Figure 8 clearly demonstrates that levobunolol does not show a strong initial burst release possibly because there are few drug molecules attached to the surface of the MPs. 85/15 PLGA is more hydrophobic than 60/40 and 75/25, and so it takes this polymer much longer to degrade, leading to a slower release of levobunolol initially (statistically significant for $t \leq 5$ days). After around three weeks, degradation of PLGA together with its bulk erosion and likely autocatalysis caused by an increase in acidity in the microparticle microenvironment can have a synergistic effect and lead to a boost in the rate of release of levobunolol.

Drug release from 75/25 PLGA is similar in profile to 60/40, but slower (statistically significant for $12 \leq t \leq 25$ days), with sustained release to 36 days. During the postoperative treatment period, elevation of ocular pressure as a side effect of steroids could happen during the second week for the patients [7, 57]. Therefore, it is highly desirable to have a high rate of release of levobunolol over this time window for the proper postoperative management of ocular surgeries. It should be noted that unlike chronic glaucoma, such as primary-open angle glaucoma, which requires lifetime treatment, postoperative glaucoma secondary to inflammation and/or steroid use is a transient condition. This postoperative change in ocular pressure is treated typically for 1–2 months only with ocular hypotensives and does not require chronic management. As a result, 75/25 PLGA has a delivery profile that is compatible with treatment for this indication.

3.6. Varying the microparticle or moxifloxacin mass to tune the daily drug release

In the previous section, the ability to finely tune the drug release profile by varying the type of polymer encapsulating levobunolol was highlighted. Another flexibility of the presented drug delivery system is the freedom to vary the mass of dexamethasone or levobunolol loaded MPs or moxifloxacin encapsulated in the same amount of hydrogel to change the daily drug release while keeping the overall release profile approximately the same. To illustrate this, two PLCL-PEG-PLCL hydrogels were synthesized. In the first hydrogel group, “full drug dosage,” contained 17 mg deprotonated levobunolol loaded MPs made with 75/25 PLGA, 7 mg dexamethasone loaded MPs made with 50/50 PLGA and 500 μ g moxifloxacin. The second hydrogel, “half drug dosage,” contained half of the MPs and moxifloxacin masses in the hydrogel. The drug release kinetics is shown in Figure 9. As can be seen in this figure, by decreasing the amount of moxifloxacin dissolved in the triblock copolymer solution or by changing the mass of levobunolol or dexamethasone loaded MPs embedded in the hydrogel, one can vary the daily drug release content of each drug

proportionally while maintaining the shape of the drug release profiles constant (The majority of timepoints were statistically significant).

3.7. Multi-drug delivery hydrogel

Figure 10 depicts the variation in percent drug release over time for three different drug molecules loaded in the multi-drug delivery platform. The present drug delivery system is designed such that two drug molecules are encapsulated in MPs and the third one is directly added to the hydrogel matrix. This specific design allows the drug delivery system to release three different drug molecules of different hydrophobicities over different durations chosen according to the application. In addition, the current design minimizes any possible drug-drug interactions which can be a common concern with multidrug delivery implants. The polymer-drug interactions with the hydrogel itself, likely through hydrogen bonding, help to slow release of drugs from the overall system. To achieve a multidrug delivery system capable of releasing desired drug molecules at desired time and rates, pilot studies were conducted which can be found in Appendix B in Supplemental Information.

Among the drug molecules chosen for this study, moxifloxacin was the most hydrophilic (water solubility: 24 mg/ml) and dexamethasone was the most hydrophobic drug (water solubility: <100 µg/mL [58]). Drug release duration and amount can be adjusted on demand through the direct addition of drug to the polymer network or encapsulating the drug molecules in MPs and embedding the MPs in the hydrogel network. While one can achieve linear release from the start of treatment (e.g. 60/40 PLGA), by utilizing a hydrophobic polymer (85/15 PLGA) one can also attain minimal drug release over early time points followed by rapid enhancement in drug release at later times.

As discussed in the introduction, several studies demonstrated that sustaining the release of drug molecules to at least a month using A-B-A triblock copolymers is quite challenging [38–40]. This trend holds regardless of the size of the therapeutic agent, as these hydrogels were not able to prolong the release of molecules as large as 150 kDa [39]. In addition, while delivering multiple drug molecules is required for several ocular disorders [2, 14, 43], previous literature has predominantly focused on single agent delivery with A-B-A triblock copolymers. In this research, a thermoresponsive hydrogel capable of releasing multiple drug molecules at different drug release rates and profiles was developed (Fig. 10). In addition, several means to finely tune the daily release and duration of drugs was illustrated by changing the type of polymer used to encapsulate the drug molecules and hydrogel as well as varying the mass of microparticles or drug molecules embedded in the hydrogel network. While an advantage of this system is its flexibility to tune the release of multiple small molecule drugs, a potential disadvantage is that formulation re-optimization is required if the type of drug or relative doses of the drugs significantly changes. The same features that enable multidrug release and precision in tuning the release rate and duration make the system more complex, which potentially could impact the clinical outcomes. Consistency of the product during scale up and manufacture would be important to minimize variability in the clinic that a multi-component product could cause.

Eye drops are the current standard of care for delivering the required drug molecules for the postoperative management following cataract surgery. However, their effectiveness is

hindered due to poor patient compliance [13, 16, 17]. Multi-drug thermoresponsive hydrogels developed in this research are easy to administer, form a depot for drug molecules at body temperature, and could enable the release of all of the drug molecules required for postoperative care of cataract surgery in accordance with clinical requirements [53, 55]. While this research demonstrates a proof-of-concept for multidrug release from thermoresponsive triblock co-polymers embedded with microparticles, additional preclinical experiments are required to evaluate the system before any clinical testing. The efficiency and safety of the developed drug delivery system for postoperative treatment following ocular surgery should be evaluated *in vivo* in multiple animal models. Important safety considerations to evaluate include no toxicity, no induction of inflammation, no increase in IOP, no wide dispersion of particles throughout the eye, and continued safety as the drug delivery system biodegrades. Incorporation of other types of anti-inflammatory (e.g. nonsteroidal anti-inflammatory drugs (NSAIDs) or immunomodulators) and ocular hypotensive drugs in the formulation should be pursued to compare their relative efficacy. Finally, the ability of the hydrogel formulation to maintain its integrity as a depot that has high safety and does not obstruct vision should also be ascertained. Following these preclinical studies, this delivery system could move from proof-of-concept to clinical candidate.

4. Conclusion

The present research reports a hydrogel-based multi-drug delivery platform that can enable postoperative management following cataract surgery. The drug delivery vehicle is a liquid at room temperature and forms a gel at body temperature, facilitating delivery via a simple intraocular injection. The delivery system releases an antibiotic, a steroid, and an ocular hypotensive, each with its own time profile and dosage tailored to clinical requirements. The antibiotic was added directly to the hydrogel network to ensure its fast release, while the steroid and ocular hypotensive were loaded in microparticles which were embedded in the hydrogel network to prolong their release duration. The complete system uses only biodegradable components.

PLCL-PEG-PLCL hydrogels were found optimal thermogels as they lead to the slowest and most linear drug release profile. The optimized PLCL-PEG-PLCL hydrogel was also able to eliminate the burst release of dexamethasone from MPs and prolong its release duration. We determined that by deprotonating the levobunolol hydrochloride, its loading into this delivery system and its daily release was enhanced considerably. The release rate of the ocular hypotensive was achieved to be slow in the beginning and to increase at later timepoints to reduce the likely elevated ocular pressure that can occur following the use of steroids. The drug dosages in this system were demonstrated to be tunable without altering the drug release profiles of the three components. This easily injectable multi-drug depot is promising for the release of multiple small molecule drugs to treat a local disease over multiple time scales, such as for postoperative treatment following ocular surgery.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

A) Overview of drug delivery platform to enable delivery of three different drug molecules. A broad-spectrum antibiotic (A) is added directly to the hydrogel network, while a potent corticosteroid (C) and ocular hypotensive agent (H) are encapsulated within microparticles and thereafter loaded into the hydrogel. B) The thermosensitive hydrogels are engineered to be liquids at room temperature, C) while they form a hydrogel network at body temperature.

Fig. 2. SEM micrographs of A) levobunolol HCl-loaded MPs with 75/25 (lactic/glycolic acid ratio) PLGA, deprotonated levobunolol-loaded MPs with B) 60/40 PLGA, C) 75/25 PLGA, D) 85/15 PLGA and E) dexamethasone-loaded MPs with 50/50 PLGA. All of the images were taken at a magnification of 1000, and the scale bar shows a length of 10 μm . The scale bar shows a distance of 10 μm .

Fig. 3.

Phase diagrams for different triblock copolymers used in this research, A) PLGA-PEG-PLGA, B) PLA-PEG-PLA, C) PLCL-PEG-PLCL. The PLGA-PEG-PLGA consisted of a 3/1 mixture of AK91 and AK12. The PLA-PEG-PLA was comprised of AK100 alone. The PLCL-PEG-PLCL consisted of a 6/1 combination of AK108 and AK109.

Fig. 4. Rheological results for different triblock copolymers; a) Storage modulus, b) Loss modulus. Results were obtained at 1Hz oscillation frequency. The PLGA-PEG-PLGA triblock copolymer was a 3/1 blend of AK91 and AK12. The PLA-PEG-PLA contained AK100 only. The PLCL-PEG-PLCL was a 6/1 blend of AK108 and AK109.

Fig. 5. Variations of A) storage and B) loss moduli with oscillation frequency at different temperatures for PLCL-PEG-PLCL polymer solutions. This polymer solution was a 6/1 mixture of AK108 and AK109.

Fig. 6.

A) Comparison of release of levobunolol with levobunolol HCl from MPs. For $t > 1$ day, all timepoints were statistically significant ($p < 0.05$). All the data points have error bars graphed for standard deviation, but many of the error bars are too small to be clearly visible. B) Release of dexamethasone from samples containing different levobunolol types. In this and following figures, mean $\pm$ standard deviation of duplicate measurements are plotted.

Fig. 7. Drug release kinetics for different drugs in different types of hydrogels, A) Moxifloxacin, B) Levobunolol and C) Dexamethasone compared to MPs alone. Data points are the average between duplicate measurements and error bars represent standard deviation. PLGA, PEG, PLA, and PLCL stand for poly(lactide-co-glycolide), poly(ethylene glycol), poly(lactic acid), and poly(lactide-co-caprolactone), respectively. The signs * and ** indicate statistical significance of PLGA-PEG-PLGA and microparticles alone relative to PLCL-PEG-PLCL group, respectively ($p < 0.05$).

Fig. 8. Effect of polymer composition used in constructing PLGA microparticles that encapsulate levobunolol on the release rate of levobunolol from PLGA MPs loaded in PLCL-PEG-PLCL hydrogels. The symbols * and ** illustrate statistical significance of 60/40 and 85/15 PLGA relative to 75/25 PLGA, respectively ($p < 0.05$).

Fig. 9. Comparison of A) mofloxacin, B) levobunolol and C) dexamethasone release profiles when the loaded drug content is decreased by a factor of 2.

Fig. 10.

An injectable multi-drug delivery PLCL-PEG-PLCL hydrogel encapsulating PLGA microparticles is capable of releasing three different drug molecules at three differing defined release rates. The signs *, **, and *** demonstrate statistical significance of moxifloxacin, 75/25 PLGA and dexamethasone relative to 85/15 PLGA, respectively ($p < 0.05$).

Table 1.

Characteristics of the synthesized microparticles.

	Number based-Particle Size (μm)	Volume Based-Particle Size (μm)	Drug Loading ($\mu\text{g}/\text{mg}$ of MPs)	Encapsulation Efficiency (%)	Zeta Potential (mV)
Levobunolol HCl 75/25 PLGA	1.3 $\pm$ 0.8	1.7 $\pm$ 2.1	2.5	2.3	-1.9 $\pm$ 0.2
Deprotonated Levobunolol 60/40 PLGA	5.3 $\pm$ 3.0	6.7 $\pm$ 7.7	6.9	6.4	-2.0 $\pm$ 0.3
Deprotonated Levobunolol 75/25 PLGA	2.3 $\pm$ 1.8	3.4 $\pm$ 4.0	6.4	6.0	-2.0 $\pm$ 0.1
Deprotonated Levobunolol 85/15 PLGA	2.7 $\pm$ 2.3	4.3 $\pm$ 5.8	3.4	3.2	-1.9 $\pm$ 0.2
Dexamethasone 50/50 PLGA	2.8 $\pm$ 1.0	3.1 $\pm$ 3.1	3.2	1.6	-1.9 $\pm$ 0.3