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Phenylboronic acid modified mucoadhesive nanoparticle drug carriers facilitate weekly treatment of experimentally-induced dry eye syndrome

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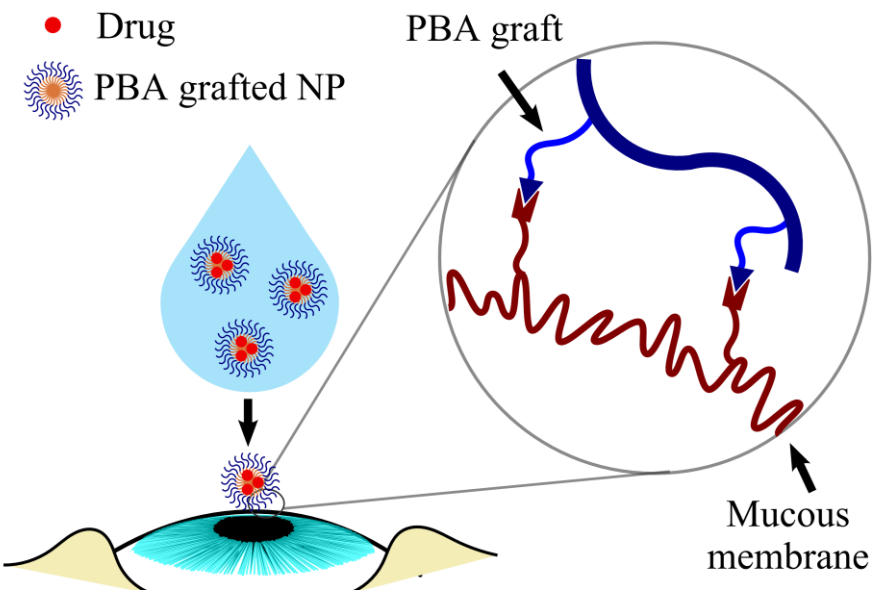

Nanoparticles self-assembled from poly(D,L-lactide)-b-Dextran (PLA-Dex) block copolymers and surface functionalized with phenylboronic acid were developed as mucoadhesive ocular drug delivery system. The *in vivo* results showed that the formulation may significantly reduce the administration dosage in effective treatment of experimental dry eye without causing adverse effects.

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ABSTRACT

Topical formulations, commonly applied for treatment of anterior eye diseases, require frequent administration due to rapid clearance from the ocular surface, typically through the lacrimal drainage system or through over-spillage onto the lids. We report on a mucoadhesive nanoparticle drug delivery system that may be used to prolong the precorneal residence time of encapsulated drugs. The nanoparticles were formed from self-assembly of block copolymers composed of poly(D,L-lactide) and Dextran. The enhanced mucoadhesion properties were achieved by surface functionalizing the nanoparticles with phenylboronic acid. The nanoparticles encapsulated up to 12 wt% of Cyclosporine A (CycA) and sustained the release for up to 5 days at a clinically relevant dose, which led us to explore the therapeutic efficacy of the formulation with reduced administration frequency. By administering CycA-loaded nanoparticles to dry eye-induced mice once a week, inflammatory infiltrates were eliminated and the ocular surface completely recovered. The same once a week dosage of the nanoparticles also showed no signs of physical irritation or inflammatory responses in acute (1 week) and chronic (12 weeks) studies in healthy rabbit eyes. These findings indicate that the nanoparticles may significantly reduce the frequency of administration for effective treatment of anterior eye diseases without causing ocular irritation.

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1. Introduction

Topical administration of an eye drop solution is the most commonly used drug delivery method for treating anterior eye diseases due to its non-invasiveness, low cost and ease of administration. Such a delivery method, however, suffers from low ocular bioavailability due to rapid clearance from the ocular surface through tear dilution and drainage through the lacrimal drainage system, resulting in less than 5% of the active drug reaching its intended target [1]. As a result, high doses of the formulations must be administered multiple times per day to achieve therapeutic efficacy, ultimately leading to a high risk of adverse effects and low patient compliance.

Much effort has been invested in improving topical formulations to overcome these shortcomings without compromising their benefits. One such approach includes the development of drug carriers developed from polymeric nanoparticles (NPs) [2]. By encapsulating therapeutic agents as their cargo, NP drug carriers enhance the solubility of drugs in water, control the release rates of these drugs, and improve the precorneal retention by targeting the ocular surface moieties [2-5]. NPs formulated from biodegradable polymers, such as poly(lactic-co-glycolic acid) (PLGA), have been studied in the delivery of ocular therapeutics to the corneal surface [6-10]. Researchers have also focused on adding poly(ethylene glycol) (PEG) on the surface of the NPs to improve the stability of the NPs in physiological environments as well as precorneal tear fluid [11-17]. In a previous study, we synthesized a Dextran based amphiphilic block copolymer, poly(D,L-lactide)-b-Dextran (PLA-b-Dex), that self-assembles into NPs with sizes ranging from 20 to 60 nm in diameter by tuning the molecular weights of PLA and Dex [18]. Dextran based NPs have shown colloidal stabilities superior to those of the PEG based NPs previously used [19]. Moreover, Dextran has abundant functional groups (i.e. OH groups) on its backbone that can be used for modification, as opposed to the single functional group on the end of PEG chains. As a result, the high density of surface functional groups in Dextran based NPs increases the efficiency of surface functionalization and consequently provides greater control over surface properties. In previous studies, researchers functionalized the surfaces of various NP drug carriers with ligands that can target the ocular mucosa to increase the precorneal retention time of the drugs [20-23]. The most common method of achieving mucoadhesive properties was through functionalizing the NPs with cationic polymers, such as chitosan, to take advantage of the electrostatic interaction between the cationic polymers and the negatively charged ocular mucin [24-28]. However, the electrostatic interaction may be partially impeded by the presence of ions in the tear fluid, resulting in the relatively rapid clearance of the drug carriers. Phenylboronic acid (PBA) molecules form a complex with *cis* diol groups of sugar residues, such as sialic acids, that are abundant on the mucin structures at physiological pH [29-34]. In addition, several studies have demonstrated biocompatibilities of PBA molecules using both *in vitro* and *in vivo* assays [34-36]. We hypothesize that the PBA functionalized PLA-b-Dex drug carriers will significantly reduce the required dosage of drugs and their administration frequency in treating anterior eye diseases by enhancing the precorneal retention of the encapsulated drugs.

The objective of this study is to develop a mucoadhesive nanoparticle drug delivery system that can reduce the administration frequency of ophthalmic drugs without compromising the therapeutic efficacy. Here, we report the formulation of PLA-b-Dex polymer nanoparticles with surfaces functionalized with PBA to achieve a mucosal-targeting drug delivery system. We

evaluated the ability of these nanoparticles to encapsulate Cyclosporine A (CycA), an immunosuppressant commonly administered for treating dry eye syndrome, and analyzed the subsequent release profile. We then tested the compatibility of these NPs by investigating both the acute and the chronic responses after administration on rabbit eyes. Finally, NP+CycA formulations with reduced administration frequencies were evaluated for treatment of experimental dry eye induced in mice.

2. Experimental

2.1. Materials

Acid-terminated poly(D,L-lactide) (PLA; Mw ~20) were purchased from Lakeshore Biomaterials (Birmingham, USA) and washed with methanol to remove monomer impurities. Dextran (Dex; Mw ~10 kDa), hydrochloric acid (HCl), triethylamine (TEA), *N*-(3-dimethylaminopropyl)-*N*-ethyl carbodiimide (EDC), 3-Aminophenylboronic acid monohydrate (PBA), sodium periodate ($NaIO_4$), glycerol, sodium cyanoborohydride ($NaCNBH_3$), and Cyclosporine A (CycA) were purchased from Sigma Aldrich (Oakville, Canada) N-Hydroxysulfosuccinimide (Sulfo-NHS) and N-Boc-ethylenediamine were purchased from CNH Technologies (Massachusetts, USA)

2.2. Synthesis of PLA-b-Dex and surface modification with PBA

The conjugation of the PLA and Dex polymer chains to form a block copolymer PLA-b-Dex and the surface modification of the PLA-b-Dex NPs with PBA were reported previously [18, 37]. Briefly, the aldehyde end group of the Dextran was conjugated with the *N*-Boc-ethylenediamine crosslinker through reductive amination with $NaCNBH_3$. After conjugation with the crosslinker, the Boc group was deprotected using HCl/TEA treatment. The deprotected amine end group is then conjugated with the carboxyl terminal group of the PLA using Sulfo-NHS and EDC as catalysts. The surface of these NPs were modified with PBA molecules by a two-step approach: the hydroxyl groups of the Dextran were oxidized to form more reactive aldehyde groups in the presence of NaIO4, and the aldehyde groups were conjugated with the amine groups of PBA molecules through reductive amination. The amount of PBA attached to the Dextran chain was quantified using UV-Vis absorption at 291 nm after obtaining the standard calibration of PBA in DMSO. The PLA-b-Dex polymers in DMSO were used as the baseline.

2.3. Characterization of PLA-b-Dex-g-PBA NPs

The NPs of PLA-b-Dex-g-PBA polymers were formed using nanoprecipitation. The sizes of the NPs were determined using Dynamic Light Scattering (DLS) by measuring the Multimode-Size Distribution (MSD) volume-averaged mean diameters using 90Plus Particle Size Analyzer (Brookhaven, λ = 659 nm at 90°). The zeta potential of the PLA-b-Dex and PLA-b-Dex-g-PBA nanoparticles were measured using Zetasizer Nano ZS (Malvern Instruments Worcestershire, U.K.). The sizes and morphology were further confirmed using Transmission Electron Microscopy (TEM) by drying the PLA-b-Dex-g-PBA NP suspension on 300 Mesh Formvar coated copper grids (Canemco and Marivac) and using phosphotungstic acid solution as the negative stain. The mucoadhesion of the PLA-b-Dex-g-PBA NPs were quantified using an *in vitro* periodic acid/Schiff (PAS) staining method described previously [37]. In brief, the NP suspension and the mucin solution were mixed and incubated at 37 °C for 1 hr. The mixture was then centrifuged and the free mucin in the supernatant was quantified using the PAS method. Mucin adsorption was calculated by subtracting the free

mucin from the initial mucin concentration. Mucin standards (0.1, 0.25, 0.5, and 0.75 mg/ml) were also used with the same procedure to obtain a calibration curve.

2.4. *In vitro* Cyclosporine A encapsulation and release

The encapsulation and the release profile of CycA with PLA-b-Dex-g-PBA NPs carriers were determined based on High-performance liquid chromatography (HPLC) quantification of the CycA [37]. In short, the NP+CycA were prepared using nanoprecipitation: 1 ml of DMSO containing 6.8 mg of PLA-b-Dex-g-PBA polymer and 3 mg of CycA (40 wt% of initial polymer) was added slowly in a drop-wise manner into 10 ml of Millipore water under mild stirring. After 30 minutes of stirring, the CycA and NP aggregates in the water suspension were removed using syringe filter (pore size = 200 nm), and the CycA that are freely suspended and loosely bound on the NP surface were removed using Amicon centrifugation units (MWCO = 10 kDa). The remaining CycA in the mixture was resuspended using acetonitrile (ACN) and quantified through HPLC (C18 HPLC column, ACN/H_2O 75:25 as the mobile phase, and UV-absorption detection at 210 nm) using a standard calibration.

The CycA release was measured by injecting 8 ml of syringe filtered NP+CycA suspension prepared using nanoprecipitation into a Slide-a-Lyzer Dialysis cassette (MWCO = 20 kDa, Fisher Scientific) and dialyzing against 200 ml of simulated tear fluid (0.436 g of $NaHCO_3$, 1.36 g of NaCl, 0.0126 g of $CaCl_2$, 0.276 g of KCl in 200 ml of Millipore water) at 37 °C. 1 ml of the release medium was extracted at each predetermined time points while 1 ml of fresh simulated tear fluid is added to the release medium. The CycA in the extracted medium was quantified similarly using HPLC. Note that the free CycA from the NP+CycA was not removed due to the irreversible aggregation caused by Amicon centrifugation. The resulting aggregated agglomerates could dramatically alter the drug release profile. Therefore, free CycA release study was performed using the same procedure to determine the total partition time of the CycA across the dialysis membrane.

2.5. Animal studies

All animal studies performed are in compliance with the guidelines of the Canadian Council on Animal Care (CCAC) and the University of Waterloo (UW) as well as regulations under the Animals for Research Act of Ontario Canada. Female New Zealand White Albino rabbits (2.5 – 3.5 kg, Charles River Laboratories, Canada) were used for both the acute and chronic ocular irritancy tests *in vivo*. Female C57BL/6 mice (Charles River Laboratories, Canada) aged around 6-8 months were used for experimental dry eye model. All animals were acclimated in the animal facility for at least one week prior to the experiments. All formulations used in this study were dialyzed, filtered, and sterilized prior to administration to the animals.

2.6. *In vivo* acute ocular irritancy test

The short-term biocompatibility of the NPs was assessed using ocular irritancy tests adapted and modified from previous studies [38, 39]. Three female rabbits were housed individually in cages on a standard laboratory diet. One eye of each of the rabbits was administered with 28 μl of the PLA-b-Dex-g-PBA NP formulation (containing about 17 μg of the NPs), while the contra-lateral eye was used as a control. Both eyes were observed using a slit lamp bio-microscope at 0 (before administration), 1, 8, 24, 48, 72, 96, 120, 144, and 168 hours after administration to examine the extent of irritancy on the ocular surface. Upon observation at

each time point, both eyes were graded using 7 categories: apparent discomfort, conjunctival redness and swelling, lid swelling, discharge, corneal opacification, and number of infiltrates from 0 (no sign) to 4 (severe) under the supervision of trained optometrists with experience working with animals [40, 41]. After 168 hrs, the rabbits were euthanized and the ocular tissues were extracted for histopathology.

2.7. *In vivo* chronic ocular irritancy test

The long-term biocompatibility of the blank NPs and the NPs with Cyclosporine A (NP+CycA) encapsulated were assessed using similar techniques described above at 0, 1, 24, and 48hr each week repeated for 12 weeks. Five female rabbits were used for blank NP irritancy test and four female rabbits were used for NP+CycA irritancy test. Similarly, one eye of each rabbit was administered with 28 μl of NP formulation (~17 μg of the PLA-b-Dex-g-PBA NPs) or 28 μl NP+CycA formulation (~17 μg of the PLA-b-Dex-g-PBA NPs and <8 μg of CycA (may also include some freely suspended CycA)), while the contralateral eye served as a control. Similarly, both eyes were examined under a slit lamp bio-microscope on the extent of irritancy based on the 7 categories described above. After 12 weeks, the rabbits were euthanized and the ocular tissues were extracted for histopathology.

2.8. Histopathology

The eyes were enucleated and collected immediately after euthanasia for histopathological evaluation by one of the authors (DH), who is a trained pathologist. The entire upper eyelids were also dissected and collected for evaluation of the tarsal conjunctiva and the underlying soft tissues. Consecutive sections of the entire ocular globe and eyelids were processed for microscopic analysis: after initial fixation in 10% neutral buffered formalin, the tissue was embedded in paraffin, serially sectioned into 5 μm thick sections, and stained with hematoxylin and eosin (H&E). The histological slides were evaluated using bright field microscopy (Leica DM1000, ICC50 HD, Leica Microsystems Inc, Canada).

2.9. Experimental dry eye model – *in vivo* efficacy test

The PLA-b-Dex-g-PBA NPs encapsulating CycA were analyzed for treating experimental dry eye syndrome in a mice model. First, the mice were induced with dry eye conditions using a previously reported method [42]. Transdermal scopolamine patches (Transderm-V, Novartis) were cut into two pieces each, wrapped around the midtails of the mice, and further secured using surgical tape. The patches were replaced every other day throughout the duration of the study. To simulate a desiccating environment, the mice cages (open-top) were placed in a fumehood for 1 hr, 3 times per day throughout the study (until day 12). After 4 days of this dry eye inducement procedure, the mice were divided into 7 different groups (Table 1) for various types of treatments on both eyes (3 mice per group; 6 eyes per group) for an additional 7 days. On day 1 (before dry eye inducement), 5 (before first administration), 8 (before second administration), and 12 (before euthanasia), tear volume measurements and corneal fluorescein staining were performed to measure the rate of tear production and analyze the ocular surface damage. Note that NP+CycA (1/wk), Saline (1/wk) was only administered on day 5, whereas NP+CycA (2/wk), Saline (2/wk), and Blank NPs were administered on days 5 and 8. Tear volume was measured by holding a phenol red dyed cotton thread (Zone-quick, White Ophthalmic Supply) with jeweler's forceps in the lateral canthus of the ocular surface for 30 seconds. Corneal fluorescein staining

was observed and photographed with a slit-lamp bio-microscope using a cobalt blue light 10 minutes after the administration of 1 μl of sodium fluorescein solution (10 mg/ml). After the 12 day period, the mice were euthanized and the ocular tissues were collected for histopathological analysis (as described in the previous section).

3. Results and Discussion

3.1. Characterization of PLA-b-Dex-g-PBA NPs

We functionalized the surface of PLA-b-Dex NPs, formed from nanoprecipitation, with the mucosal-targeting ligand PBA (Scheme 1). The characterization of the PBA functionalization, the CycA loading, and diameters of the NPs are summarized in Table 2. The amount of PBA density on the Dextran surface was 17.6 ± 2.7 mol% (mol of PBA/mol of Dextran monomers). NP diameter decreased from 45.8 nm to 28.6 nm likely due to the functionalization with PBA, and was also observed in a similar previous study [37]. The size decrease is possibly a result of the reduced hydrophilicity of the Dextran surface or the change in the packing density of the nanoparticles due to the volume change of the hydrophilic chains. We found that having excess PBA (i.e. more than 23 mol% PBA/Dextran) on the NP surface may cause the NPs to be too hydrophobic and thus compromise its colloidal stability in our previous study [37]. Therefore, PBA density must be tuned to balance between mucoadhesion and colloidal stability. Another possibility for decreased size may be due to the crosslinking of Dextran by PBA due to the boronate complexation. PBA can covalently bind to the *cis*-diol groups of carbohydrates, and such reaction could not only lead to aggregation between nanoparticles, but also decrease nanoparticle mucoadhesion affinity with the precorneal tear film. Previous studies have reported that this complexation occurs preferentially at pH above the pKa of the PBA (around 9), but such complexation is unstable at physiological pH [34, 36, 43, 44]. We also performed an independent study where we incubated Dextran and PBA (Dextran monomer: PBA = 1:1 molar ratio) in Millipore water. After removing the unbound PBA using dialysis, there was negligible amount of PBA remaining, analyzed with UV-vis. These studies suggest that the complexation between Dextran and PBA is unfavourable at physiological pH of the ocular surface and at neutral pH of the nanoparticle formulation. The NPs exhibited a spherical morphology as observed in TEM imaging (Figure 1). The zeta potential of the PLA-b-Dex NPs is close to 0, but when PBA is grafted onto the surface, the potential has dropped to near – 30 mV (Table 2). The pKa of the PBA molecules are near 8.6 [45], thus at pH ~7.4 (at which the measurements were taken) some percentage of the PBA molecules would be in their deprotonated state, contributing to the overall negative surface charges of the nanoparticles; similar range of zeta potentials were measured on nanoparticles with PBA molecules on the surface previously [46]. The PBA functionalized NPs showed *in vitro* mucoadhesion properties, resulting in mucin binding of 1.18 ± 0.02 mg/mg of NPs, which is much higher than the values obtained for other types of mucoadhesive NPs such as chitosan based NPs (~ 0.25 mg/mg of NPs) and thiolated NPs (~ 0.13 mg/mg of NPs) [47]. From these preliminary findings we postulate that the PBA functionalized NPs may exhibit strong binding affinity toward the ocular mucosa under physiological environment

3.2. *In vitro* Cyclosporine A encapsulation and release

We evaluated the encapsulation efficiency and the release profile of CycA in the PLA-b-Dex-g-PBA NPs using HPLC. The NPs were able to encapsulate about 11.9 ± 1.6 wt% of CycA with respect to the weight of the NPs without drastically altering the

diameter of the particles (Table 2). The particle size increase is most likely due to the inclusion of the CycA in the PLA core of the NPs. Compared to the commercial product, RESTASIS, an equal volume of PLA-b-Dex-g-PBA formulation is able to encapsulate a comparable amount of CycA and the therapeutically effective dosage can be obtained by tuning the polymer and/or drug concentration in the formulation. The *in vitro* release study showed that the CycA release was maintained for up to 5 days (Figure S1 in the Electronic Supplementary Material (ESM)) which is significantly higher than other types of nanoparticles for topical ocular drug delivery [24, 48-52]. Two different phases were shown in the release profile depending on the release rate. This biphasic release profiles were often observed in literatures involving PLA based nanoparticle drug carriers [53, 54]. First phase showed a burst release in the first 12 hrs, most likely due to the freely suspended non-encapsulated CycA and also some CycA that may be loosely attached on the surface of the nanoparticles. Previous studies showed that there is very little association between Dextran and CycA [55], and that the majority of CycA partitions into the hydrophobic core of the NPs due to their low water solubility [56-58]. Thus, most of the burst release is most likely contributed by the release of freely suspended non-encapsulated CycA. The burst release of free drug was further confirmed by running a parallel drug release study using only Cyclosporine A to determine their partition time across the dialysis membrane. We used this as a reference to determine the total release of the free drug from the dialysis membrane, which was determined to be 12 hr in the case for CycA). The second phase of the release profile shows a relatively slow diffusional release of CycA from the PLA-b-Dex-g-PBA NPs after the initial burst release. Together with the enhanced mucoadhesion results, the CycA release profiles provide the potential for these NPs to be a long-lasting drug delivery platform administered with a reduced frequency compared to RESTASIS which requires twice daily administration.

3.3. *In vivo* acute ocular irritancy test

Acute responses after one-time administration of PLA-b-Dex-g-PBA NPs were observed using a slit-lamp bio-microscope by grading both the NP administered and contralateral control rabbit eyes for 7 categories over a week (Figure 2). We observed no corneal opacification and infiltrates in any of the eyes throughout the study. All of the other 5 categories—discomfort, conjunctival redness and swelling, lid swelling, and discharge—also showed no significant difference between the administered and the control eyes, similar to what has been observed from chitosan nanoparticles [38]. Most categories showed grades between 0 (no sign) and 1 (mild) with the exception of conjunctival redness. As both the NP administered and control eyes scored relatively higher grades (between 1 and 2) for conjunctival redness, it is highly probably that environmental stress underlies the high scores rather than the administration of NPs. From the slit-lamp examination alone, the NPs seem well-tolerated by the rabbits' eyes, but closer examination of the ocular tissues is necessary to further validate the findings.

After the slit-lamp examination for a week, the ocular tissues of the rabbits were collected for histopathology. Histopathological evaluation revealed the presence of normal ocular surface structures in both control and NP administered eyes (Figure 3). All eyes demonstrated preserved architecture and morphology in the anterior segment. Corneas of NP administered and control eyes displayed normal numbers of cell layers with appropriate morphology (Figure 3, top row). We observed no signs of inflammation, altered layer integrity, or the presence of residual particles in any of the eyes. Hyperplastic changes, hyperkeratosis,

or other alterations in the process of normal epithelial maturation and renewal were also not noted. The bulbar and tarsal conjunctiva of both NP administered and control eyes revealed findings within normal limits. NP administered and control eyes also showed adequate numbers of goblet cells with preserved morphology and abundant secretory products (Figure 3, middle and bottom rows). No differences were observed in the size or location of the conjunctiva-associated lymphoid tissues. Individual scattered lymphocytes and single polymorphonuclears and eosinophils were occasionally observed, predominantly in the tarsal conjunctival epithelium and stroma, in both administered and non-administered eyes (Figure 3, bottom row). These findings represent normal variations in the tissue characteristics of exposed mucosal membranes expected for healthy subjects. There were no inflammation, edema, residual particles, epithelial or vascular abnormalities. The ocular angle demonstrated usual architecture. Both the slit-lamp examination and histopathological evaluations suggest that the NP formulation causes no observable acute irritation on the rabbit eyes. We expected the appropriate compatibility of NPs, as these NPs were composed of a biodegradable polymer chain PLA and a natural polysaccharide Dextran, which have both shown *in vitro* and *in vivo* compatibilities in our previous study [18]. In addition, the biocompatible nature of PBA targeting ligands has also been demonstrated previously and has since been widely used in *in vivo* molecular targeting of sialic acids expressed on cell surfaces [59]. This study confirms that the combination of the biocompatible NPs surface functionalized with biocompatible PBA does not cause any short term adverse effects on the ocular surface of the rabbits.

3.4. *In vivo* chronic ocular irritancy test

In addition to the acute irritancy study, it is essential to analyze the chronic ocular response to the repeated administration of NPs since eye drops are often applied periodically for a prolonged duration of treatment. The chronic response on the ocular surface after weekly administration for NPs or NP+CycA for up to 12 weeks was also examined using a slit-lamp bio-microscope and the same grading system of 7 categories described above. No sign of conjunctival swelling, corneal opacification nor infiltrates were observed in any of the eyes throughout the 12 week study. From weekly administration of PLA-b-Dex-g-PBA NPs, discomfort, conjunctival redness, discharge and lid swelling levels were mostly in the mild region (between score of 0 and 1), with no significant deviation between the NP administered and contralateral control eyes (Figure 4). We also made similar observations after weekly administration of CycA encapsulated NPs (NP+CycA): we observed a slow increase of lid swelling near the end of the study (week 9 to week 12) to moderate scores of about 1.5 with no apparent deviation between the administered and non-administered eyes, suggesting the slow increase is likely due to environmental stress (Figure 5).

Similarly, after the 12 week study, all ocular tissues of the rabbits were collected for histopathological analysis. Histopathological evaluation showed all the signs representative of healthy eyes similar to the acute irritancy test above (Figure 6). Both NP and NP+CycA treated eyes and their corresponding contralateral control eyes demonstrated normal ocular surface structures and anterior eye segments with preserved morphology and architecture. All corneas displayed normal numbers of cell layers with appropriate morphology without any sign of inflammation, altered layer integrity, or presence of residual particles. Hyperplastic changes, hyperkeratosis, or other alterations in the process of normal epithelial maturation and renewal were also not noted. Bulbar and tarsal conjunctiva of all eyes revealed findings within normal limits: they all showed similar adequate numbers of goblet cells

with preserved morphology and abundant secretory products. No differences in the size or location of the conjunctiva-associated lymphoid tissues were observed. Individual scattered lymphocytes and single polymorphonuclears and eosinophils were occasionally observed predominantly in the tarsal conjunctival epithelium and stroma in all the eyes. No inflammation, edema, epithelial or vascular abnormalities were found in either set of rabbits.

The repeated dosage of NPs may increase the potential for adverse effects as their corneal exposure time is increased. The study showed that the NPs were well-tolerated for up to 12 weeks, thus demonstrating the long-term compatibility of the carrier system. It was also paramount to perform the same test on NP formulation with the drugs inside, since the drug encapsulating NPs may exhibit an increased diameter due to the increase in the hydrophobic core size, which may or may not lead to a varied ocular response. The current study demonstrated that the inclusion of the drug in the NPs did not significantly alter the ocular response. These two studies indicate that the NP formulation is a promising long term drug delivery system for treating anterior ocular diseases. However, it is not clear how much nanoparticles remain after each dosing of the formulation. Therefore, one of the ongoing studies is to investigate the dosage-dependency of the ocular compatibility of the nanoparticles by studying their ocular biodistribution at predetermined time intervals.

3.5. *In vivo* efficacy test – experimental dry eye

Experimental dry eye was induced in mice and 7 different treatments (Table 1) were applied to the mice groups to analyze the effect of the treatments against dry eye. Tear volume measurements on day 1 and 5 (before DE inducement and before 1st admin) suggest that the tear volumes were significantly reduced ($p < 0.05$) (Figure 7) as a result of the combination of application of anticholinergic drug, scopolamine, and the simulation of desiccating environment [42]. The dryness of the eyes were also observed in corneal fluorescein staining imaging to varying degrees (Figure S2, between the 1st and 2nd columns): after 4 days of dry eye inducement, less of the administered fluorescein was cleared from the ocular surface compared to day 1 possibly due to the surface damage caused from lack of surface lubrication and/or the reduced tear clearance rate. The healthy group maintained a normal tear volume production rate with minimal or no ocular surface damage, which resulted in clearance of the majority of the fluorescein within 10 mins (Figure S2). Saline (1/wk) and (2/wk) did not show any significant improvement in the tear volume production or the fluorescein clearance from day 5 to 12. NP+CycA (1/wk) and NP+CycA (2/wk) were the only groups that showed a slight increase in tear volume as well as improved fluorescein clearance on day 12 compared to day 5. RESTASIS, administered 3 times per day for 7 days, did not show any improvement in tear production on day 12 compared to day 5. Moreover, the fluorescein images show that less fluorescein was cleared on day 12 compared to day 5, which is most likely due to the ocular surface damage as a result of frequent administration. Blank NPs (2/wk), while showing a certain degree of improvement in terms of fluorescein clearance, were unable to clear as much fluorescein as those administered with NP+CycA (1/wk) and NP+CycA (2/wk) on day 12. The tear volume measurements suggest that NP+CycA formulations showed improved performance in reducing the symptoms of dry eye conditions, however, we note that none of the formulations were able to restore the tear volume production of day 1 by the end of this study (day 12). We postulate that it may be due to the fact that we are constantly inducing dry eye symptoms using anticholinergic agents and therefore it may outweigh the effects of the Cyclosporine A

treatment. Similar observations were made in a previous study where they showed that although the treatment of Cyclosporine A itself showed restored lacrimation but when treated alongside anticholinergic agents, the lacrimation was not restored [60]. Thus, closer examination of the immunosuppression, which is the main function of Cyclosporine A, is needed to evaluate the efficacy of the formulations.

Histopathological evaluation of mice in the healthy group showed bulbar and tarsal conjunctiva with stratified squamous epithelium, an adequate number of goblet cells with abundant secretory products, and complete lack of inflammation (Figure 8, A). The stroma showed occasional single lymphocytes and eosinophils within the normal range. The experimental dry eye treated with Saline (1/wk) and (2/wk) groups showed pronounced inflammatory changes ranging from focal mild infiltrates to severe inflammation (Figure 8, B and C). The inflammatory infiltrates, consisting predominantly of lymphocytes with occasional polymorphonuclears and eosinophils, were located in the bulbar and tarsal conjunctiva. The infiltrates involved the conjunctival epithelium as well as the subepithelial stroma of lamina propria. In addition, substantial areas demonstrated a markedly reduced number or complete lack of goblet cells, thereby significantly reducing the mucin secretion and eliminating its lubricating and surface protecting effects [61]. Similarly, mice with experimental dry eye treated with Blank NPs showed mixed inflammatory infiltrates composed of polymorphonuclears and lymphocytes, with occasional plasma cells and eosinophils (Figure 8, D). The bulbar and tarsal conjunctival epithelium both showed markedly reduced number of goblet cells. RESTASIS treatment demonstrated clearing of the intraepithelial and subepithelial inflammatory infiltrates (Figure 8, G). However, substantial residual areas showed complete lack of goblet cells, and goblet cells in isolated areas also exhibited altered morphology with scarce secretory products, which explains why the majority of the fluorescein was not cleared at the end of the treatment (day 12). Treatment with NP+CycA (1/wk) and (2/wk), like RESTASIS, also reversed the inflammatory processes in experimental dry eye induced mice (Figure 8, E and F). No residual particles, epithelial or vascular abnormalities were found. Individual scattered lymphocytes and single polymorphonuclears and eosinophils were observed predominantly in the tarsal conjunctival epithelium and stroma at levels similar to those found in Healthy group. The NP+CycA treated mice showed adequate number of goblet cells on the ocular surface with normal morphology and abundant secretory products.

Dry eye is characterized as tear deficiency caused by ocular surface inflammation that leads to injury to the ocular surface [61]. The histologic features of dry eye include abnormal proliferation of the ocular surface epithelium, different degrees of inflammatory changes, and decreased production of mucus, manifested by the reduction or absence of goblet cells on the ocular surface epithelium [42]. In all three studies, the tear volume measurement, fluorescein clearance, and histopathology, the Saline (1/wk) and (2/wk) showed no sign of mitigating the symptoms of dry eye conditions such as lowered tear production rate or ocular surface inflammation. This suggests that simple hydration of the ocular surface using saline, at the frequency of the administration specified, was not enough to reduce the inflammation nor recover the integrity of the ocular surface. Blank NP treatment, similar to saline treatment, showed lack of treatment effects of dry eye conditions, but the reduction of goblet cells in the Blank NP group was not as drastic as the reduction of goblet cells in the Saline group. We postulate that the mucoadhesive NPs, once adhering to the ocular mucosa, may improve the hydration of the ocular surface with tear fluid due to the high hydrophilicity of the Dextran. This may also explain why the NP+CycA groups also resulted

in the preservation of the ocular surface, demonstrated by the abundance of goblet cells, while RESTASIS treated eyes showed insufficient number of goblet cells and somewhat slower recovery of the ocular surface. Another study reports a significant increase in goblet cell density after 3 months of treatment with RESTASIS [62], so it is likely that the recovery effect was not observable within the time frame of the current study due to insufficient ocular retention of Cyclosporine A from RESTASIS administration. Although the three times daily treatment of RESTASIS effectively eliminated the inflammatory infiltrates from the eyes, the NP+CycA treatment achieved both the elimination of inflammatory infiltrates as well as the complete recovery of the ocular surface with only a single administration per week in the duration of the study. This further implies the long retention of these mucoadhesive NPs on the ocular surface, thus immensely enhancing the ocular bioavailability and efficacy of the encapsulated drugs.

Although the current formulation achieved efficacy in treatment of experimental dry eye, there is room for further improvement. One of the ongoing studies analyzes the effect of PBA surface density on the in vivo retention of the NPs on the ocular mucous membrane, to find the optimal PBA density that will maximize its in vivo mucoadhesion without compromising the particle stability. In addition, finding the nanoparticle:drug ratio that maximizes the total release of the drugs at a therapeutically effective rate is also a significant improvement on the current formulation.

Conclusion

Nanoparticles (NPs) self-assembled from PLA-b-Dex copolymers and surface functionalized with PBA ligands were formulated as a mucoadhesive drug carrier for topical ocular drug delivery application. The NPs encapsulated large dose of Cyclosporine A and sustained the release at a clinically relevant dose for a prolonged period of time. Neither the blank NP carriers nor NPs encapsulating Cyclosporine A caused any observable irritation or inflammatory responses after weekly administration for up to 12 weeks observed both by slit-lamp examination and histopathology. The NPs encapsulating Cyclosporine A also showed efficacy in treating experimental dry eye in mice: while thrice a day administration of RESTASIS only showed clearance of the inflammatory processes, the once a week administration of Cyclosporine A loaded NPs demonstrated both the elimination of inflammatory infiltrates and the complete restoration of the ocular surface. The current study provides promising results for the potential application of PLA-b-Dex-g-PBA NPs to dramatically improve the efficacy of ocular therapeutics for treating anterior eye diseases.

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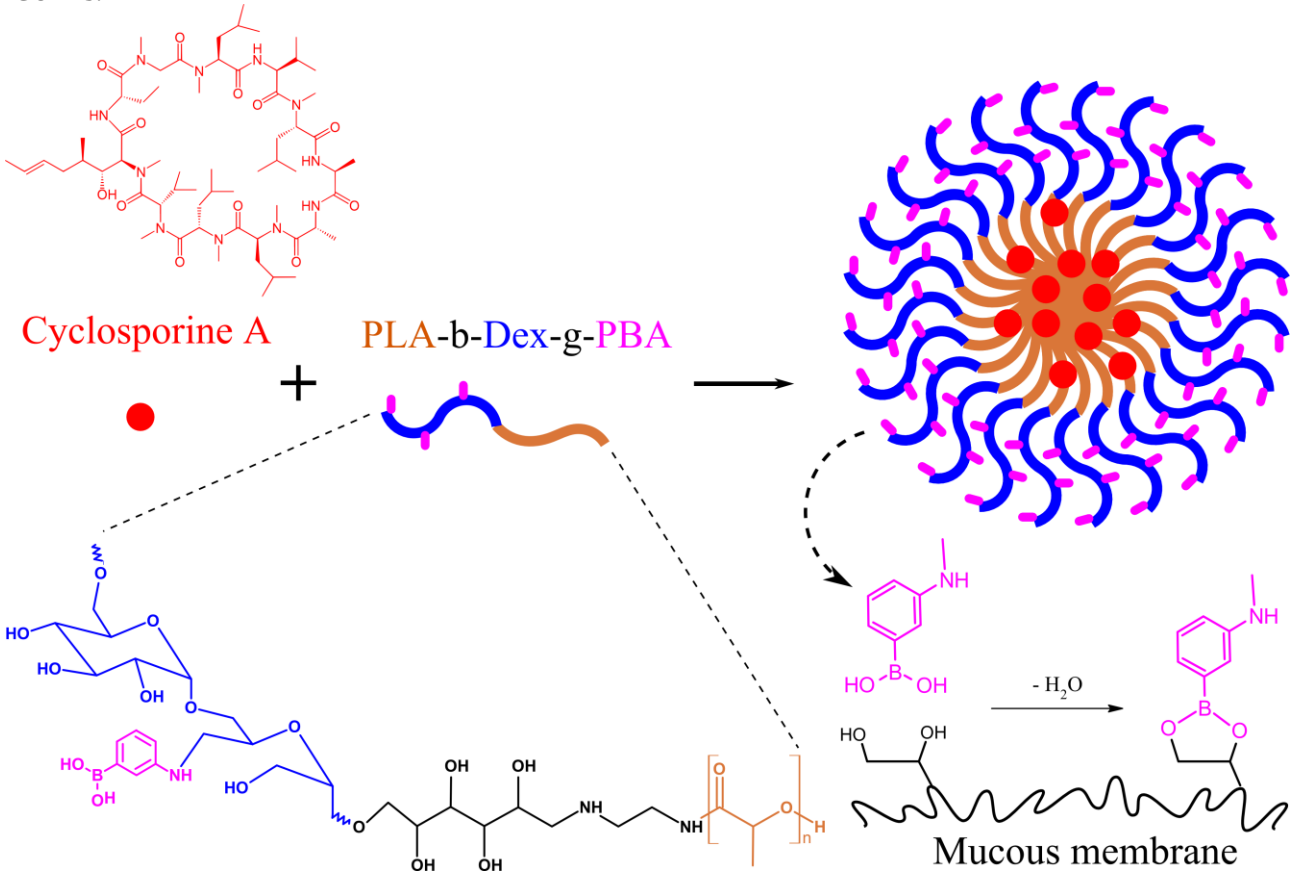
FIGURES.



Scheme 1. Schematic illustration of the self-assembly process to form Cyclosporine A loaded PLA-b-Dex-g-PBA nanoparticles, and the mucoadhesion mechanism of the grafted PBA's.

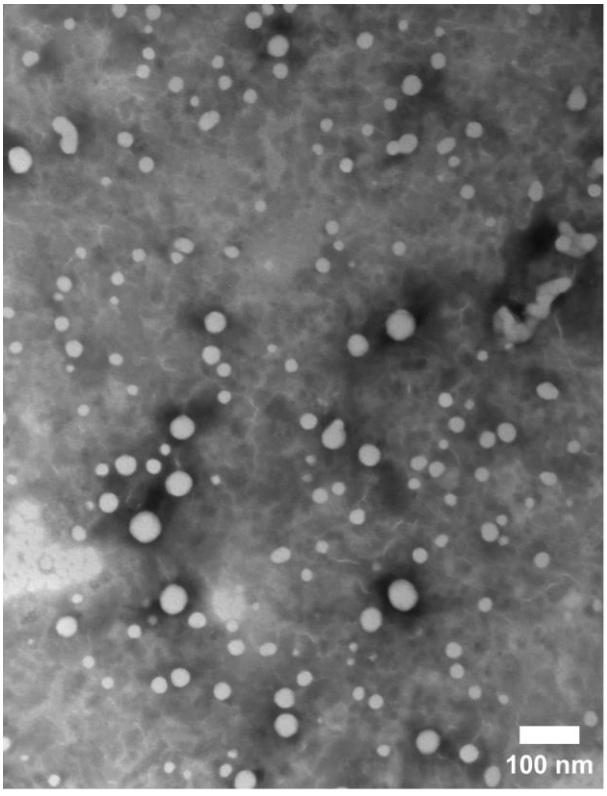


Figure 1. TEM image of PLA-b-Dex-g-PBA NPs.

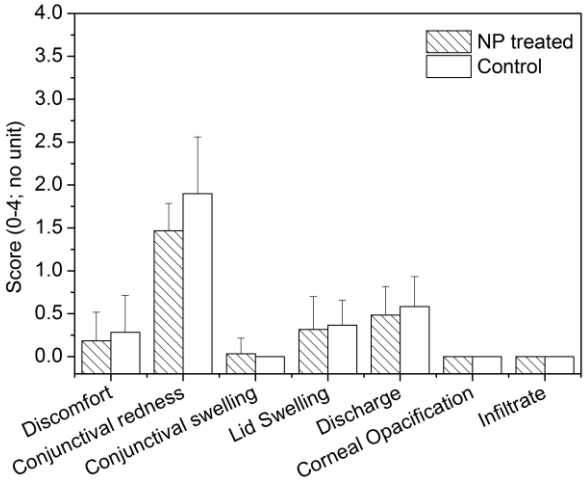


Figure 2. In vivo acute ocular irritancy test grading obtained through slit-lamp examination.

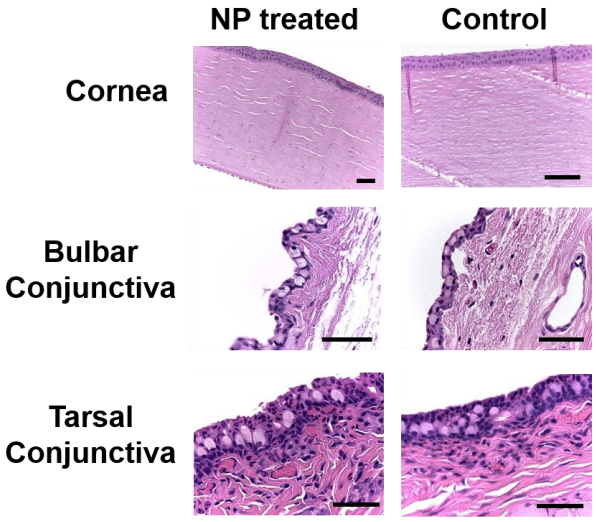


Figure 3. Histopathology analysis of both NP administered and control eyes in in vivo acute ocular irritancy test reveal no significant differences in the corneal and conjunctival morphological characteristics and architecture.

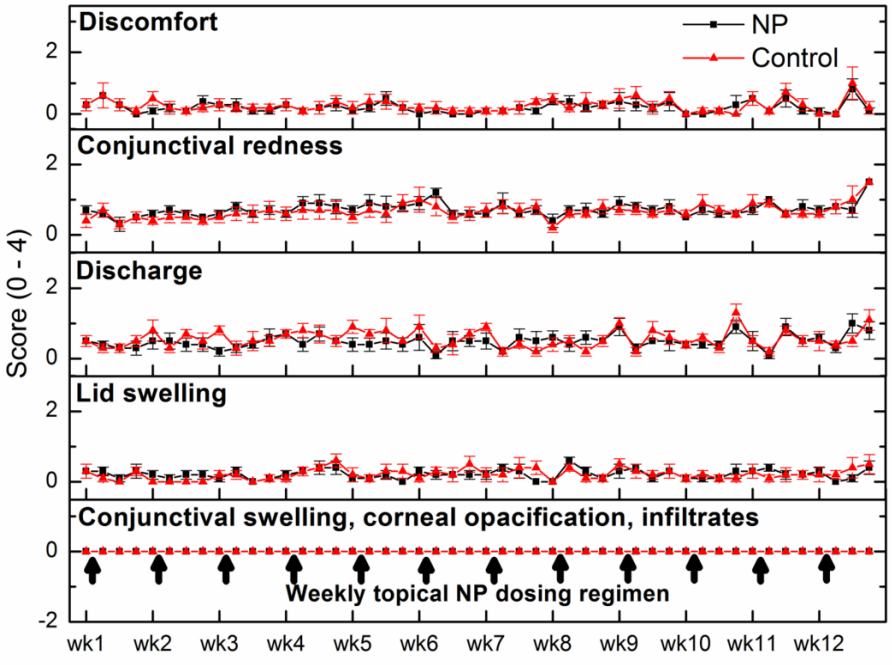


Figure 4. In vivo chronic ocular irritancy test for weekly administration of NP formulation for up to 12 weeks (each data point shown as mean ± s.e.m. (standard errors of mean); n = 5).

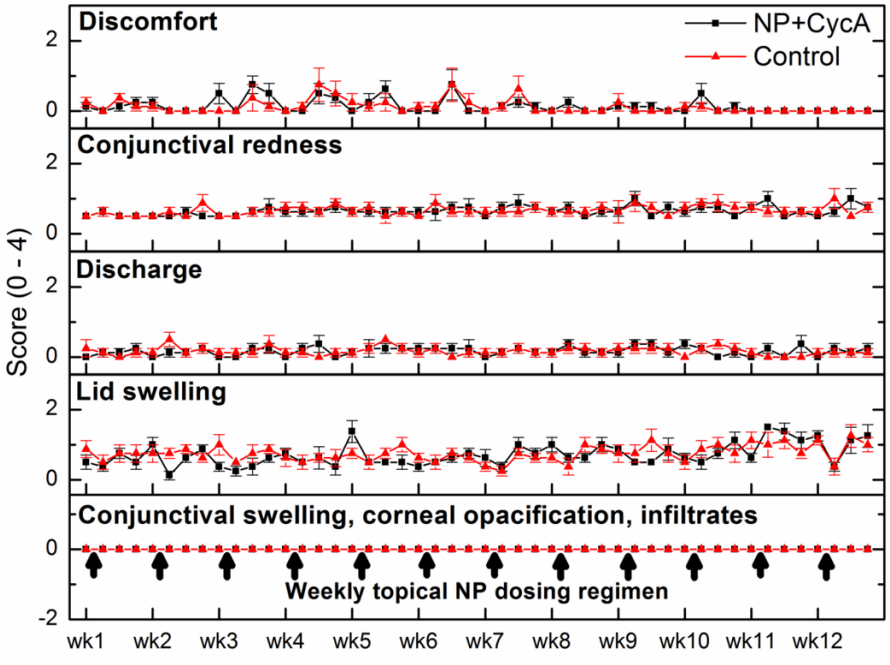


Figure 5. In vivo chronic ocular irritancy test of weekly administration of NP+CycA formulation for up to 12 weeks (each data point shown as mean ± s.e.m.; n = 4).

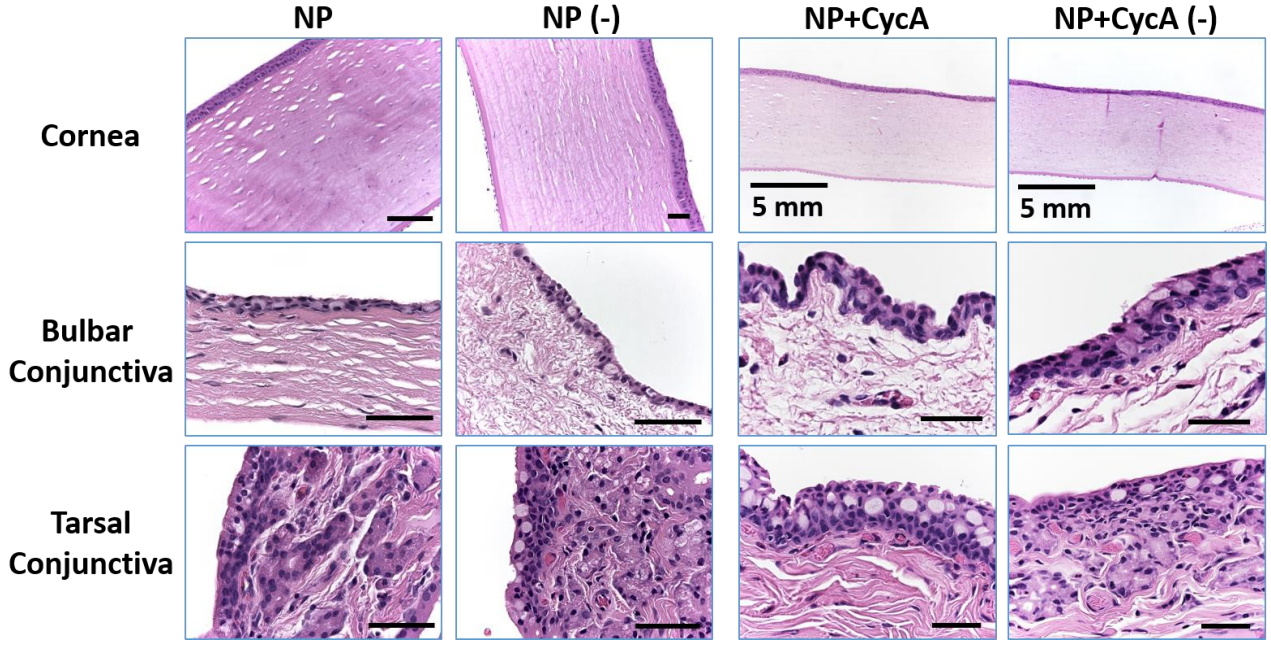


Figure 6. Histopathology study after in vivo chronic ocular irritancy tests with weekly administration of NP and NP+CycA. NP (-) and NP+CycA (-) represent the contralateral control eyes of the rabbits administered with NP or NP+CycA formulations.

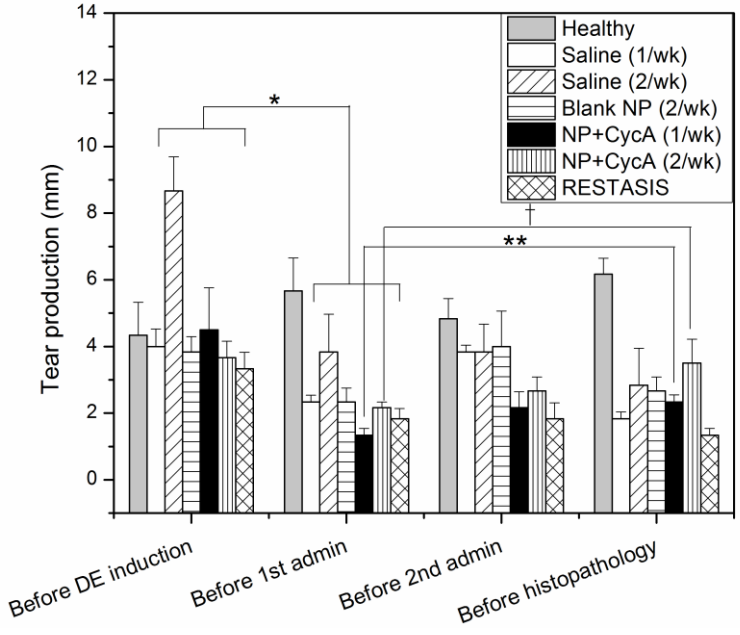


Figure 7. Tear volume measurements of 7 different treatment groups at 4 time points: before dry eye (DE) induction (day 1), before 1st administration (day 5), before 2nd administration (day 8), and before histopathology (day 12). Each data point is shown as mean ± s.e.m., n = 6. Statistical symbols for t-test: † for $0.05 < p \leq 0.1$, * for $0.01 < p \leq 0.05$, ** for $0.001 < p \leq 0.01$, and *** for $p < 0.001$.

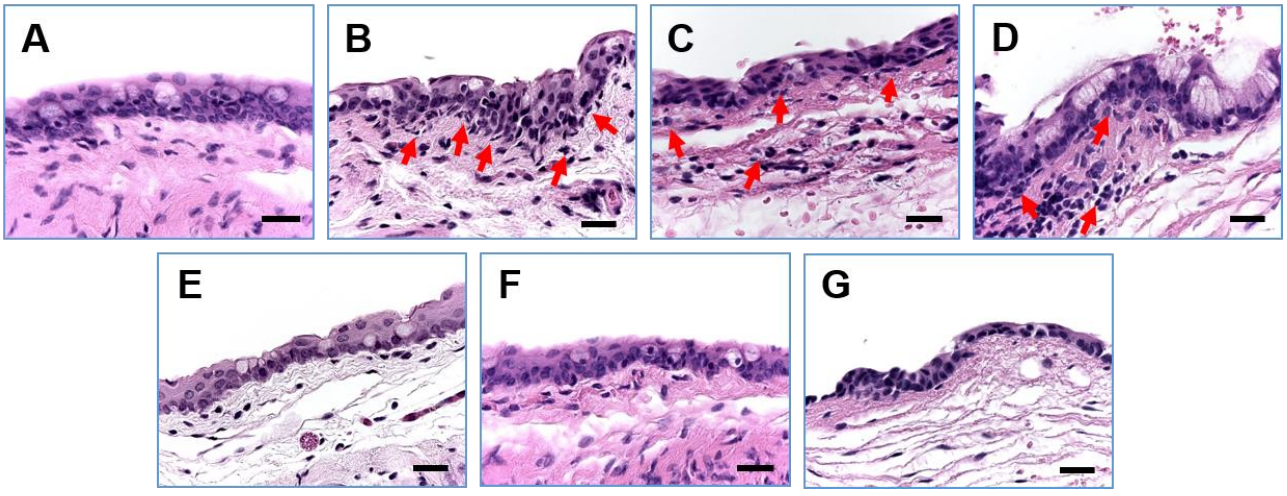


Figure 8. Histopathology analysis of ocular tissues of mice after 7 different treatment types: A) Healthy, experimental dry eye treated with B) Saline (1/wk), C) Saline (2/wk), D) Blank NPs, E) NP+CycA (1/wk), F) NP+CycA (2/wk), and G) RESTASIS. The scale bars (black) are 300 μm in length. The arrows (red) represent some of the inflammatory infiltrates such as lymphocytes, polymorphonuclears and eosinophils observed.

TABLES.

Table 1. 7 different treatment groups applied to the mice for experimental dry eye treatment study in mice.

Treatment group	Dry eye induced	Admin.	Admin. volume (μl)	NP (μg)	CycA (μg)	Admin. frequency
Healthy	No	N/A	0	0	0	
Saline (1/wk)	Yes	Saline	7	0	0	x1/week
Saline (2/wk)	Yes	Saline	7	0	0	x2/week
Blank NP (2/wk)	Yes	Water	7	4	0	x2/week
NP+CycA (1/wk)	Yes	Water	7	4	<1.5	x1/week
NP+CycA (2/wk)	Yes	Water	7	4	<1.5	x2/week
RESTASIS	Yes	N/A	3	0	1.5	x3/day

Table 2. PLA-b-Dex-g-PBA characterization: PBA surface functionalization, Cyclosporine A loading, and diameter.

Sample	PBA/Dextran (mol%)	CycA load (wt%)	Mean diameter (nm)	ζ potential (mV)
PLA-b-Dex	0	0	45.8 ± 1.5	- 2.63 ± 1.55
PLA-b-Dex-g-PBA	17.6 ± 2.7	0	28.6 ± 3.1	-31.3 ± 1.5
PLA-b-Dex-g-PBA with CycA	17.6 ± 2.7	11.9 ± 1.6	35.6 ± 7.4	

Note that the average and the standard deviations were calculated using measurements of three batches of the nanoparticles

Electronic Supplementary Material

Phenylboronic acid modified mucoadhesive nanoparticle drug carriers facilitate weekly treatment of experimentally-induced dry eye syndrome

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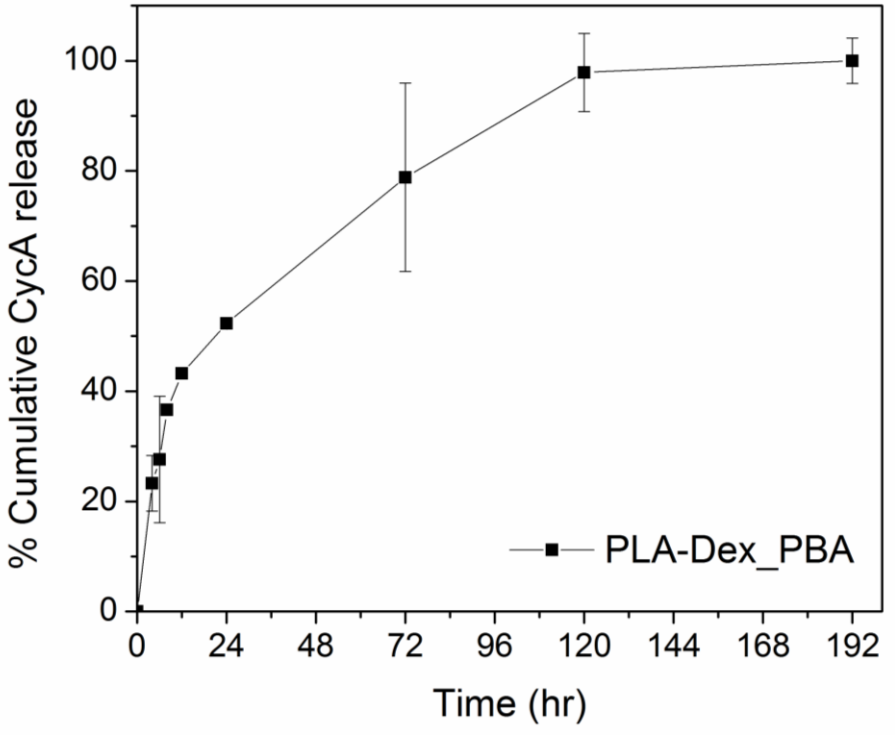

Figure S1. *In vitro* release of CycA from PLA-b-Dex-g-PBA in simulated tear fluid.

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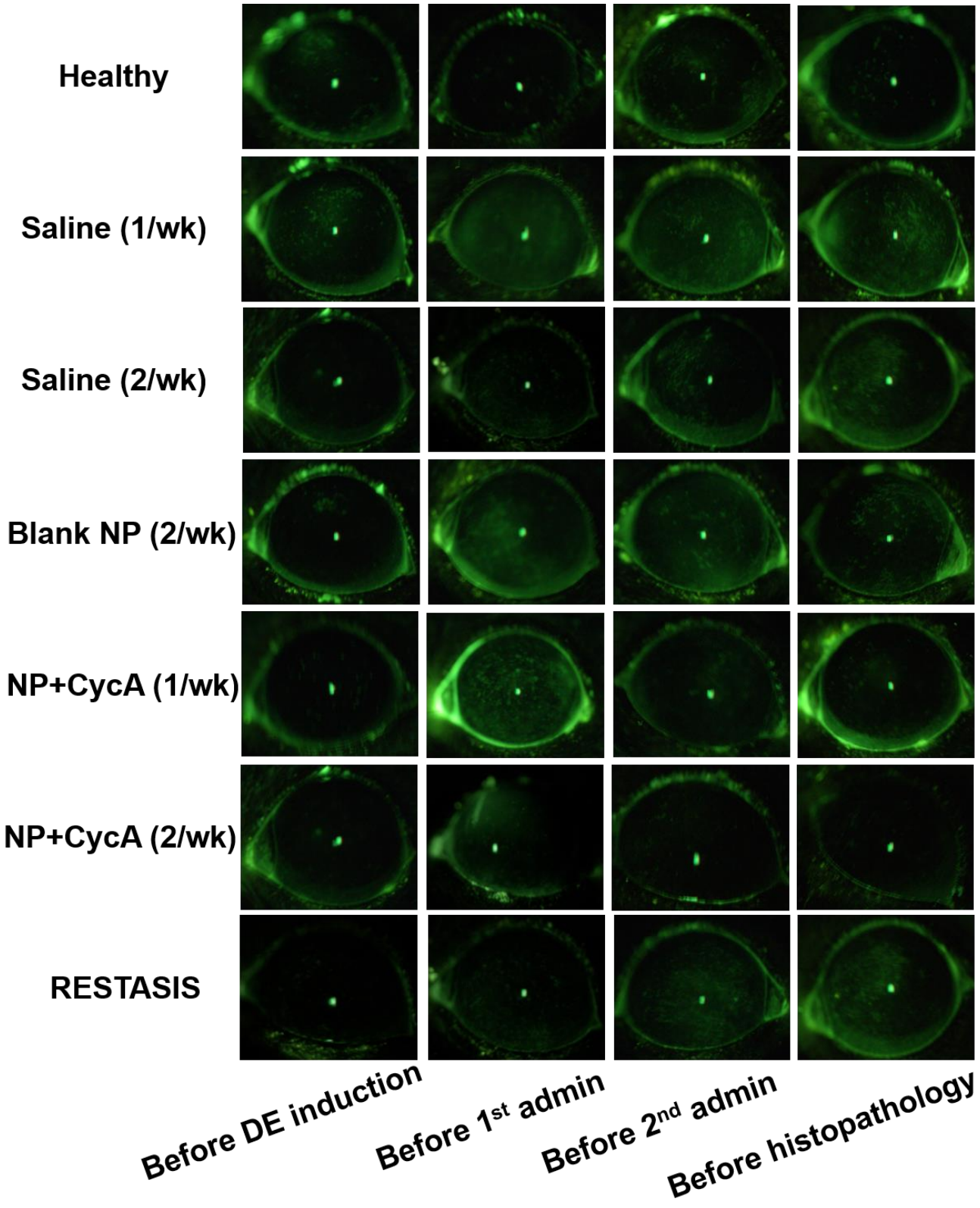

Figure S2. Corneal fluorescein staining images of 7 different treatment groups obtain at 4 time points: before dry eye (DE) induction (day 1), before 1st administration (day 5), before 2nd administration (day 8), and before histopathology (day 12). The images were obtained 10 minutes after the application of sodium fluorescein. Note that the corneal fluorescein present on the edges of the lids were

due to the local structure and has little to do with the tear production or ocular surface damage, thus they were not taken into consideration for the fluorescein clearance analysis.