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Hybrid Dendrimer Hydrogel/PLGA Nanoparticle Platform Sustains Drug Delivery for One Week and Antiglaucoma Effects for Four Days Following One-Time Topical Administration

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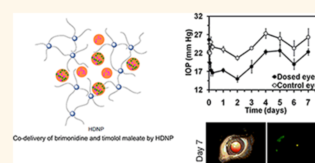
ABSTRACT We report a novel hybrid polyamidoamine (PAMAM) dendrimer hydrogel/poly(lactic-*co*-glycolic acid) (PLGA) nanoparticle platform (HDNP) for codelivery of two antiglaucoma drugs, brimonidine and timolol maleate. This platform was not cytotoxic to human corneal epithelial cells. Cellular uptake of Nile red-encapsulating PLGA nanoparticles was significantly increased by dendrimer hydrogel. A prolonged residence time of nanoparticles was demonstrated through investigation of FluoSpheres loaded into dendrimer hydrogel. Both brimonidine and timolol maleate were slowly released *in vitro* over a period of 28−35 days. Following topical administration of one eye drop (30 μL of 0.7% w/v brimonidine and 3.5% w/v timolol maleate) in normotensive adult Dutch-belted male rabbits, the HDNP formulation resulted in a sustained and effective IOP reduction (18% or higher) for 4 days. Furthermore, the HDNP maintained significantly higher concentrations of brimonidine in aqueous humor and cornea as well as timolol maleate in the aqueous humor, cornea, and conjunctiva up to 7 days as compared to saline, DH, and PLGA nanoparticle dosage forms, without inducing ocular inflammation or discomfort. Histological analysis of the cornea and conjunctiva did not reveal any morphological or structural changes. Our work demonstrated that this new platform is capable of enhancing drug bioavailability and sustaining effective IOP reduction over an extended period of time. This newly developed platform can greatly reduce dosing frequency of topical formulations, thus, improving long-term patient compliance and reducing enormous societal and economic costs. Given its high structural adaptability, many other chronic ocular diseases would benefit from long-lasting drug delivery of this new platform.



KEYWORDS: dendrimer · glaucoma · nanotechnology · ophthalmic solution · PLGA · controlled release

Glaucoma is a neurodegenerative disease with high intraocular pressure (IOP) being the most important risk factor. It is a leading cause of visual impairment and blindness in the world, according to the World Health Organization.[1] In the U.S.A. alone, over 2.2 million people suffer from glaucoma.[2] It was estimated that this disease could pose an economic burden of over 1.50 billion dollars annually in social security benefits, lost revenue from income tax, and health care expenditures. Various classes of antiglaucoma agents have been developed to lower IOP, including beta blockers (e.g., timolol), prostaglandin analogs (e.g., latanoprost, travoprost), α-adrenergic agonists (e.g., brimonidine), and carbonic anhydrase inhibitors (e.g., dorzolamide). Due to the precorneal tear clearance and the highly permi-selective corneal epithelium, antiglaucoma drugs in the form of eye drops usually have low bioavailability (less than 5% or even below 1%) and a short duration of activity, thus, requiring daily administration. For instance, timolol (Timoptic) has to be applied twice daily and brimonidine (Alphagan P) three times daily. Thus, patient noncompliance has been cited as the leading problem in treating glaucoma.[3]

Although injectable drug delivery systems and implantable, refillable devices have been actively explored and are able to sustain release and efficacy of antiglaucoma drugs for several months[4] or even years,[5] eye drop administration is by far the most convenient administration route and perhaps the only practical way for aging populations and patients in developing countries. To meet rapidly increasing clinical need for treating eye diseases and to overcome the shortcomings of conventional dosage forms, there is a great deal of interest in developing new topical formulations for glaucoma treatment. They are expected to not only increase the ocular bioavailability and sustain therapeutic efficacy of topically applied drugs over an extended period of time but ideally be less invasive to reduce

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frequent dosing as well as increase patient compliance. In the past, a variety of unconventional dosage forms, such as mucoadhesive hydrogels, colloidal dosage forms, *etc*., have been explored to prolong drug residence time on the cornea and increase the bioavailability of antiglaucoma drugs. For example, Lele and Hoffman developed a mucoadhesive formulation based on an ionic complex of partially neutralized poly(acrylic acid) for delivery of levobetaxolol.[6] *In situ*-forming hydrogels such as Gelrite (deacetylated gellan gum) solution[7] can undergo phase transition in the ocular cul de sac to form visco-elastic hydrogel in response to environmental changes for sustained drug release. Colloidal dosage forms including nanoparticles and microparticles have been shown to be capable of sustaining drug release or enhancing drug bioavailability.[8] A microparticulate ophthalmic delivery system, Betoptic S, containing betaxolol 0.25%, has been approved for commercial use for glaucoma in the U.S.A.[9]

Polyamidoamine (PAMAM) dendrimers have emerged as an ideal class of nanoparticles for drug delivery due to their unique structural features and properties.[10,11] PAMAM dendrimers have been found to possess bioadhesive properties and exert strong interaction with the surface of cornea, with which the residence time of pilocarpine can be prolonged.[12] Recently, we have developed a photocurable PAMAM dendrimer hydrogel (DH) platform, made from PAMAM dendrimer tethered with polyethylene glycol (PEG) acrylate.[13] It possesses structural characteristics and properties derived from integration of dendrimer nanoparticles and PEG network. Our previous *in vitro* and *ex vivo* work has shown that DH can be applied to fabricate a liquid ophthalmic formulation for enhanced antiglaucoma drug delivery.[14] This enhanced delivery was attributed to the prolonged interaction of the DH formulation with the cornea as well as its greater drug-loading capacity. To further enhance antiglaucoma drug delivery and enable better control over drug release, we designed a hybrid PAMAM dendrimer hydrogel/poly(lactic-*co*-glycolic acid) (PLGA) nanoparticle platform (HDNP) and formulated it into an aqueous eye drop formulation for codelivery of two antiglaucoma drugs, brimonidine and timolol maleate. Biocompatible and biodegradable PLGA nanoparticles are safe for delivery of ophthalmic agents and are capable of sustained delivery of antiglaucoma agents.[4] This hybrid platform is novel as it possesses two types of drug delivery vehicles with a greater control over drug release and integrates the properties of light-induced gelling, mucoadhesive nanoparticles, and PEG hydrogel. The cytocompatibility of the HDNP formulation, the uptake of PLGA nanoparticles, and drug release kinetics mediated with this new platform were examined. Furthermore, *in vivo* drug efficacy and absorption in ocular tissues as well as formulation safety assessment were analyzed and reported herein.

RESULTS

Preparation of Drug Formulations. A DH platform has been developed by us.[13] This platform is capable of simultaneous delivery of both hydrophobic and hydrophilic drugs due to its amphiphilic structure conferred by the hydrophobic interior core of PAMAM dendrimer[15,16] and the hydrophilic PEG network. By tuning the degree of PEGylation, PEG chain length, and the density of acrylate groups on the dendrimer surface, photocurable dendrimer-PEG-acrylate can form either a completely solidified hydrogel network or a partially cross-linked viscous solution.[13] To allow eye drop instillation, PAMAM dendrimer G3.0 possessing an average of three PEG-acrylate chains was employed to make liquid antiglaucoma drug formulations. The preparation and characterization of the DH platform was reported in detail in our previous work.[13] To take advantage of the adaptable structure of the DH network, we designed a hybrid platform in this study by integrating PLGA nanoparticles into the DH platform, as shown in Figure 1. The so-called hybrid dendrimer HDNP utilizes biodegradable PLGA nanoparticles as a primary vehicle for drug loading and a partially cross-linked dendrimer hydrogel network for dispersion of drug-loaded nanoparticles. It was expected to achieve a greater control over drug delivery and release and further enhance bioavailability and therapeutic effectiveness of drugs to be delivered.

Due to the multifactorial nature of glaucoma, a single drug is often insufficient to control IOP regardless of the duration of action of the drug. A study shows that nearly 40% of glaucoma patients require concomitant use of two or more antiglaucoma drugs to achieve IOP reduction by year 5.[17] A combination of two or more antiglaucoma drugs can achieve a better and quicker control on IOP reduction and help reduce the number of dosages and the amount of preservative for patient compliance improvement. Several combination medications, such as dorzolamide/timolol maleate, latanoprost/timolol maleate, and brimonidine tartrate/timolol maleate, have been developed and released to the market. For instance, COMBIGAN is an ophthalmic solution containing brimonidine tartrate 0.2% and timolol maleate 0.5% and dosed twice a day. Following this approach, we prepared combination drug formulations containing both brimonidine and timolol maleate. Brimonidine is a relatively selective α-2 adrenergic receptor agonist, while timolol maleate is a nonselective β-adrenergic receptor inhibitor. Typically, daily administered ophthalmic solutions have a concentration of 0.1 or 0.2% for brimonidine and 0.25 or 0.5% for timolol maleate. Given that the new platform has a large loading capacity and the ability to sustain drug release, we prepared combination formulations containing high doses of drugs (0.7% brimonidine and 3.5% timolol maleate) and evaluated their sustained

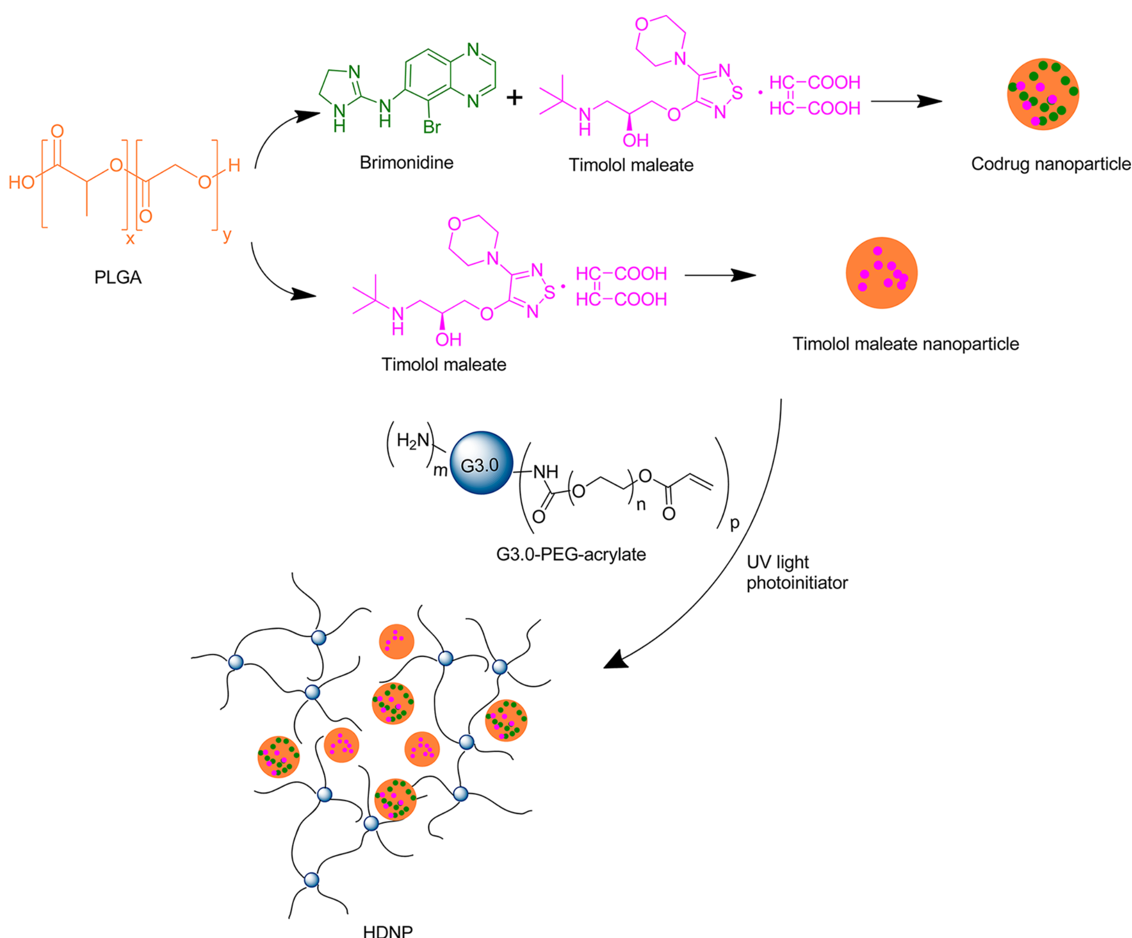

Figure 1. Hybrid dendrimer hydrogel/nanoparticle platform (HDNP). Nanoparticles loaded with brimonidine and timolol maleate were entrapped in the dendrimer hydrogel by subjecting the dendrimer-PEG acrylate to UV light in the presence of photoinitiator.

release profiles and IOP-lowering effects in this work. Brimonidine and timolol maleate loaded combination particles had a diameter of 258 nm and a zeta-potential of −28.8 mV. Drug loading in these particles was 7.51% for brimonidine and 5.85% for timolol maleate. Timolol maleate alone loaded nanoparticles had a diameter of 287 nm and a zeta-potential of −27.4 mV. The drug loading in timolol maleate particles was 7.82%. Timolol maleate alone particles were used in the appropriate proportion to prepare the combination formulation containing 0.7% brimonidine and 3.5% timolol maleate. The polydispersity index of the combination and timolol maleate nanoparticles was 0.113 and 0.383, respectively.

Cytocompatibility. It is essential to develop a new drug formulation that has no or minimal toxicity effects to the eye. To this end, the potential toxicity of the drug formulations prepared was evaluated *in vitro*. Because a single dose volume (30 μL) of a particular eye drop formulation would be instilled topically to rabbits in animal studies, the effects of the instilled volume containing various drug formulations were studied. MTT and protein assays were applied to measure the enzymatic activity (viability) and protein content of human corneal epithelial cells (HCETs), both of which are

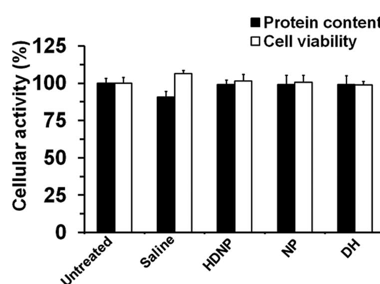

Figure 2. Lack of cytotoxicity of hybrid dendrimer hydrogel-nanoparticle formulation in human corneal epithelial cells. All formulations were prepared in PBS and contained both brimonidine and timolol maleate. Data are expressed as mean ± SD for *n* = 6.

indicative of cellular activity and function. According to our measurements (Figure 2), the viability and protein content level of the cells treated with saline, DH, NP, or HDNP drug formulations were comparable to those of the untreated cells. Therefore, the formulations were considered to be nontoxic and used in the rest of the experiments.

Enhanced PLGA Nanoparticle Uptake. To quantify PLGA nanoparticle uptake, Nile red-loaded PLGA nanoparticles were prepared and used in uptake studies. The amounts of nanoparticles in cell lysates were

quantified and normalized to protein content. As shown in Figure 3, the level of nanoparticle uptake aided with dendrimer hydrogel (HDNP) is significantly higher than nanoparticles in saline solution at the indicated time points. Following a brief incubation for 5 min, the nanoparticle uptake was 197.0 μg/mg for HDNP, which represented a nearly 7-fold increase as compared to the uptake for the nanoparticle in saline solution ($p < 0.001$). Longer incubation resulted in more nanoparticle uptake. Particularly, the nanoparticle uptake for HDNP increased by 26.2% to 248.7 μg/mg following a 1 h incubation and by 88.1% to 370.6 μg/mg following a 3 h incubation. A more pronounced increase in particle uptake for NP was observed with increasing incubation time. The particle uptake for NP increased by 2-fold to 77.3 μg/mg following 1 h incubation and by 3-fold to 102.7 μg/mg following a 3 h incubation. Nonetheless, the actual particle uptake for HDNP was still 2.2-fold higher than NP at 1 h ($p < 0.05$) and 2.6-fold higher at 3 h ($p < 0.05$). It is reasonable to believe that more nanoparticle uptake would lead to more drug absorption because drug molecules are stably encapsulated by the particles.

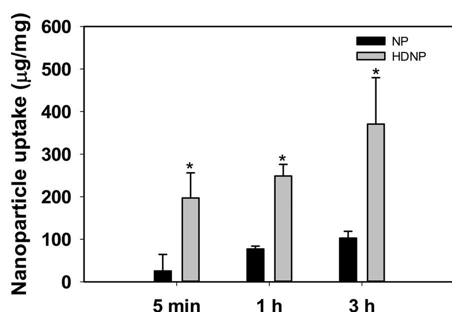

Figure 3. Enhanced uptake of nanoparticles by dendrimer hydrogel in human corneal epithelial cells. Data are expressed as mean ± SD for $n = 6$. *Indicates $p < 0.05$ compared to NP formulation at the indicated time points.

Sustained *In Vitro* Drug Release. Under the perfect sink condition, the release of brimonidine and timolol maleate was greatly extended by HDNP and NP as compared to the drug release from saline solution and DH reported in our previous work.[14] Four different models, eqs 1−4, were fit to the experimental data, and the results are summarized in Table 1. AIC test or F-test was conducted to determine the model that best describes drug transport. According to our recent studies, nearly 100% of drug molecules were released from saline solution within 90 min.[14] Both brimonidine and timolol maleate release patterns were best described by the Higuchi model, which suggests that the drug transport in this case was driven by Fickian diffusion only. As for DH formulation, 50% drug release occurred in a short duration (2 h for brimonidine and 1 h for timolol maleate) possibly due to burst release.[14] However, the time taken for 100% drug release was much longer: 72 h for brimonidine and 48 h for timolol maleate. This suggests that DH formulation helped hinder the release of entrapped drug molecules. The first 60% of the drug release was processed for modeling because of their high adjusted R^2 values in each fitting function. Again, Fickian diffusion was attributed to the controlled drug release for both brimonidine and timolol maleate as the Higuchi model was the most appropriate to fit the data set for DH formulation.

A more strikingly extended release of brimonidine and timolol maleate was enabled by NP and HDNP. The drug molecules were slowly and evenly released over a period of 28 days for NP (Figure 4A) and 35 days for HDNP (Figure 4B). A 50% drug release for both brimonidine and timolol maleate from HDNP formulation took 10 days. The drug release period lasted as long as 5 weeks with minimum burst release. The release of brimonidine from NP and HDNP was best described by the Ritger-Peppas equation. The value of diffusional exponent (n) in the equation is used to determine drug

TABLE 1. Parameters, Adjusted R^2, and AIC Values Estimated by Fitting Drug Release Data to Four Mathematical Models[a]

		mathematical models														
		Higuchi			Ritger-Peppas				Peppas-Sahlin					zero-order		
drug	formulation	k	adj. R^2	AIC	k	n	adj. R^2	AIC	k_1	k_2	m	adj. R^2	AIC	k	adj. R^2	AIC
brimonidine	saline[d]	10.19	0.98	25.13	7.41	0.58	0.99	42.93	3.18	−0.03	0.89	1.00	NA[c]	1.24	0.87	35.37
	DH[b,d]	35.52	1.00	10.92	34.73	0.52	1.00	12.76	20.39	14.04	0.39	1.00	25.64	26.12	0.85	34.71
	NP	16.74	0.90	52.19	6.07	0.85	0.98	38.85	6.52	1.17	0.59	0.98	42.74	3.84	0.97	39.01
	HDNP	17.22	0.97	47.10	11.97	0.62	0.99	41.24	9.30	−0.18	0.79	0.99	43.86	3.57	0.90	63.09
timolol maleate	saline[d]	12.39	0.82	37.82	34.55	0.25	0.90	53.50	10.19	−0.25	0.73	0.94	NA[c]	1.43	0.31	44.64
	DH[b,d]	51.53	0.97	24.43	51.20	0.36	1.00	30.69	73.99	−21.76	0.54	1.00	NA[c]	47.05	0.62	35.38
	NP	16.86	0.96	39.48	13.08	0.59	0.97	41.05	11.66	2.43	0.44	0.97	43.99	3.79	0.85	52.52
	HDNP	15.63	0.98	37.66	15.02	0.51	0.98	41.18	13.16	2.94	0.38	0.98	44.39	3.20	0.81	66.20

[a] Four models were fit to the experimental data. AIC test or F-test was conducted to determine the model that best describes drug transport. [b] The first 60% release was fit to the models. [c] AIC (Akaike Information Criterion) was not obtained due to insufficient information. F-test was used instead for model comparison between the model with the lowest AIC value and the Peppas-Sahlin model. [d] Data used for model fitting were taken from our previous publication.[14]

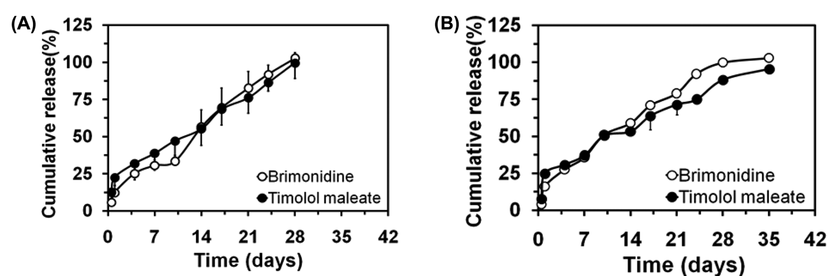

Figure 4. *In vitro* release of brimonidine and timolol maleate from PLGA nanoparticles (A) and HDNP formulation (B) at 37 °C in PBS pH 7.4. Data are expressed as mean ± SD for $n = 3$.

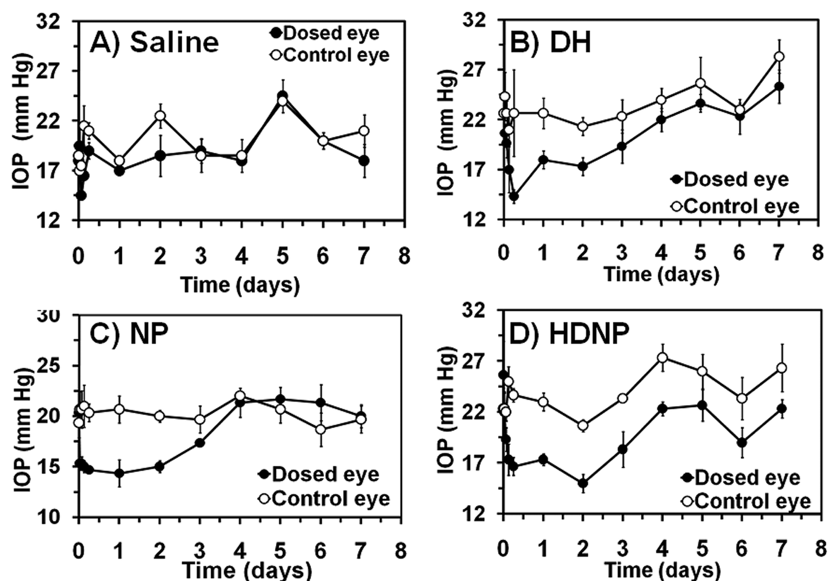

Figure 5. HDNP sustains reduction in IOP in Dutch-belted rabbits. The IOP reduction observed after administration of brimonidine and timolol maleate in (A) saline, (B) dendrimer hydrogel, (C) nanoparticle, and (D) hybrid dendrimer nanoparticle formulations was assessed. Data are expressed as mean ± SEM for $n = 3$.

transport mechanism. When n is 0.5 for thin film or 0.43 for sphere, the drug release mechanism is Fickian diffusion.[18] When n is 1 for thin film or 0.85 for sphere, the drug release mechanism is Case II transport, in which the macromolecular chain relaxation heavily affects drug release. When n falls between these threshold values, an anomalous transport mechanism is observed, accounting for the coupled effects of Fickian diffusion and macromolecular chain relaxation. Given that n is 0.62 in the case of brimonidine release from HDNP and 0.85 in the case of brimonidine release from NP, anomalous transport is presumed to be responsible for brimonidine transport. In contrast, the Higuchi model is still the best to describe the release of timolol maleate from NP and HDNP. Nonetheless, both brimonidine and timolol maleate were released at a similar rate, thus, ensuring them to achieve sustained release and complementary therapeutic efficacy.

Enhanced Drug Absorption and Extended IOP Control. The IOP-lowering effect of all formulations was examined following one topical eye drop administration. IOP was measured in both eyes of the rabbits for 7 days. A mean reduction of 15% or higher was regarded to be effective in IOP control.[19] In each animal, the right eye was treated with a given drug formulation, while the left eye was undosed for IOP comparison. The brimonidine/timolol maleate saline formulation began to result in an effective IOP reduction at 1.5 h and quickly reached its peak effect at 3 h, reducing IOP by 18.0% (Figure 5A). At 6 h and later time points, the saline formulation failed to effectively reduce IOP in the dosed eyes. In contrast, brimonidine/timolol maleate DH formulation started lowering IOP effectively in the dosed eyes in less than 30 min, which resulted in a maximum IOP reduction of 37.6% at 6 h, and sustained an effective IOP reduction until 48 h (Figure 5B). Following the administration of the brimonidine/timolol maleate NP formulation, an effective IOP reduction (25.2%) was first observed at 30 min (Figure 5C). The NP formulation maintained a high IOP reduction ranging from 24.8 to 30.5% until 48 h ($p < 0.05$ for IOP between dosed and undosed eyes), in which the peak effect was observed at 24 h. More strikingly, brimonidine/timolol maleate HDNP formulation resulted in a longer and more effective IOP reduction (Figure 5D). This formulation had an onset at 3 h, reducing IOP by 29.5%, which was

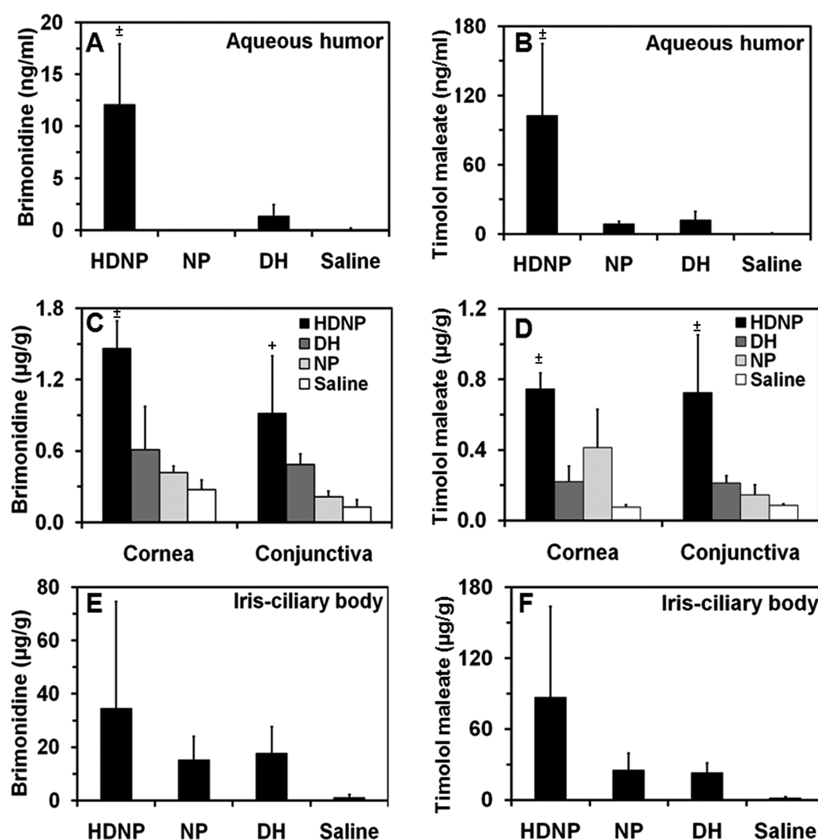

Figure 6. At the end of 7 days postdosing, drug levels in eye tissues including aqueous humor (A , B), cornea and conjunctiva (C , D), and iris ciliary body (E , F). Data are expressed as mean ± SD for $n = 3$. ± indicates $p < 0.05$ compared to NP, DH, and saline group, and + indicates $p < 0.05$ compared to saline group only. For iris-ciliary body, due to high standard deviation in HDNP group, no statistical significance was observed with any group.

also the peak effect. This peak effect was maintained up to 6 h. The IOP reduction was then sufficiently maintained as high as above 18% until 96 h. Furthermore, the IOP of the dosed eyes was significantly lower than that of the undosed eyes at any time point from 3 to 96 h ($p < 0.05$).

At day 7 postdosing, the rabbits were sacrificed and the levels of brimonidine and timolol maleate in the eye tissues were assessed. Comparison between DH and saline formulations for drug levels in eye tissues showed that there was no statistically significant difference in the concentration of either brimonidine or timolol maleate in the corresponding eye tissue. In contrast, as a result of the application of HDNP formulation, the concentration of brimonidine became 7.8-fold higher in aqueous humor ($p = 0.009$; Figure 6A) and 1.4-fold higher in cornea ($p = 0.006$; Figure 6C) when compared with the levels of brimonidine achieved with DH formulation. Brimonidine levels in iris/ciliary body were also detectable (Figure 6E); however, there was not a statistically significant difference among the treatment groups ($p = 0.347$). Both formulations also resulted in a similar concentration of brimonidine in conjunctiva ($p = 0.114$; Figure 6C). As for timolol absorption, HDNP formulation significantly enhanced its absorption in aqueous humor, cornea, and conjunctiva as compared to the other formulations ($p < 0.05$). In particular, when compared to DH formulation, the concentration of timolol maleate following HDNP treatment was 7.2-fold higher in the aqueous humor ($p = 0.023$; Figure 6B), 241.6% higher in the cornea ($p < 0.001$), and 240.7% higher in the conjunctiva ($p = 0.017$; Figure 6D).

It is believed that the IOP-lowering effect of brimonidine is related to its concentrations in the aqueous humor and ciliary body, although the peak concentration in aqueous humor is relatively lower than in iris and ciliary body.[20,21] Acheampong *et al.* reported that, following a single dose of instillation of 35 μL of 0.5% brimonidine tartrate, brimonidine reached a peak concentration of 19.1 μg/g in ciliary body and a peak concentration of 1.26 μg/mL in aqueous humor at 1.5 h in pigmented rabbits.[20] In our work, the respective brimonidine concentrations in iris/ciliary body and aqueous humor were 34.63 ± 40.02 μg/g and 12.11 ± 5.83 ng/mL at day 7 in Dutch-belted rabbits topically treated with HDNP application. Although the brimonidine concentration in aqueous humor at day 7 postdosing of HDNP formulation was only 0.96% of the peak concentration (i.e., 1.26 μg/g) at 1.5 h postdosing in Acheampong *et al.*'s work,[20] its level was still significantly higher than its levels in the aqueous humor

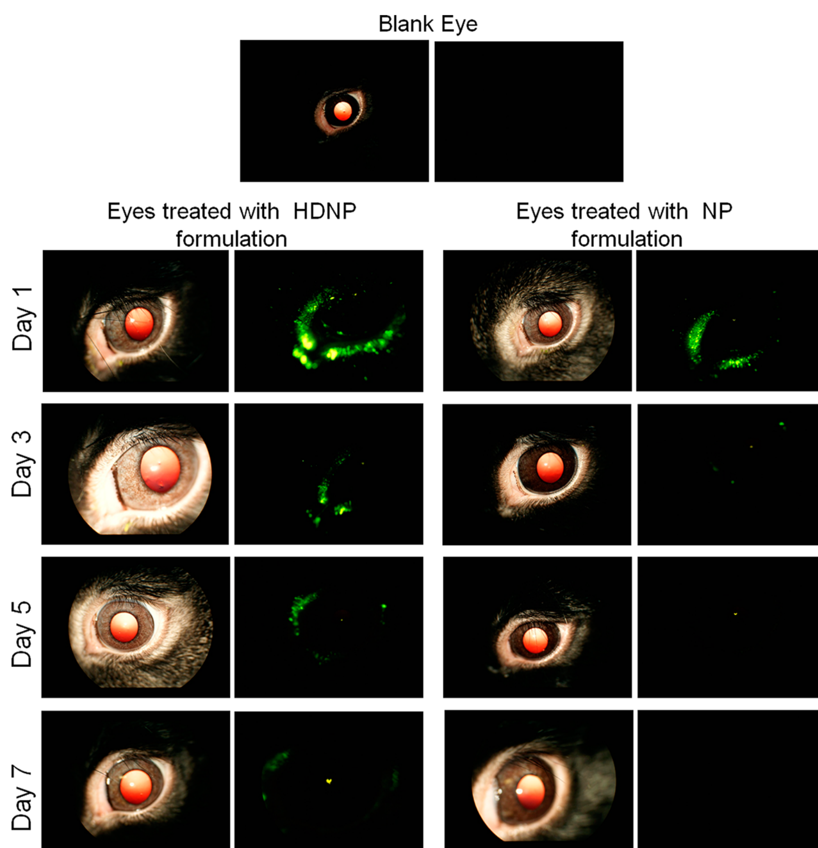

Figure 7. Representative fundus camera images of rabbits instilled with HDNP and NP formulations. FluoSpheres were entrapped in dendrimer hydrogel or PBS pH 7.4 instead of drug-encapsulating PLGA nanoparticles. Images were taken at the end of days 1, 3, 5, and 7 of instillation of formulations. Left panel of each group is the regular fundus camera image of the eyes, and right panel is a fluorescent fundus camera image.

of the rabbit eyes treated with the other formulations. Furthermore, timolol concentration in aqueous humor was significantly enhanced by HDNP as compared to the other formulations. Therefore, we believe that the sustained IOP-lowering effect by HDNP was attributed to its capability of enhancing brimonidine and timolol concentrations and preventing their quick decline in aqueous humor.

Prolonged Particle Retention and Safety Assessment. Following instillation of FluoSpheres dispersed in pH 7.4 PBS, the fluorescence signal on the rabbit eye surface dropped dramatically the next day, became negligible on day 3, and completely vanished on day 5 (Figure 7). In contrast, on the surface of the eye instilled with FluoSpheres HDNP, fluorescence signal sustained until the end of observation (i.e., day 7), although fluorescence declined gradually over time. Nonetheless, the presence of FluoSpheres was still prominent at day 7, indicating that the particles gained a significantly prolonged residence time with the use of a dendrimer hydrogel.

During the course of observation (7 days), the eyes receiving treatment of different drug-encapsulating formulations were examined for signs of inflammation including anterior chamber cells and flare and pupil dilation as well as signs of discomfort such as tearing and blinking. HDNP neither induced chamber cells and flare and pupil dilation nor caused abnormal tearing and blinking. According to histological examination of the cornea (Figure 8) and the conjunctiva (Figure 9) of the rabbit eyes at the end of day 7, no morphological or structural changes were observed. This confirmed that all these formulations, including HDNP, did not induce any gross toxicity in the ocular tissues, which is consistent with the *in vitro* cytotoxicity results.

DISCUSSION

Our goal was to develop a topical delivery system that can sustain antiglaucoma drug release and efficacy for as long as possible. Although prostaglandin analogs such as latanoprost are known to be slightly more efficacious in reducing IOP than brimonidine and timolol maleate, it is believed that the prostaglandin analogs may themselves exert prolonged effects following a single drop.[22] As compared to latanoprost, brimonidine and timolol have a shorter duration of effects (less than a day)[23,24] and thus are more ideal to assess our proposed delivery system to sustain drug delivery and effects. Intraperitoneal water loading elevates the pressure in the rabbit eyes, resulting in a greater decrease in IOP in response to the above drugs. However, we chose normotensive rabbits as opposed

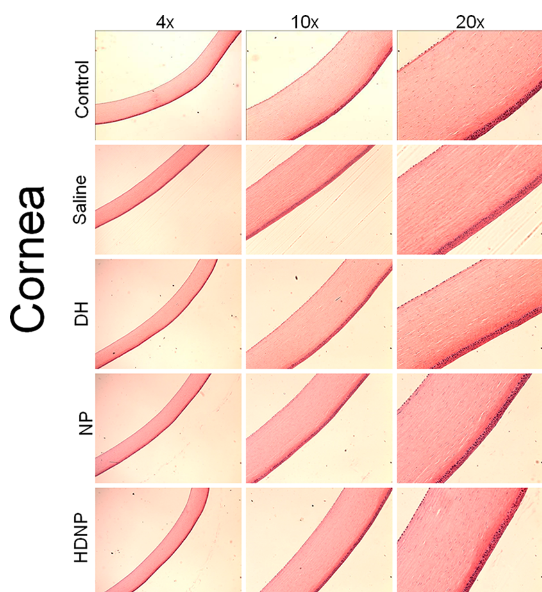

Figure 8. Cornea of rabbit eyes instilled with different formulations loaded with brimonidine and timolol maleate. Rabbits were sacrificed at the end of the study and the eyes were enucleated. The eyes were fixed in 10% formalin solution for 24 h and 5 μm sections were prepared. The sections were stained using hematoxylin and eosin. Images were captured at three different magnifications of 4×, 10×, and 20× using a light microscope.

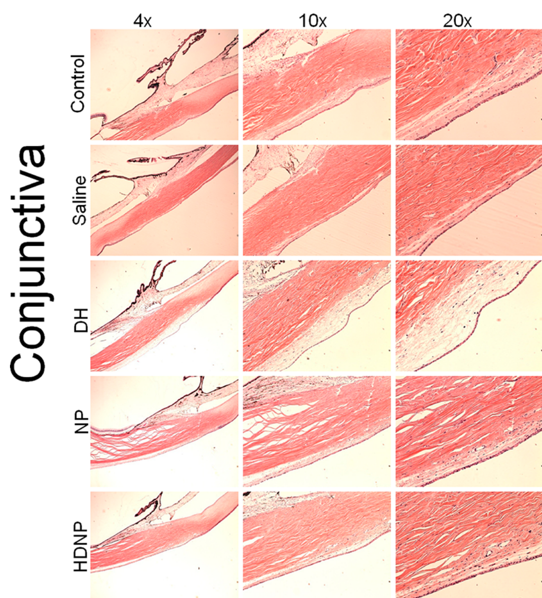

Figure 9. Conjunctiva of rabbit eyes instilled with different formulations loaded with brimonidine and timolol maleate. Rabbits were sacrificed at the end of the study and the eyes were enucleated. The eyes were fixed in 10% formalin solution for 24 h and 5 μm sections were prepared. The sections were stained using hematoxylin and eosin. Images were captured at three different magnifications of 4×, 10×, and 20× under a light microscope.

to ocular hypertensive, water-loaded rabbits because of the following considerations. First, the one-week duration of our study would require repeated water loadings in the same animal, which would be stressful for the animals. Second, these drugs provide only modest improvements in IOP reductions in water-loaded models. Furthermore, both brimonidine[23] and timolol[24] have been shown to reduce intraocular pressure in normotensive rabbits.

Following a single eye drop administration, individual DH and NP formulations were able to deliver a sustained IOP-lowering effect in Dutch-belted rabbits up to 48 h, which has surpassed the short duration of action of a saline formulation. More astonishingly, the HDNP formulation maintained an effective IOP reduction for 4 days. We attribute this greatly sustained IOP-lowering effect to the prolonged precorneal residence time, enhanced bioavailability, and well-controlled drug release as a result of integrating DH and NP into a single platform. Our work confirmed that HDNP significantly enhanced the absorption of both brimonidine and timolol maleate in aqueous humor and cornea as well as the absorption of timolol maleate in conjunctiva. Partially cross-linked PAMAM dendrimer G3.0-PEG-acrylate carries a large number of amine groups and, thus, confers mucoadhesiveness upon the formulation for strengthened interaction with the eye surface. Meanwhile, PVA-stabilized PLGA nanoparticles are anionic and nonmucoadhesive in nature. The *in vitro* particle uptake studies showed that PLGA nanoparticle uptake by cells was greatly enhanced by entrapment in PAMAM-G3.0-PEG-acrylate hydrogel. The enhanced uptake was observed at all time-points studied (5 min, 1 h, and 3 h). Drug-encapsulating PLGA nanoparticles can be taken up by cells through rapid endocytosis. PLGA polymers are used in the fabrication of several FDA-approved sustained release injectable products including Lupron Depot, Nutropin Depot, Zoladex, and Sandostatin LAR Depot.[25]

HDNP sustained drug release for 5 weeks without apparent burst release. The *in vitro* release kinetics correlated well with the extended drug efficacy *in vivo*. This sustained release of drug also helped minimize the potential toxicity of drugs due to burst release. Although drug release from biodegradable PLGA nanoparticles can be governed by passive diffusion and the degradation of the carrier,[26] our release kinetics studies showed that Fickian diffusion governs timolol maleate release from HDNP and contributes to the anomalous transport of brimonidine from HDNP along with macromolecular chain relaxation. Furthermore, a prolonged residence time of FluoSpheres on the eye surface mediated with dendrimer hydrogel is attributed to strong binding of positively charged PAMAM dendrimer to abundant mucins on the eye surface.

Our *in vitro* and *in vivo* studies showed that HDNP can make brimonidine and timolol maleate achieve extended release and sustain an effective IOP-lowering effect (18% reduction or higher, $p < 0.05$) for 4 days. The application of this new platform can potentially reduce dosing frequency of topical formulations. This would

significantly improve long-term patient compliance and reduce medical costs and societal burden. To further explore and validate this new platform, several issues that have not been adequately addressed in the current studies must be studied in future work. Future studies will include dose optimization for brimonidine and timolol maleate, mechanistic understanding of drug delivery and absorption, fine-tuning of the structure and properties of HDNP, examination of accumulation of the dendrimer in the eye, systemic drug exposure, long-term effectiveness of the formulation, distribution, long-term safety, and systemic effects of the platform, *etc*., all of which will be based on a large-scale animal study. More parameters of ocular response such as hyperemia, chemosis, lens opacity, and corneal defects will be examined to ensure its safety. Another issue with antiglaucoma drug eye drops is that patients can be very sensitive to the preservatives used in those drugs. Whether or not preservatives will be needed and whether they might irritate the eye should be taken into account. We assessed a single dose of the combination, which resulted in superior delivery for one week and sustained IOP reduction for 4 days. Further optimization of the system and dosing is needed for one-week effects with a single application. Dosing frequency reduction, for instance, from one drop daily to one drop per four days or one week, not only requires patient education on the importance of dosage regimen and employment of a reminder system to reduce nonadherence, but also increases the need for dosing and delivery precision. Delivery devices that can simplify self-administration and provide precise doses for the end user would be desirable to reduce the risk of IOP variation, which would be seen if a dose was not instilled properly. Solutions to patient noncompliance and nonadherence must be pursued when this platform is further evaluated for clinical application.

CONCLUSIONS

A hybrid dendrimer hydrogel/PLGA nanoparticles platform was developed and used to fabricate a brimonidine/timolol maleate combination formulation for topical application. Our work demonstrated that this new platform is capable of enhancing drug bioavailability and substantially sustaining drug activity following topical administration. This newly developed platform can greatly reduce dosing frequency of topical formulations, potentially improving long-term patient compliance and reducing enormous societal and economic costs. Given its high structural adaptability, many other chronic ocular diseases would benefit from long-lasting drug delivery of this new platform.

MATERIALS AND METHODS

Preparation of Drug Formulations. Four combination drug formulations having 0.7% w/v brimonidine (Sigma-Aldrich, St. Louis, MO) and 3.5% w/v timolol maleate (Sigma-Aldrich, St. Louis, MO) were prepared and evaluated in this work. All formulations were prepared under aseptic conditions to ensure sterilization and used immediately. No preservative was added to the prepared formulations in this work to avoid its potential cross effect in assays.

Saline Solution Formulations. The saline control formulations were prepared by dispersing 3.5 mg of brimonidine and 17.5 mg of timolol maleate in 500 μL of phosphate buffer saline (PBS, pH 7.4).

Dendrimer Hydrogel Formulations (DH). To prepare DH formulations, 3.5 mg brimonidine and 17.5 mg timolol maleate were dispersed in 500 μL of solution of G3.0-PEG-acrylate (8.1% w/v in PBS). Following addition of 25 μL of eosin Y photoinitiator solution (0.1% w/w eosin Y, 40% w/w triethanolamine TEOA, and 4% w/w 1-vinyl-2 pyrrolidinone), the solution was then exposed to long-wavelength (365 nm) UV light for 30 min and kept overnight under ambient light prior to use.

Nanoparticle Formulations (NP). The preparation is illustrated in Figure 1. Drug-encapsulating nanoparticles were prepared using the conventional o/w solvent evaporation method.[27] Resomer RG 503 H (PLGA, 50:50, M_w 24000−38000, Boehringer Ingelheim, Inc.) was used to make nanoparticles for drug encapsulation. Briefly, a PLGA solution was prepared by dissolving 100 mg of PLGA in 1 mL of dichloromethane. Brimonidine (20 mg) and timolol maleate (40 mg) were then dispersed in the PLGA solution. The polymer/drug dispersion was added to 10 mL of an aqueous solution containing 2% polyvinyl alcohol (PVA) as emulsifier, followed by a 1 min sonication with a power input of 10 W. The primary emulsion formed was transferred to 50 mL of PVA solution and then subjected to sonication for 3 min at a power input of 30 W. The secondary emulsion formed was kept under stirring at room temperature for 3 h. The nanoparticles formed were centrifuged at 2×10^4 g for 15 min. Upon the removal of the supernatant, nanoparticle pellets were dispersed in 25 mL of distilled water, followed by another round of centrifugation and dispersion in distilled water. The obtained brimonidine- and timolol maleate-encapsulating PLGA nanoparticles were freeze-dried. A nanoparticle formulation containing timolol maleate alone was prepared similarly. Particle size and zeta potential were measured with Malvern Nanosizer (Malvern Inc., Westborough, MA). Drug contents in both nanoparticle formulations were estimated using LC-MS/MS. The two formulations were combined to achieve 0.7% w/v brimonidine and 3.5% w/v timolol maleate in the final formulation in sterile PBS.

Hybrid Dendrimer Hydrogel/Nanoparticle Formulations (HDNP). PLGA nanoparticles equivalent to 17.5 mg of timolol maleate and 3.5 mg of brimonidine were prepare following the method described above and dispersed in 500 μL of PBS solution containing 8.1% w/v G3.0-PEG-acrylate and mixed with 25 μL of eosin Y photoinitiator solution. This dispersion was exposed to UV light for 30 min and then kept overnight under ambient light prior to use.

Cytotoxicity Assay. To test cytotoxicity of the drug formulations prepared, human corneal epithelial cells (HCETs) were plated in a 96-well plate at a seeding density of 5000 cells/well and allowed to grow for 24 h in serum containing medium. The medium was then replaced with 200 μL of serum free medium. The cells were either left untreated or incubated with 30 μL of drug eye drop formulation ($n = 6$) for 24 h. At the end of 24 h, the medium in each well was replaced with 200 μL of fresh serum-free medium. To each well 20 μL of 5 mg/mL MTT solution in pH 7.4 PBS was added. Following incubation for 3 h at 37 °C, the medium was aspirated out and the formazan crystals formed in the well were dissolved in 200 μL of dimethyl sulfoxide (DMSO). The absorbance of the dissolved formazan crystals in DMSO was recorded at 570 nm and used to estimate cell viability relative to control cells.

Protein contents in the cell lysates were quantified using the Micro BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL). Briefly, a Micro BCA working reagent was prepared by mixing Mirco BCA reagents A and B at 50:1 ratio. Afterward, 25 μL of the solution from the MTT assay was mixed with 200 μL of the Micro BCA working reagent and kept at 37 °C for 30 min. The absorbance of the mixture solution was measured spectrophotometrically at 562 nm for protein concentration determination. Bovine serum albumin was used as a standard in this assay.

***In Vitro* Cellular Uptake of Nanoparticles.** To investigate cellular uptake of PLGA nanoparticles, Nile red-loaded PLGA nanoparticles were first prepared using the o/w/w emulsion method.[28] Nile red-loaded PLGA nanoparticles (100 μg) were dispersed in 500 μL of PBS solution containing 8.1% w/v G3.0-PEG-acrylate to formulate an HDNP formulation. A saline solution of Nile red-loaded nanoparticles suspended in pH 7.4 PBS (100 μg per 500 μL; *i.e.*, NP) was prepared and used as a control.

The uptake of nanoparticles by HCETs was estimated. HCETs were seeded in 48-well plates at a density of 2×10^4 cells/well. The cells were allowed to adhere for 48 h to reach 80% confluence. Then, the growth medium in each well was replaced with fresh serum free medium. After 30 min, 30 μL of either HDNP or NP formulation containing 100 μg of Nile red nanoparticles was added to each well (n = 6). The cells were incubated with each formulation at 37 °C for 5 min, 1 h, or 3 h. At the end of each specified time interval, the cells were washed twice with 0.25 mL of acidic PBS (pH 5) and twice with 0.25 mL of pH 7.4 PBS to remove any loosely bound particles and then treated with 0.25 mL of PBS containing 2% v/v triton-X 100 to obtain cell lysate. Particles in cell lysate were quantified by reading the fluoresce intensity of encapsulated Nile red in a plate reader at an excitation of 485 nm and an emission of 608 nm. Nile red-loaded nanoparticle standards in pH 7.4 PBS ranging from 7.8 μg/mL to 1 mg/mL were used for the standard curve. Nanoparticle cell uptake was normalized to protein content in cell lysate. Protein content was quantified with Micro BCA Protein Assay Kit.

***In Vitro* Drug Release Studies.** Drug-loaded formulations (100 μL of saline or DH or 1 mL of HDNP or PBS containing 2 mg of drug-encapsulating PLGA nanoparticles) were placed in dialysis bags (Spectra/por 7 membrane, MWCO = 2000 Da). Each dialysis bag was completely immersed in 1.5 mL of dissolution medium (pH 7.4 PBS containing 0.05% sodium azide) in a 2 mL centrifugal tube. The tubes were constantly shaken in a shaker incubator, in which temperature was maintained at 37 ± 2 °C. At predetermined time intervals, the drug release medium was completely replaced with 1.5 mL of fresh medium pre-equilibrated to 37 ± 2 °C to maintain the sink condition. The amount of drug released to the dissolution medium was quantified with LC-MS/MS.

To elucidate the drug transport mechanism, the following four mathematical models were applied to fit the experimental data:[29]

$$M_t/M_\infty = kt^{1/2} \quad (1)$$

where M_t/M_∞ is the fractional drug release, k is a kinetic constant, and t is the release time. eq 1 is known as the Higuchi equation, which describes the Fickian diffusion of a drug.

$$M_t/M_\infty = kt^n \quad (2)$$

eq 2 is known as the Ritger-Peppas equation, in which n, a diffusional exponent, is used to describe drug transport mechanism: 0.5 (thin film) or 0.43 (sphere) for Fickian diffusion; 1 (thin film) or 0.85 (sphere) for Case II transport; and between 0.5 and 1 (thin film) or between 0.43 and 0.85 (sphere) for anomalous transport.

$$M_t/M_\infty = k_1 t^m + k_2 t^{2m} \quad (3)$$

where k_1 and k_2 are kinetic constants, and m is the diffusional exponent. eq 3 is also called the Peppas-Sahlin equation, which describes the drug transport as a result of Fickian diffusion (the first term) and Case II transport (the second term).

$$M_t/M_\infty = kt \quad (4)$$

eq 4 is the zero-order equation, in which drug transport is independent of drug concentrations.

To determine a model that best fits experimental data, fitting models were compared by running Akaike information criterion (AIC) test. Models with lower AIC values are more likely to be correct. In some cases, F-test was used as an alternative method for model comparison when AIC was unavailable due to limited data. OriginPro 8 software was used for fitting and model comparison.

***In Vivo* Drug Administration and IOP Measurements.** Normotensive adult Dutch-belted male rabbits (Mrytle's rabbitry, TN), 1.5–2.0 kg, were used in this work. The rabbits were provided with free access to food and water in a temperature-controlled room (18–24 °C). All rabbits used in this work were housed under proper conditions at the University of Colorado, Denver. The procedures conducted were approval by the IACUC of University of Colorado, Denver.

Three rabbits were used for each formulation (n = 3). Each formulation (30 μL) was instilled topically into the upper quadrant of the right eye using a positive displacement pipet (Gilson, Inc., WI) and the eye manually blinked three times. The IOP was measured at several time intervals (0.5 h before dosing; 0.5, 1.5, 3, and 6 h postdosing; and 1, 2, 3, 5, 6, and 7 days postdosing) using Tono-Pen AVIA Applanation Tonometer (Reichert Technologies, NY). IOP was also measured in the undosed left eye at all mentioned time intervals.

At the end of 7 days, animals were euthanized by intravenous injection of sodium pentobarbitone (150 mg/mL) in marginal ear vein. Eyes were enucleated and frozen immediately on dry ice: isopentane bath. Eyes were dissected in frozen condition to isolate different ocular tissues.

Drug Extraction and Recovery. Drug content in isolated tissue samples were estimated after liquid–liquid extraction of the drug from tissues. Briefly, the ocular tissues were mixed with 490 μL of 0.1 M NaOH and 10 μL of 12.5 μg/mL dorzolamide (internal standard) in 10 mL glass tubes, vortexed for 10 min, and then homogenized using a hand homogenizer on an ice bath. Basic NaOH solution was used here to keep basic molecules of analytes in un-ionized state. To this tissue homogenate, 4 mL of ethyl acetate/dichloromethane mixture (1:1 v/v) was added and samples were vortexed for 15 min and then subjected to centrifugation at 3000 g for 10 min to separate the organic layer. The separated organic layer was evaporated under nitrogen at 40 °C. The residue after evaporation was reconstituted in 1 mL of acetonitrile/water (75:25 v/v) and subjected for LC-MS/MS analysis. The liquid–liquid extraction method for extraction of brimonidine and timolol from bovine ocular tissue was validated to determine the extraction recovery using three different concentrations (low, medium, and high) to cover the entire range of expected concentrations of brimonidine and timolol.[14]

To analyze drug levels in aqueous humor, 40 μL of aqueous humor was diluted 2.5 times with acetonitrile–water mixture (75:25) containing dorzolamide as internal standard and subjected to LC-MS analysis.

Examination of Particle Retention and Distribution and Histological Analysis. FluoSpheres carboxylate-modified nanospheres (200 nm), yellow-green fluorescent, 2% solids (simply referred to as FluoSpheres; Invitrogen, Grand Island, NY) as a surrogate of PLGA nanoparticles were concentrated and then loaded into dendrimer hydrogel or plain pH 7.4 PBS to prepare FluoSpheres HDNP and NP formulations containing 20% FluoSpheres. They were applied to evaluate residence time on the eye surface for particles. The concentration of FluoSpheres in HDNP and NP (20%) was comparable to that of PLGA in HDNP and NP drug formulations.

NZW satin pigmented strain of rabbits (Western Oregon Rabbit Company, Philomath, OR) was used in the study. Drug-encapsulating HDNP or NP (30 μL) was instilled topically into the upper quadrant of one eye (right) and the eye manually blinked three times. The FluoSpheres hydrogel formulation (20%, 30 μL) or Fluospheres suspension in pH 7.4 PBS (20%, 30 μL) was instilled topically onto the left eye (n = 2 eyes). Evaluation of both eyes was performed before instillation, 2 h and 1–7 days after instillation. Eyes were dilated with 1% tropicamide solution before evaluations. The left eye was evaluated for fluorescence

using fundus camera (Kowa Optimed Inc., CA). A slit lamp (SL-D7, Topcon Medical Systems, NJ) was used to examine ocular inflammatory response such as anterior chamber cells and flare and pupil dilation as well as signs of discomfort such as tearing and blinking.

Rabbits were sacrificed at the end of the study. The eyes were enucleated, and a terminal blood sample was collected. The eyes that were instilled with drug encapsulating HDNP or NP formulation were fixed in 10% formalin solution for 24 h and 5 μm sections were prepared. The sections were stained using hematoxylin and eosin. The cornea and conjunctiva isolated from the treated eyes were studied for toxicity examination under a light microscope (Olympus BX41 laboratory microscope) fitted with a camera (Diagnostics Instruments, Inc.).

LC-MS/MS Analysis. The concentration of brimonidine and timolol in study samples were measured by means of LC-MS/MS. An API-3000 triple quadrupole mass spectrometry (Applied Biosystems, Foster City, CA) coupled with a PerkinElmer series-200 liquid chromatography (Perkin-Elmer, Walthm, MA) system was used. Analytes were separated on Zorbax extended C18 column (2.1 × 50 mm, 5 μm) using 5 mM ammonium formate in water (A) and acetonitrile (B) as mobile phase. The linear gradient elution at a flow rate of 0.3 mL/min, with a total run time of 6 min was as follows: 60% A (0−1.0 min), 10% A (2.0−4.0 min), and 60% A (4.5−6.0 min). Brimonidine, timolol, and dorzolamide (internal standard) were analyzed in positive ionization mode with the following multiple-reaction monitoring (MRM) transitions: 292 → 212 (brimonidine); 317 → 261 (timolol); and 325 → 199 (dorzolamide). The assay's detection limits for quantitation of brimonidine and timolol maleate were 5.8 and 0.725 ng/mL, respectively. Plasma levels of drugs at the end of the study were below detection limits in all groups.

Statistical Analysis. Data were reported as mean ± SD and analyzed with one-way analysis of variance (ANOVA) followed by *t* test for pairwise comparison of subgroups. *P* values <0.05 were considered statistically significant.

Conflict of Interest: The authors declare no competing financial interest.

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