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Daylight-Mediated, Passive, and Sustained Release of the Glaucoma Drug Timolol from a Contact Lens

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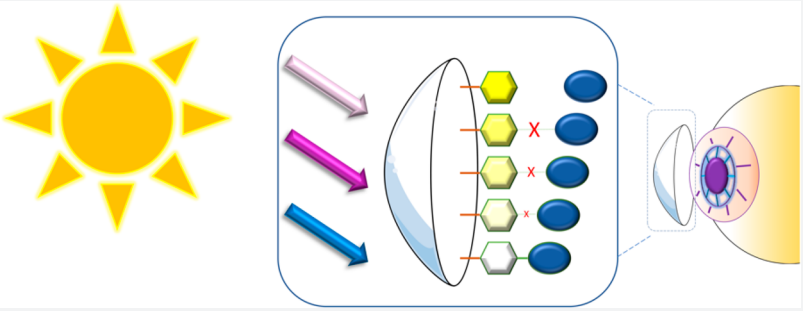
Supporting Information



ABSTRACT: Timolol, a potent inhibitor of β-adrenergic receptors (βARs), is a first-line drug for decreasing the intraocular pressure (IOP) of patients with glaucoma. Timolol is administered using 0.5% eye-drop solutions at >3 × 10^7 times the inhibitory concentration (k_i) for βARs. This high dose is wasteful and triggers off-target effects that increase medication noncompliance. Here, we introduce contact lenses that release timolol to the eye throughout the day during passive exposures to natural daylight at a more therapeutically relevant concentration (>3000 k_i). Timolol is coupled to the polymer of the contact lens via a photocleavable caged cross-linker and is released exclusively to the surrounding fluid after the 400−430 nm mediated cleavage of the cross-linking group. Studies conducted in a preclinical mouse model of glaucoma show photoreleased timolol is effective as authentic timolol in reducing IOP. Our studies highlight several advantages of daylight-mediated release of timolol from lenses compared to eye-drops. First, fitted contact lenses exposed to natural daylight release sufficient timolol to sustain the inhibition of βARs over a 10 h period. Second, the contact lenses inhibit βARs in the eye using only 5.7% of the timolol within a single eye-drop. Third, the lenses allow the patient to passively control the amount of timolol released from the lens—for example, early morning exposure to outdoor sunlight would release enough timolol to maximally reduce the IOP, whereas subsequent periodic exposures to indoor daylight would release sufficient timolol to overcome the effects of its spontaneous dissociation from βARs. Fourth, our lenses are disposable, designed for single day use, and manufactured at a low cost.

INTRODUCTION

Glaucoma is a neurodegenerative ocular disease characterized by the progressive death of retinal ganglion cells, irreversible loss of the visual field, and blindness.[1] Glaucoma risk factors include aging, diabetes, and cardiovascular conditions, and it is projected to affect >100 million people by 2040.[2,3] The onset and progression of glaucoma are accompanied by an elevation of the intraocular pressure (IOP),[4] which is managed by administering drugs to decrease the production of the aqueous humor. Among these drugs are potent inhibitors of the nonselective β-adrenergic receptors (βARs),[5] including timolol. Currently, timolol is administered to patients in the form of concentrated eye-drops (0.5 wt %; 15.8 mM or 0.425 mg in 85 μL), which is notoriously inefficient and wasteful.[8] The concentration of timolol in a single eye-drop is ∼3 × 10^7 higher than the inhibitory concentration for βARs (k_i = 0.5−0.6 nM),[7,6] and although sufficient to inactivate βARs in the eye, inhibition is temporary owing to spontaneous dissociation of the drug from timolol−βARs complexes.[7] Interestingly, spikes in the IOP during sleep[9] are not reduced by timolol. More than 80% of the timolol delivered from an eye-drop collects in the nasolacrimal duct, where it is absorbed through nasal mucosa and enters the bloodstream, reaching a serum concentration of 0.4 ng/mL (∼1.3 nM).[10,11] Because this concentration exceeds the k_i for βARs in the heart and lung, it may result in cardiac and respiratory dysfunctions.[7,10,12−15] Finally, topical application of 0.5% timolol is also known to induce migraines, burning-sensations, and blurred-vision that collectively undermine medication compliance.[6,16] Although drug-soaked contact lenses have been reported to release drugs to manage glaucoma, they offer few benefits over traditional eye-drops because the drug molecules rapidly diffuse out of the lens.[17−21] To reduce these timolol overdose-related effects, we have engineered contact lenses to sustain the release timolol during exposures to daylight at a more therapeutically relevant

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dose (>3000 k_i) compared to eye-drops. Specifically, our engineered contact lenses are designed to passively release timolol from their surfaces during exposures to blue−violet wavelengths (400−430 nm) of natural light for at least 10 h (Figure 1). The contact lenses are composed of a hydrogel

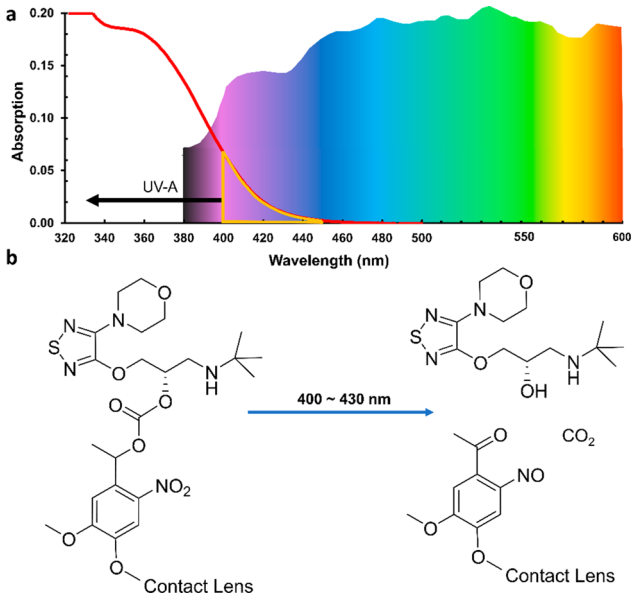

Figure 1. (a) Overlay of the intensity−wavelength distribution of daylight and the action spectrum of caged timolol shown in orange. (b) Structure of the caged timolol covalently bonded to a contact lens and the products of the photocleavage reaction triggered by 400−430 nm light. The daylight spectrum was adapted from the source.[37]

polymer that is chemically linked to ∼200 μg (0.27 μmol) of timolol via a photolabile cross-linking group (caged timolol) (Figure 1b). The synthetic schemes used to prepare the reactive caged timolol cross-linkers are shown in Figure 2. We chose the dimethoxy-substituted 2-nitrobenzene caged group over other photocleavable groups because its action spectrum overlaps favorably with the violet-blue wavelengths of daylight.[22,23] Contact lenses coupled with caged timolol, such as the one shown in Figure 4, are fabricated within a few hours using a 2-component cast system (Supporting Information, Figure S1). The copolymerization reaction generates a transparent lens that harbors a uniform concentration of caged timolol and absorbs ∼44% of photons at 400 nm and the majority of photons over the UV-A range.[23,24] Exposure of the lens to 405 nm or indoor daylight excites the caged group, and is followed by a clean excited-state photoisomerization reaction that results in the cleavage of the carbonate bond, releasing active timolol from both surfaces of the lens (Figure 1a). The 2-nitrosobenzaldehyde photoproduct, on the other hand, remains covalently attached to the polymer, and its yellowish tinge provides measure of the amount of timolol released from the lens (Figure 4b).

Our studies highlight several advantages of contact lenses that passively release timolol and related therapeutics during exposures to daylight compared to treatments that employ eye-drops or drug-soaked lenses. For example, lenses exposed to indoor daylight would release sufficient timolol to sustain the inhibition of βARs in the eye over a 10 h period, corresponding to ∼5.7% of the timolol present in a single eye-drop. Moreover, the patient may control the amount of drug released from the lens, for example, by venturing outdoors on a sunny day, which would release higher levels of timolol. Thereafter, exposures to indoor daylight would release lower levels of timolol to compensate for the effects of spontaneous dissociation of timolol from βARs. Finally, our single-use lenses are produced at low cost (∼$0.50).

RESULTS AND DISCUSSION

Guiding Calculations on the Loading of Caged Timolol and Photorelease of Timolol from Fitted Contact Lenses. The timolol−βAR complex has a half-life of several hours,[7] and so in the absence of a fresh source of timolol, a single early morning dose of timolol would result in appreciable reactivation of βARs by the end of the day. Our lenses are designed to replenish timolol in the eye between successive blinks to sustain the inhibition of βARs for up to 10 h. Arbitrarily setting the percentage of timolol molecules photoreleased from the lens that inhibit βARs at a very low value of 0.1%, the lens should release timolol between successive blinks at 1000 times the k_i (500 nM). This concentration would correspond to the release of $\sim 1.8 \times 10^{-8}$ mol or 5.7 μg of timolol over a 10 h period. This quantity is considerably less than the amount of caged timolol that we routinely couple to our lenses (200 μg or 0.27 μmol), of which approximately half would be exposed to light once fitted to the eye. Another factor to consider in the design of our lens is the probability of light-absorption by caged timolol molecules bonded to the lens, and the efficiency of the photocleavage reaction that releases timolol. According to the Beer−Lambert law, a transparent 55 μL contact lens (radius 0.6 cm) of 0.5 mm thickness loaded with 200 μg of caged timolol (2.7×10^{-7} mol) would absorb ∼44% of the photons between 400 and 430 nm, and the majority of photons in the UV-A region, i.e., <400 nm (Figure 1a).[23,24] Given a quantum yield for the photoisomerization of the dimethoxy-2-nitrobenzene of ∼0.1,[23,24] a caged timolol-coupled lens would generate the k_i concentration of timolol (5×10^{-15} mol) in a 10 μL volume following an exposure to $\sim 3 \times 10^9$ photons between 400 and 430 nm. We have estimated a fitted contact lens exposed to natural light would be impacted by $\sim 10^{14-16}$ photons between successive blinks (10 s), of which ∼10% would be in the blue−violet range (400−430 nm; Figure 1a). Using the lower rate of exposure to blue−violet photons, i.e., 10^{13}/10 s, the lens could release 3300 times the k_i concentration of timolol in a 10 μL volume between successive blinks. We note higher levels of timolol would be released from the lens during exposures to direct sunlight, a consequence of unavoidable exposures to UV-A wavelengths and the generally higher intensities of photons over the 400−430 nm region of sunlight (<399 nm; Figure 1).

Design of Daylight-Mediated, Timolol-Releasing Contact Lenses. Our approach to release active timolol from both surfaces of the contact lens during exposures to blue−violet light is schematized in Figure 4. By immobilizing caged timolol throughout the polymer backbone, we can realize four important design goals for a light-activated therapeutic contact lens. First, since caged timolol is inactive,[25] and moreover physically isolated from target βARs, the inhibition of βARs will be controlled exclusively by daylight-mediated photo-uncaging of the 2-nitrobenzyl group (Figures 1b and 4). Second, contact lenses exposed to daylight (400−430 nm) should release timolol to the eye over a 10 h period via a first-order reaction and at a more therapeutically relevant

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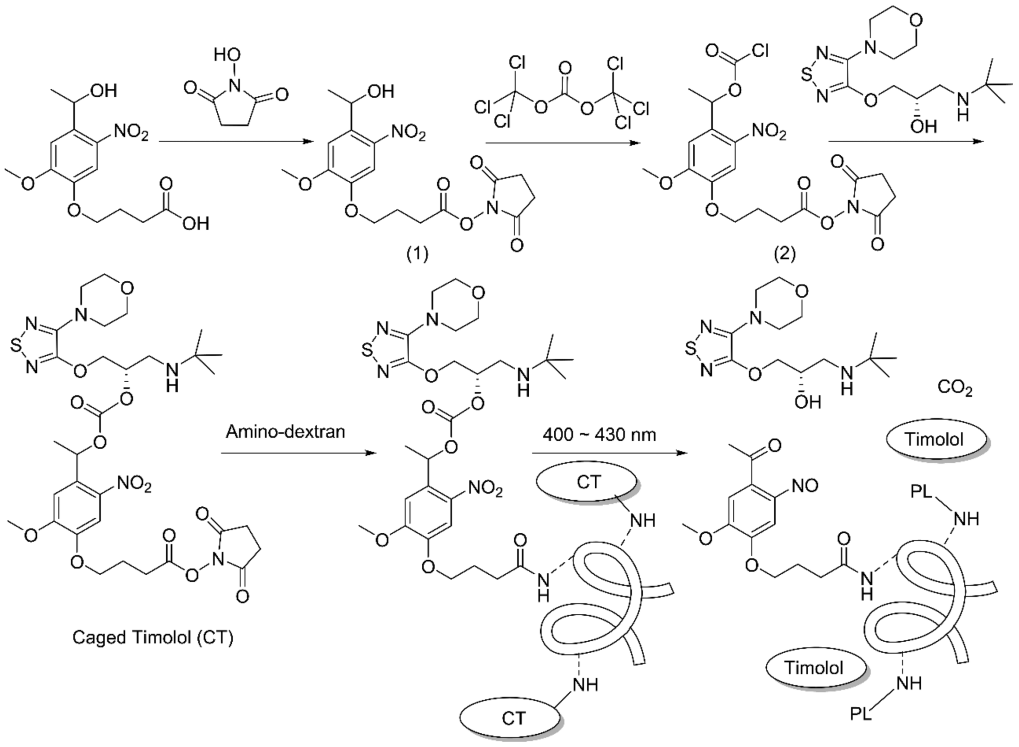

Figure 2. Synthetic protocols used to prepare caged timolol and its conjugation to amino-dextran. The carboxylic group of hydroxyethyl photolinker (PL) was first esterified with *N*-hydroxysuccinimide (NHS) to form the activated succinimidyl ester (compound **1**). Triphosgene was used to react with the hydroxyethyl group of PL to form a chloroformate (compound **2**), which was coupled to timolol via a carbonate linkage to form caged timolol. The reactive NHS-functionality was used to couple caged timolol to primary amine groups on amino-dextran. Photoisomerization of the caged timolol cross-linker triggered by the blue−violet light (400−430 nm) component of sunlight releases active timolol from the dextran, while the photoproduct remains chemically attached to dextran.

dose compared to a single eye-drop (>3000k_i versus $3 \times 10^7 k_i$, respectively). Third, the 2-nitrosobenzaldehyde photoproduct of the uncaging reaction should remain covalently bound to the polymer of the disposable contact lens (Figure 4). Fourth, the lens should allow the patient to control the amount of timolol released from the lens; for example, he/she may want to boost the amount of released timolol to achieve full inhibition of βARs by venturing out earlier on a sunny day, and to spend periods indoor to decrease the amount of photoreleased timolol to a level that overcomes the effects of spontaneous dissociation of timolol molecules from βARs.[7]

Characterization of the Photo-Uncaging of Timolol Using a Model Conjugate. To evaluate the photo-uncaging of timolol from caged timolol on the contact lens, we performed photochemical and chemical characterization studies using an amino-dextran conjugate of caged timolol as a model of the hydrogel (Figure 2). We prepared the dextran conjugate by adding the NHS-ester of caged timolol (14.8 mg or 20 μmol) from a *N*,*N*′-dimethylformamide stock (DMF) to 100 mg or 2.5 μmol of amino-modified dextran (40 kDa with an average of 20 amino groups) in 3 mL of phosphate buffered saline (PBS), followed by 5 μL of triethylamine. After incubating the mixture in the dark for 30 min at room temperature with gentle shaking, we removed unreacted caged timolol, DMF, and other small molecules by dialyzing the reaction mixture against water using a 10 000 MW cutoff membrane. After passing the dextran conjugate through a 0.22 μm micron filter, we subjected 3 mL aliquots of the conjugate to light. First, we recorded the absorption spectrum (Figure 3a) of the caged timolol conjugate before and after defined exposures to a 405 nm light source [a Cairn Research 395 nm LED whose output was filtered through a Schott UG390 nm filter to remove UV-A (<400 nm; power = 0.2 mW/cm^2; Supporting Information, Figure S2)]. Analysis of the intensity of the 350 nm absorption of the preirradiated sample using an extinction coefficient of 5000 M^{-1} cm^{-1}[24,26] showed the dextran conjugate contained 130 μM caged timolol; i.e., the 3 mL solution contained 3.9×10^{-7} mol (255 μg) of caged timolol. The absorption spectra of the caged timolol conjugate as a function of the 405 nm LED-exposure time are shown in Figure 3a. These overlaid spectra reveal two isosbestic points at 300 and 425 nm, a finding that suggests the photo-uncaging reaction conducted over 26 min is clean and leads to defined changes of the populations of three absorbing species in the sample, namely, caged timolol (which shows a decrease at 350 nm), the uncaged photoproduct (whose spectrum extends beyond that of caged timolol), and the photoreleased timolol (which shows an increase at 295 nm). A plot of the absorption value at 350 nm as a function of 405 nm LED-exposure time shows the uncaging reaction proceeds at an initial rate of 0.25 nmol/s (Figure 3a insert). Next, we dialyzed the postirradiated (405 nm) solution (3 mL) against 3 changes of water (20 mL each) using a 3000 MW cutoff dialysis membrane to identify small molecule products of the uncaging reaction in the dialysate. After reducing the volume of the combined water dialysate to 3 mL, we filtered the solution through a 0.22 μm micron filter and subjected the sample to mass spectrometry. We found the dialysate contained a single species with m/z of 317.1642, which corresponds precisely to that expected for timolol (Supporting Information, Figure S4). Using an

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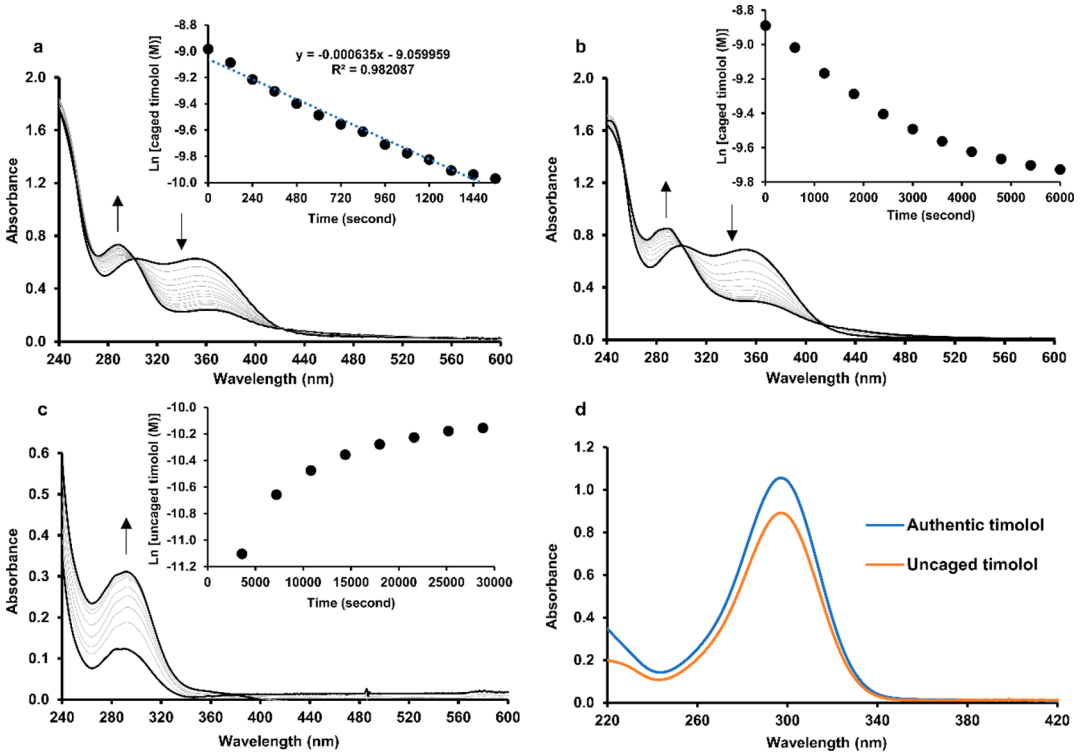

Figure 3. (a, b) UV−vis spectroscopic studies of 405 nm and daylight-triggered release of timolol from amino-dextran-conjugated caged timolol. The caged timolol−dextran conjugate was dialyzed against water using a 10 kDa cutoff membrane. Two aliquots were withdrawn and exposed to 405 nm and natural daylight. The absorption spectra of the samples were recorded at intervals initially every 2 min for 26 min for part a, and then every 10 min for 100 min for part b until no further change was observed. The insert of each plot shows the change in the natural logarithm of the concentration of amino-dextran-conjugated caged timolol converted from absorption intensity at 350 nm versus time, suggesting the uncaging reaction proceeds via a first-order reaction. (c) UV−vis spectroscopic study of daylight-triggered release of timolol from an engineered lens that was exposed to indoor sunlight—the spectra were recorded at intervals of 1 h for 8 h. The insert plot shows the change in the natural logarithm of the concentration of photoreleased timolol in the bathing solution converted from absorption intensity at 295 nm versus time. Timolol was shown to be the only small molecule released from the contact lens as made evident by (d) UV−vis spectroscopy, and mass spectroscopy and ^{1}H NMR (see the Supporting Information, Figures S6 and S13, respectively).

extinction coefficient for timolol as 7924 M^{-1} cm^{-1} at 295 nm,[27] and an absorption value at 295 nm of 0.54, the calculated concentration of photoreleased timolol in the 3 mL solution of 6.8×10^{-5} M indicates the exposure to 405 nm released $\sim 2.0 \times 10^{-7}$ mol or ~63.3 μg of soluble timolol from the conjugate, corresponding to ~51% of the original 0.39 μmol of the caged timolol dextran conjugate.

Next, we exposed the second 3 mL solution of the caged timolol−dextran conjugate to indoor sunlight—we conducted these studies indoors on typical Spring mornings on the University of California—Berkeley campus (Supporting Information, Figure S3). We recorded the absorption spectra of the solution as a function of the exposure time. The absorption spectra recorded for one of these time-series studies (n = 3) shown in Figure 3b reveal the same isosbestic points and spectral shift to that recorded for the 405 nm exposed caged timolol−dextran conjugate (Figure 3a). After a 100 min exposure to indoor daylight, we dialyzed the sample and processed the dialysate precisely as detailed for the 405 nm LED study—once again, we established from the mass spectroscopic and ^{1}H NMR analyses that the dialysate contained pure timolol (Supporting Information, Figures S5 and S13, respectively). From the absorption intensity at 295 nm of 0.58, we calculated the 3 mL solution contained 7.3×10^{-5} M of photoreleased timolol (2.2×10^{-7} mol or 69.6 μg), corresponding to ~56.4% of the original 0.39 μmol of caged timolol. The nonlinear release kinetics recorded for daylight-exposed caged timolol−dextran (Figure 3b) may have resulted from a decrease in the intensity of indoor daylight over the 100 min study, as we note the same analysis conducted for the sample exposed to the constant energy 405 nm LED was linear, and best described as a first-order reaction (Figure 3a). Our experiments show similar amounts of timolol were released from the dextran conjugate during exposures to the 405 nm LED light and indoor daylight. We calculated the amount of timolol released during an exposure of caged timolol−dextran to indoor daylight would correspond to the release of a fresh ~6 μM dose of timolol at 12 000k_i in the 10 μL volume of every tear-film over a 10 h period (3600 blinks).

Characterization of the Photo-Uncaging of Timolol in a Contact Lens. Having demonstrated that exposures of caged timolol−dextran conjugates to 400−430 nm light release pure timolol to the bathing solution, we investigated the release of timolol from contact lenses exposed to indoor daylight. After testing a number of copolymer systems for the fabrication of the caged timolol-coupled contact lens, we finally selected a hydrogel copolymer composed of 2-hydroxyethyl methacrylate (HEMA), ethylene glycol dimethacrylate (EGDMA), and *N*-vinyl-2-pyrrolidone (NVP).[19] This type of copolymer is similar in composition to the cosmetic contact lenses popular with young people in Asia. As detailed in the Materials and Methods section and in Figure 4, we reacted the

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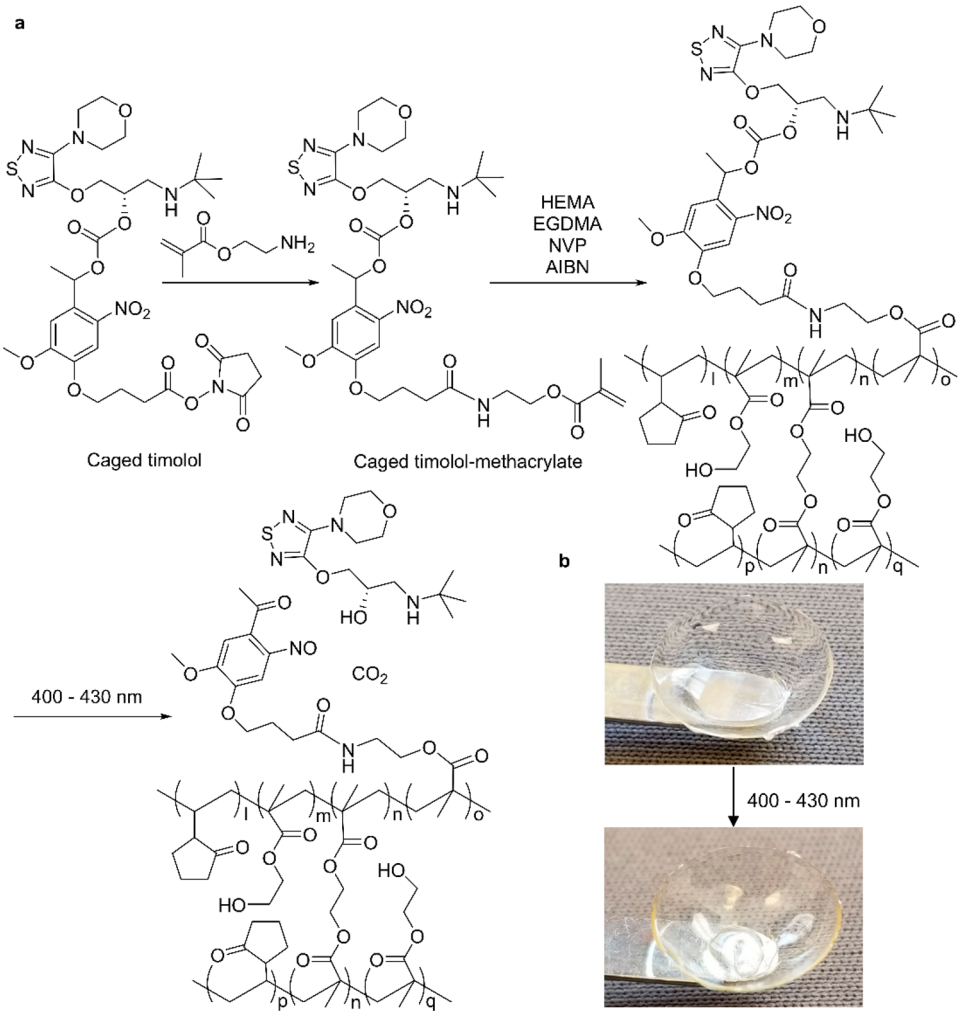

Figure 4. (a) Synthetic protocol used to introduce the methacrylate group to caged timolol and its subsequent integration for the fabrication of the engineered contact lens, including details of the photoisomerization reaction triggered by blue−violet light (400−430 nm) of daylight to release timolol. The methacrylate group was introduced to caged timolol by the reaction of the NHS-ester of timolol with 2-aminoethyl methacrylate. This compound was mixed with HEMA, NVP, and EGDMA and copolymerized in a 2-component cast to fabricate caged timolol-conjugated contact lenses. The polymerization was initiated thermally by the addition of AIBN at 90 °C for 2 h. Exposure to the daylight led to the release of timolol, while the uncaged product remained chemically bound on the contact lens. (b) The engineered contact lens was immersed in PBS bath and exposed to the daylight for 8 h; its color changed from transparent to slightly yellow (the color of uncaged photolinker) after the exposure.

NHS-ester of caged timolol with 2-aminoethyl methacrylate to generate a free methacrylate group, which we subsequently copolymerized with the (meth)acrylate groups of HEMA, EGDMA, and NVP. We initiated the polymerization reaction thermally using azobis(isobutyronitrile) (AIBN) as a catalyst—a soft yet pliable polymer formed in about 2 h at 90 °C. Next, we conducted the same polymerization reaction within a 2-component cast that is used to fabricate cosmetic contact lenses (Supporting Information, Figure S1). In some cases, we also grafted the surface of the lens with a PEG brush to mask surface-exposed caged timolol and its photoproduct from any interacting cells.[28] In particular, after removing the contact lens from the cast, it was immersed in 3 mL of 20% 4-Arm-PEG-acrylate at room temperature for 24 h in the dark and transferred to 3 mL of water. Next, we added ammonium persulfate and *N*,*N*,*N*′,*N*′-tetramethylethylenediamine to initiate its polymerization at the surface. After a 30 min reaction, we washed the lens five times with 10 mL of distilled water over 24 h in the dark to remove unreacted monomers and caged timolol, and to fully hydrate the lens. Next, we investigated whether caged timolol, timolol, or other UV−vis absorbing component was capable of leaking from the contact lens by recording the absorption spectrum of the bathing solution of a lens immersed in 2 mL of PBS in the dark for 3 days. As can be seen in the spectrum of Figure S7, we did not find any evidence of absorbing species (250−500 nm) in the bathing solution that correspond to caged timolol or timolol (Supporting Information, Figure S7).

Next, we exposed transparent contact lenses loaded with 200 μg of caged timolol and suspended in PBS (2 mL) to indoor daylight for 8 h and recorded absorption spectra of the bathing solution at defined time-points. Analyses of the overlaid absorption spectra from this study (Figure 3c) show the intensity of the 295 nm peak increased in an exposure-time-dependent manner. Next, we determined from UV-absorption (Figure 3d) and mass spectroscopy that timolol was the only molecule released to the bathing solution during the exposure to indoor light (Supporting Information, Figure S6). The absence of any absorption intensity beyond 350 nm in the bathing solution strongly suggests the red-shifted 2-nitro-

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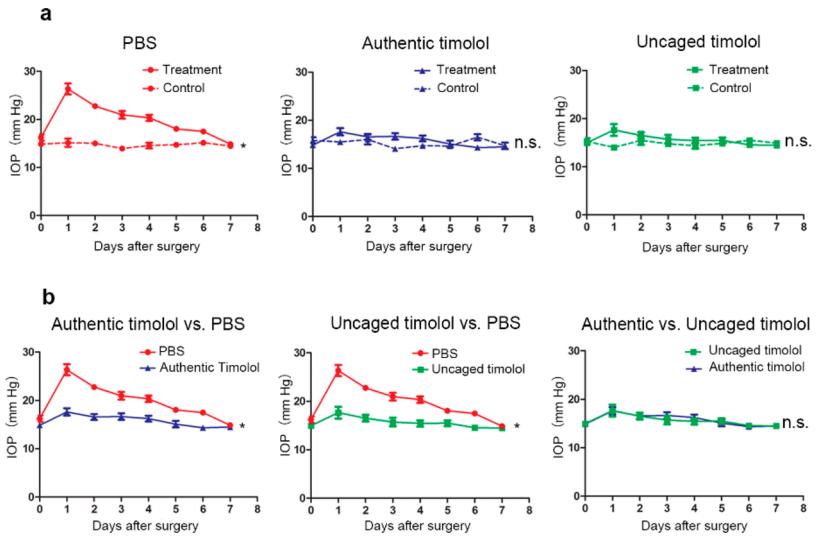

Figure 5. Comparative analysis of elevation of intraocular pressure (IOP) between different mouse groups. (a) Comparison between lasered (right) eye and control left eye in three treatment conditions: PBS (negative control), 0.5% timolol maleate in PBS (positive control), and 0.5% solution of uncaged timolol in PBS. (b) Comparison between the three treatment conditions in the lasered right eyes. Our results show uncaged timolol is as efficient as authentic timolol in reducing the IOP in comparison to control PBS which demonstrated no effect on IOP. *p < 0.05. n.s., not significant (n = 7/group).

sobenzaldehyde photoproduct remains chemically fixed to the polymer of the contact lens—the pale-yellow color of the daylight-exposed contact lens supported this conclusion (Figure 4b). Based on our analysis of the kinetics of the uncaging reaction shown in Figure 3a,b, we conclude the photorelease of timolol from the contact lens proceeds via a first-order reaction mechanism—we believe the progressive decrease in the reaction rate over the 8 h study, evident in the plot shown in Figure 3c, is due to the time-dependent decrease in the intensity of the 400−430 nm component of daylight. Using the intensity of the 295 nm absorption (0.308) recorded at the end of the 8 h exposure of the lens to daylight, we calculated the lens released ∼77.5 nmol or ∼24.4 μg of timolol to the 2 mL bathing solution (38.8 μM). Spread over a period of 10 h, we calculated the lens would have released timolol at an average of ∼4305 times the k_i concentration for βARs between successive (10 s) blinks. Moreover, the amount of timolol released from the lens represents ∼12.2% of the 200 μg of caged timolol coupled to the lens. We estimate the amount of caged timolol coupled to the lens could be reduced by a factor of 3 without affecting the effectiveness of the photorelease of timolol from the lens. The amount of timolol released from the contact lens over the 10 h study (∼24.4 μg) represents ∼5.7% of the amount of timolol within a single eye-drop (85 μL; 0.5% timolol; 425 μg). In summary, our studies demonstrate the feasibility of using caged timolol-coupled contact lenses for daylight-mediated, passive, and sustained release of timolol for at least 10 h at a more therapeutically relevant dose than that possible using eye-drops.

In Vivo Analysis of the Functional Activity of Uncaged Timolol. To investigate the functional activity of optically released timolol in reducing elevated IOP, we performed in vivo assays using a well-established preclinical mouse model of glaucoma induced via laser-mediated photocoagulation of the episcleral veins for 1 week.[29] As we reported previously, we performed the surgery on the right eye of each mouse, while using the left untreated eye as a control.[29] For the experiments in this study, we first concentrated and filtered the water dialysate of the 405 nm LED-exposed caged timolol to achieve a 0.5% (15.8 mM) solution, i.e., identical to that used in eye-drops. Next, we applied 10 μL of the uncaged timolol, authentic timolol (both at 0.5% or 15.8 mM), or control PBS to the laser-treated eye of each mouse three times per day for 7 days and recorded the IOP of both eyes for each mouse at daily intervals. Moreover, to investigate the therapeutic effects on glaucoma parameters of corneal edema and retinal nerve fiber layer (RNFL) thinning, we also monitored the mouse eyes in vivo using the anterior and posterior-segment optical coherence tomography (OCT), respectively. Since retinal ganglion cell (RGC) death is another important parameter of glaucomatous damage, we assessed it as well by ex vivo immunofluorescent microscopic assays. RGCs were detected using a specific antibody against Brn3a, a common marker for RGCs,[30] which was recognized by a Cy3-conjugated secondary antibodies for imaging. We recorded a significant increase in IOP in the right eyes of the mice after laser photocoagulation, which was unaffected by PBS administration (Figure 5). In contrast, IOP of laser-treated eyes receiving purified (uncaged) timolol decreased at a similar rate to that recorded in mice treated with authentic timolol. While the lasered eyes of control PBS-treated groups showed corneal edema, RGC death and RNFL thinning due to elevated IOP, all three parameters were significantly reduced in the uncaged timolol or authentic timolol-treated eyes (Figure 6). Thus, uncaged timolol is indistinguishable from authentic timolol in its ability to decrease the IOP and to protect the ocular structures of a mouse model of glaucoma. We elected to forego studies to evaluate the effectiveness of our lenses fitted to the eyes of the mouse model of glaucoma, as one could not rule out the possibility that a change in the IOP resulted from a non-timolol mechanism, for example, stress and efforts by the mouse to physically remove the lens. We are currently engaged in discussions to evaluate the effectiveness of daylight-mediated release of timolol from our lenses on human subjects.

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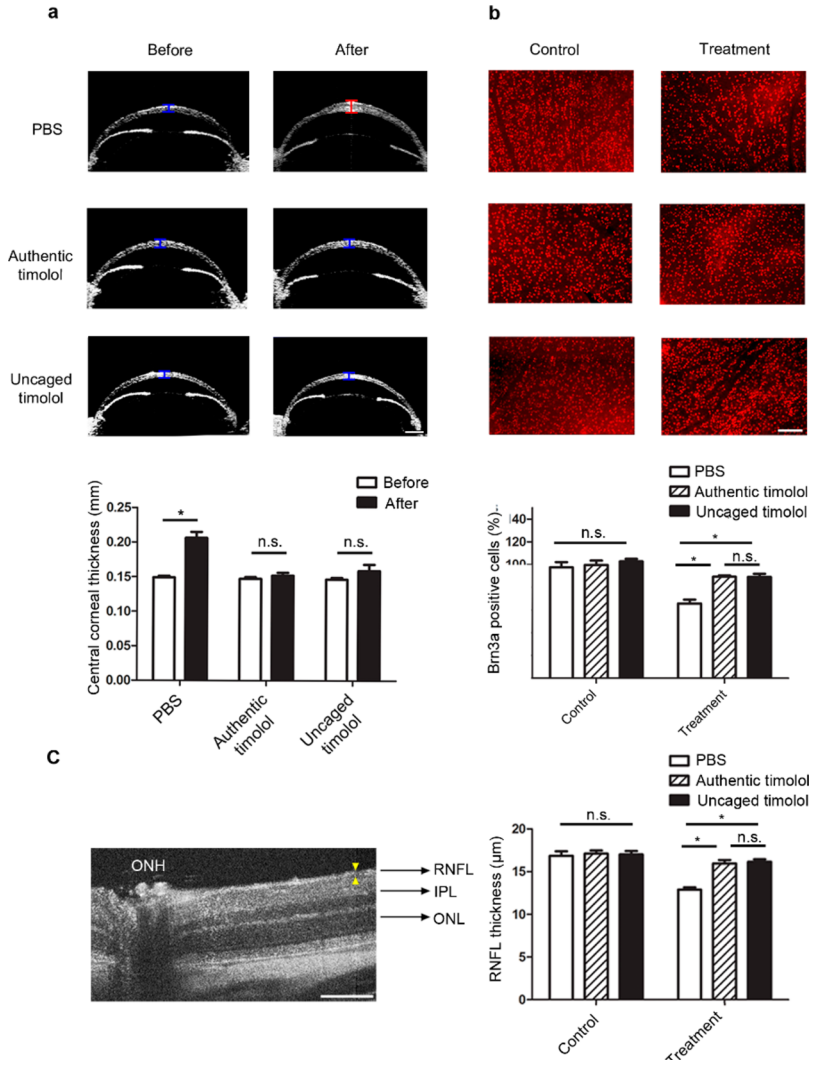

Figure 6. Uncaged timolol protected ocular structures and significantly reduced corneal edema, retinal ganglion cell loss, and retinal nerve fiber layer thinning associated with glaucoma. (a) In vivo anterior-segment OCT analysis showing the central cornea thickness (indicated by red or blue brackets) was significantly reduced at day 3 post laser treatment in uncaged timolol and authentic timolol-treated eyes, compared to PBS control condition. Scale bar: 0.5 mm. *$p < 0.05$. n.s., not significant ($n = 7$/group). (b) Ex vivo immunofluorescent microscopic analysis showing RGC loss was significantly reduced at day 7 post laser treatment in the uncaged timolol and authentic timolol-treated eyes. Red: Brn3a. Scale bar: 100 μm. *$p < 0.05$. n.s., not significant ($n = 6$/group). (c) In vivo posterior-segment OCT analysis showing RNFL thinning was significantly reduced at day 7 post laser treatment in the uncaged timolol and authentic timolol-treated eyes. RNFL, retinal nerve fiber layer; IPL, inner plexiform layer; ONL, outer nuclear layer; ONH, optic nerve head; scale bar: 100 μm. *$p < 0.05$. n.s., not significant ($n = 7$/group).

CONCLUSIONS

We have developed a contact lens that releases timolol during exposures to natural daylight at more therapeutically relevant doses (>3000 times the k_i concentration of timolol for βARs) compared to that within a single eye-drop ($\sim 3 \times 10^7 k_i$). Optically triggered release of timolol from the contact lens to the eye affords several benefits over eye-drop approaches. First, daylight-exposed contact lenses are designed to passively release a fresh dose of timolol to as much as ~4305 times the k_i for βARs between successive blinks over a 10 h period. Second, the concentration of timolol released to the eye between successive blinks is ~10 000 times less than that contained within a single 0.5% eye-drop, which should help to reduce off-target effects and low patient compliance associated with eye-drop delivery.[6,16] Importantly, the total amount of timolol released from the lens represents only ~5.7% of that contained in a single 0.5% eye-drop solution. Third, disposable caged timolol-coupled contact lenses are fabricated at low cost, estimated at ~$0.50 (excluding labor and capital investments). Unlike more expensive extended wear drug-releasing lenses that may suffer from contamination and require the patient to recharge lenses with timolol every day,[31] the low cost of our lenses compared to eye-drop solutions ($185 for a 1 month supply)[8] would allow the patient to use a fresh caged timolol loaded lens each day. Fourth, sustained release of timolol from lenses exposed to daylight would allow the patient to maintain full inhibition of βARs until they are removed after sunset; i.e., once the fitted lens has released sufficient timolol to decrease the IOP, the patient would only need to seek additional daylight exposure to release an amount of timolol that compensates for spontaneous dissociation of timolol molecules from βARs.[7] In this regard, one might recommend to the patient that he/she boosts timolol release to the eye soon after fitting the lenses by venturing outside on a sunny day for 30

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min, and thereafter and until sunset to periodically expose the lens to indoor daylight to sustain the inhibition of βARs.

Looking ahead, we envisage three technological developments to improve the performance and functions of light-mediated drug-releasing contact lenses. The first would be to integrate a recently reported sensor of IOP in a contact lens[32] coupled with caged timolol. A theranostic contact lens that integrates IOP-sensing with passive release of timolol would allow the patient to passively self-medicate by controlling their exposures to daylight, as described earlier. Second, we note it should possible to red-shift the action spectrum to uncage timolol to the blue−green region of the wavelength spectrum (450−500 nm) by introducing additional *n*- or π-bonding units to the 2-nitrobenzyl group.[22,24,26,33] Red-shifted caged cross-linkers would also make it possible to release the drugs from the lens during passive exposures to indoor lighting systems, for example, fluorescent and LEDs. The third development would be to adapt the caged cross-linking coupling-strategy to introduce other small molecule drugs to manage or treat ocular conditions, including prostaglandins and antibody therapeutics[23,24] against vascular epithelia growth factor receptors (VEGFRs), including Avastin, Eylea, and Lucentis, to manage age-related macular degeneration (AMD).[34,35]

MATERIALS AND METHODS

Materials. The 4-[4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (hydroxyethyl photolinker) was purchased from Novabiochem; dextran (40 kDa, 20 mol of amine per mole) was purchased from Invitrogen Molecular Probes. All of the other chemical reagents were purchased from Sigma-Aldrich. No unexpected or unusually high safety hazards were encountered during the course of our studies.

Synthetic Procedures. The reactions were conducted in the dark and under N_2 atmosphere protection, and the rate of stirring was set to 120 rpm. Unless specified, all experiments were conducted at room temperature.

Synthesis of Compound 1. Compound **1** was synthesized using the following literature-derived procedures with slight modification.[22,36] The 4-[4-(1-hydroxyethyl)-2-methoxy-5-nitrophenoxy]butanoic acid (120 mg, 0.40 mmol) was totally dissolved in 7 mL of dry DMF at 0 °C; subsequently *N*-(3-(dimethylamino)propyl)-*N*′-ethyl carbodiimide hydrochloride (EDC-HCl, 1.5 equiv, 115 mg, 0.60 mmol) and *N*-hydroxysuccinimide (NHS, 1.5 equiv, 69 mg, 0.60 mmol) were added. The resulting mixture was stirred for 1 h at 0 °C and then 16 h at room temperature in the dark under a nitrogen gas atmosphere. The crude product was obtained as a slightly yellow solid after the solvent was removed under high vacuum at 50 °C. To the residue, 20 mL of CH_2Cl_2 was added and extracted with water (3 × 15 mL). The organic layer was dried over $MgSO_4$ and filtrated, and the solvent was removed under vacuum to yield the desired product as pale-yellow solid. The purity of the product was assured by TLC eluting with EtOAc/hexanes (6/4, v/v), showing only a single spot. Yield: 142 mg, 89.6%. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 7.52 (s, 1H), 7.34 (s, 1H), 5.47 (d, *J* = 4.36, 1H, −OH), 5.23 (q, *J* = 5.87, 1H), 4.10 (t, *J* = 6.40, 2H), 3.88 (s, 3H), 2.83 (m, 2H), 2.79 (s, 4H, NHS), 2.06 (quint, *J* = 6.88, 2H), and 1.33 (d, *J* = 6.20, 3H). Please see Figure S10 in the Supporting Information for the ^{1}H NMR resonance assignment.

Synthesis of Compound 2. Compound **1** (50.0 mg, 0.13 mmol) and *N*,*N*-diisopropylethylamine (DIEA, 63 μL, 0.34 mmol) were added in 8 mL of CH_2Cl_2. After stirring for 15 min at 0 °C, triphosgene (48.6 mg, 0.17 mmol) was added. The resulting mixture was kept stirring at 0 °C for 1 h and then 15 h at room temperature before the solvent was removed under vacuum to give a residue. The crude product was dissolved in 30 mL of CH_2Cl_2, extracted with brine (3 × 15 mL), and dried over $MgSO_4$. The solvent was evaporated under vacuum to give a yellow solid as compound **2**. The purity of the product was confirmed by TLC (EtOAc/hexanes, 6/4, v/v). Yield: 49.5 mg, 0.11 mmol, 82.7%. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 7.52 (s, 1H), 7.31 (s, 1H), 5.81 (q, *J* = 6.64, 1H), 4.13 (t, *J* = 6.38, 2H), 3.93 (s, 3H), 2.83 (t, *J* = 7.44, 2H), 2.79 (s, 4H), 2.06 (quint, *J* = 6.82, 2H), and 1.85 (d, *J* = 6.68, 3H). Please see Figure S11 in the Supporting Information for the ^{1}H NMR resonance assignment.

Removal of the Maleate Salt of Timolol. *S*-(−)-1-(*t*-Butylamino)-3-[(4-morpholino-1,2,5-thiadiazol-3-yr)oxy]-2-propanol maleate salt (timolol maleate salt, 25 mg, 0.058 mmol) was treated with 15 mL of NaOH aqueous solution (2 M), and then the solution was extracted with CH_2Cl_2 (3 × 15 mL). The organic layers were combined and dried over $MgSO_4$. After the solvent was removed under vacuum, the pure timolol without maleate was obtained. Yield: 17.6 mg, 0.056 mmol, 96.6%. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm 4.99 (s, 1H, −NH), 4.38−4.34 (dd, *J* = 10.50, *J* = 4.06, 1H), 4.28−4.24 (dd, *J* = 10.60, *J* = 6.06, 1H), 3.78 (quint, *J* = 4.80, 1H), 3.66 (t, *J* = 4.74, 2H), 3.42 (t, *J* = 4.74, 2H), 2.52 (d, *J* = 5.94, 2H), 1.39 (s, 1H), and 0.97 (s, 6H). Please see Figure S9 in the Supporting Information for the ^{1}H NMR resonance assignment.

Synthesis of Caged Timolol. Timolol (17 mg, 0.054 mmol) and DIEA (1.2 equiv, 11.3 μL, 8.37 mg, 0.0648 mmol) were dissolved in 8 mL of anhydrous CH_2Cl_2. After stirring for 15 min at 0 °C, compound **2** (1.5 equiv, 37.1 mg, 0.081 mmol) was added. The resulting mixture was kept stirring for 15 min at 0 °C and then 6 h at room temperature. The solution was diluted with $CHCl_3$, extracted with brine (3 × 15 mL), and dried over $MgSO_4$. The solvent was evaporated under vacuum to give crude product as yellow oil, which was purified by silica gel column chromatography eluting with a mixture of EtOAc/hexane (9/1, v/v). The solvent was evaporated under vacuum to give a slightly yellow solid. Yield: 25.9 mg, 0.035 mmol, 64.9%. ^{1}H NMR (400 MHz, DMSO-d_6): δ ppm, 7.50 (s, 1H), 7.32 (s, 1H), 5.86 (q, *J* = 6.66, 1H), 5.15 (m, 1H), 4.64−4.60 (dd, *J* = 11.58, *J* = 2.67, 1H), 4.49−4.44 (dd, *J* = 11.40, *J* = 6.89, 1H), 4.08 (t, *J* = 6.44, 2H), 3.94 (s, 3H), 3.65 (t, *J* = 4.45, 2H), 3.42−3.34 (m, 2H), 2.71 (d, *J* = 5.92, 2H), 1.99 (quint, *J* = 7.33, 2H), 1.88 (d, *J* = 6.63, 3H), and 0.98 (s, 6H). Please see Figure S12 in the Supporting Information for the ^{1}H NMR resonance assignment.

Preparation of Caged Timolol−Amino-Dextran Conjugate. To a solution of 100 mg of amino-dextran (40 kDa, 20 mol of amino per mol) dissolved in 2 mL of PBS, 148 μL of caged timolol DMSO solution (0.10 mg/μL in DMSO, 14.8 mg, 0.02 mmol) was added. After the mixture was shaken in the dark at room temperature for 15 min, DMF (∼1 mL) was added dropwise until the solution became clear. After the mixture was shaken in the dark for 15 min, it was injected into a Slide-A-Lyzer 10K dialysis cassette and dialyzed against 500 mL of agitated water 5 times in the dark (5 changes of 500 mL of water over course of 16 h) to remove the unreacted caged timolol and other small molecules. The volume of dialysate increased to 6.5 mL, from which two 3 mL aliquots were

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withdrawn for the studies of 405 nm LED light and sunlight-triggered release of timolol.

Preparation of Caged Timolol−Aminoethyl Methacrylate Conjugate. To 20 μL of 2-aminoethyl methacrylate hydrochloride PBS stock solution (10 mg/mL in PBS, pH 8, 1.21 μmol), 5 μL of caged timolol DMSO stock solution (40 μg/μL in DMSO, 200 μg, 0.27 μmol) was added. The mixture was kept at room temperature in the dark for 10 min, and HEMA (10 μL) was added to get a clear solution. The solution was sonicated for 10 min.

Preparation of Hydrogel Contact Lens Conjugated with Caged Timolol. Hydrogel contact lenses were prepared by mixing the above prepared caged timolol−aminoethyl methacrylate conjugate (30 μL) with 2-hydroxyethyl methacrylate (100 μL), ethylene glycol dimethacrylate (5 μL), *N*-vinyl-2-pyrrolidinone (15 μL), and azobis(isobutyronitrile) (1 mg). The final mixture was transferred to a contact lens mold, and a contact lens shape transparent hydrogel was obtained after heating the mold for 2 h at 90 °C. The contact lens was purified by soaking in 10 mL of water 3 times over 24 h to remove unreacted materials.

UV−Vis Spectroscopic Studies. Light-triggered release of timolol from caged timolol−amino-dextran conjugate and from hydrogel contact lens to its bathing solution was analyzed using UV−vis absorption spectroscopy recorded by an Agilent 8453 UV−vis spectrophotometer (Agilent Technologies). Absorbance spectra were recorded on a 3 mL solution of 130 μM caged timolol−amino-dextran conjugate before and after 2 min exposures to 405 nm over the course of 26 min. The light source is a 395 nm LED (Cairn Research, UK) filtered through a Schott UG390 nm filter to remove the UV-A (<400 nm; power = 0.2 mW/cm^2). A parallel experiment was conducted on an identical sample exposed to indoor daylight on a typical sunny Spring morning on the University of California—Berkeley (UCB) campus (10 min per exposure). The intensities of the LED blue-light and daylight were measured using an Ohir meter manufactured by Laser Measurement Group.

The photochemical release of timolol from contact lens to its bathing solution was also investigated by UV−vis spectroscopy. Contact lens loaded with caged timolol was immersed in 2 mL of PBS in a glass vial sealed with a cap that was exposed to indoor daylight on a typical sunny Spring morning on the UCB campus. The absorption spectra of the bathing solution were recorded every 1 h over an exposure period of 8 h.

In Vivo Animal Assays. *Animals.* Eight week old CD1 mice (Charles River Laboratories, Wilmington, MA) were used in the study. All animals were treated according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research, and all procedures were approved by the animal care and use committee of the institute. Mice were anesthetized using a mixture of ketamine, xylazine, and acepromazine (50 mg, 10 mg, and 1 mg/kg body weight, respectively) before each experiment, and topical anesthesia was complemented with 0.5% proparacaine hydrochloride ophthalmic solution (Bausch & Lomb, Rochester, NY). Antibiotic ointment was applied after laser treatment.

Measurement of Intraocular Pressure. Intraocular pressure (IOP) was measured using a TonoLab rebound tonometer (Icare Lab, Helsinki, Finland) under light general anesthesia with 2% isoflurane. Each instrument-generated average was derived from six effective IOP measurements, and the measurement was performed three times for each eye.

Laser Photocoagulation. As we reported previously,[29] mice were randomized to receive unilateral episcleral vein coagulation by laser photocoagulation (532 nm, OcuLight TX; IRIDEX Corporation, Mountain View, CA) on the right eye, and the left eye was used as control. Mice of technical failure (i.e., hyphema) were excluded from the study.

Pharmaceutical Intervention. Mice post laser photocoagulation were randomized to receive eye-drops (10 μL) of 0.5% timolol maleate ophthalmic solution, photoreleased timolol, or control PBS immediately after the procedure on the cauterized eye and then 3 times per day for 7 days. IOP was measured before and after laser treatment every day in both eyes.

Anterior and Posterior-Segment Optical Coherence Tomography. The cornea thickness analysis was performed as we reported previously.[29] Briefly, an anterior-segment OCT (Visante OCT MODEL 1000; Carl Zeiss Meditec, Dublin, CA) was used to evaluate central corneal thickness. Quadrant-scans along four axes were performed to ensure scanning through the central cornea, and data along the 0−180° axis were used for analysis. For the evaluation of RNFL thickness, upon pupil dilation with 1% tropicamide ophthalmic solution (Akorn, Inc., Lake Forest, IL), retinal cross-section images were captured with a posterior-segment Phoenix Image-Guided OCT instrument and analyzed using InSight software (Pleasanton, CA). RNFL thickness was measured at 400 μm from the center of the optic nerve head within 4 quadrants (nasal, temporal, superior, and inferior) and averaged to a single thickness value.

Assessment of Retinal Ganglion Cell Density. The experiment was performed as we described previously.[29] Briefly, whole-mount retinae were harvested at day 7 postprocedure, fixed in 4% paraformaldehyde, and sequentially incubated with a goat anti-Brn3a (Santa Cruz Biotechnology, Santa Cruz, CA) primary antibody, and Cy3-conjugated donkey antigoat secondary antibody (Abcam, Cambridge, MA). Samples were examined by an AxioImager M1 epifluorescence deconvolution microscope with AxioVision 4.8 software (Carl Zeiss AG). For Brn3a-labeled RGC counting, eight areas (688 × 545 μm) of each retina were randomly selected at a distance of 850 μm from the optical disc. Digital images were analyzed using ImageJ software (http://imagej.nih.gov/ij/; provided in the public domain by the National Institutes of Health, Bethesda, MD). The percentage scores were obtained by normalizing to control condition defined as being 100%.

Statistical Analysis. Data were reported as mean ± SEM. The statistical significance between two groups was assessed by Prism software (GraphPad, La Jolla, CA). IOP and cornea thickness data were analyzed using two-way repeated measures ANOVA with a Bonferroni post hoc test, and RNFL thickness and RGC data were assessed by paired *t* test. *P* less than 0.05 was considered significant.

■ ASSOCIATED CONTENT

Supporting Information

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Additional data and figures including photographs, high resolution electrospray ionization mass spectroscopic spectra, UV−vis absorption study, ^{1}H NMR data, and resonance assignments (PDF)

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Author Contributions

C.M. designed synthetic protocols, performed experiments, collected and analyzed data, and wrote the manuscript. M.S. performed in vivo mice experiments, collected and analyzed data, and wrote the in vivo assay section of the manuscript. P.L. assisted in developing polymers at the early stage of the study. L.C. designed and supervised the in vivo assays. G.M. proposed the study, supervised the entire research project, directed the experiments, performed critical data analysis, wrote the core content, and edited the manuscript with input from all other authors. All authors read, discussed, and corrected the manuscript.

Notes

The authors declare the following competing financial interest(s): Work detailed in the manuscript has been submitted to the USPTO as a provisional patent.

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References 6 and 8 have been transposed, the corrected references reposted November 30, 2018.

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