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Research Article

Biodegradable Thermosensitive PLGA-PEG-PLGA Polymer for Non-irritating and Sustained Ophthalmic Drug Delivery

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Abstract. Challenges of ophthalmic drug delivery arise from not only the limited solubility of hydrophobic therapeutics, but also the restricted permeability and fast clearance of drugs due to the complex anatomy and physiology of the eyes. Biodegradable thermosensitive polymer, poly(dl-lactide-co-glycolide-b–ethylene glycol-b-dl-lactide-co-glycolide) (PLGA-PEG-PLGA) is a desirable ophthalmic drug delivery system because it can be formulated into injectable solution which forms gel *in situ* to provide prolonged drug release. In this study, excellent biocompatibility of blank PLGA-PEG-PLGA (1800-1500-1800) thermogel was demonstrated with insignificant difference from saline noted in rat eye enucleation test, *in vivo* inflammation test upon topical instillation, and subconjunctival injection. After subconjunctival injection, thermogel formulations loaded with hydrophilic (rhodamine B) or hydrophobic (coumarin 6) fluorescent dyes were retained up to 4 weeks in eye tissues and significantly higher level was detected than rhodamine B solution or coumarin 6 suspension in weeks 3 and 4. Moreover, *in vivo* whole body imaging showed that dye-loaded (sulfo-cyanine 7 NHS ester, Cy7; or cyanine 7.5 alkyne, Cy7.5) thermogels had longer retention at the injection site and retarded release to other body parts than dye solutions. Generally, the release rate of hydrophobic dyes (coumarin 6 and Cy7.5) was much slower than that of the hydrophilic dyes (rhodamine B and Cy7) from the thermogel. In summary, the thermogel was safe for ophthalmic drug delivery and could deliver both hydrophobic and hydrophilic compounds for sustained drug release into eye tissues with single subconjunctival injection for better patient compliance and reduced risks on repeated injection.

KEY WORDS: biocompatibility; *in situ* thermosensitive hydrogel; ocular delivery; PLGA-PEG-PLGA; subconjunctival injection.

INTRODUCTION

Ophthalmic drug delivery is challenging due to various physicochemical hurdles of therapeutic agents (e.g., poor solubility, lack of sustained drug action) and physiological barriers of the eyes (e.g., fast drug clearance after topical dosing from the cul-de-sac of the eyes, blood-retinal barrier protecting the eye from xenobiotics) (1–5). Several strategies have been attempted to overcome these challenges via applying various administration routes and delivery systems for ophthalmic drug delivery.

In terms of administration routes, while topical formulations are feasible for delivering therapies targeting posterior segment of the eyes (6–9) with recent research advancement, the delivery efficiency of therapeutic agents to ocular tissues is very limited (1,10). High-dose and frequent administration are therefore needed for therapeutic effects, which often cause adverse side effects (11) and higher risk to poor patient compliance with the treatment. Local delivery with intravitreal injection allows direct reach of the posterior segment. However, this invasive procedure may cause threats to vision and ocular ischemic change (12) in the long term with repeated dosing and increased risk of post-surgery complications. Subconjunctival injection is another local delivery strategy, which has better patient compliance than intravitreal injection and higher drug delivery efficiency than systemic administration. It has been shown to achieve high local drug concentrations and sustained drug release effect (13–15), thus allowing less-frequent dosing (injections) and reduced systemic side effects compared with systemic administration (16).

Besides alternating administration routes, novel delivery systems have been developed by exploiting sol–gel transition

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property of thermosensitive polymers to address the challenges in ophthalmic drug delivery (17). Due to the facile synthesis process, capability of incorporating a wide range of therapeutic agents and controllable biodegradation rate (18–20), poly(dl-lactide-co-glycolide-b–ethylene glycol-b-dl-lactide-co-glycolide) (PLGA-PEG-PLGA) thermogel has gained increasing attention for ophthalmic drug delivery (21–27). Most research has been focused on topical and intravitreal injection applications. Subconjunctival injection of ReGel® (PLGA-PE-PLGA 1500-1000-1500; LA:GA ratio = 3:1) (28) has been reported to prolong the release of ovalbumin up to 14 days (29). However, the drug-loaded ReGel® has a sol–gel transition temperature below room temperature (around 20°C for 23% *w/v*), which might not be suitable for administration (30) through fine needles for opthalmic injection at ambient room conditions. In fact, the thermosensitive sol–gel transition windows of the many PLGA-PEG-PLGA block copolymers are rather narrow and far outside the physiological temperature range, thus limiting their application as drug carriers. Previous attempts have been made by tuning of block ratios with mixture of more than one PLGA-PEG-PLGA (25,27). However, this created extra uncertainties in formulations as no systematic approach was available in the choice of block lengths and ratios of polymers involved which could only be obtained by trial-and-error leading to limited options.

The use of PLGA-PEG-PLGA as a novel carrier for ophthalmic drug delivery through either topical or intravitreal injection has been reported in a few recent studies (21–27). However, these studies focused mainly on the therapeutic effect enhancement. The investigation about the sustained release of specific therapeutic agents and fundamental understanding about the use of this thermosensitive polymer as a carrier for ophthalmic drug delivery, especially on local drug delivery via subconjunctival injection, is limited. Also, the versatility of compounds compatible with this thermogel and the corresponding efficiency in delivering therapeutic agents into eye tissues have not been adequately studied for a rational design of delivery system using PLGA-PEG-PLGA in future application. To this end, a commercially available PLGA-PEG-PLGA (1800-1500-1800; LA:GA ratio = 3:1), with sol–gel transition temperature of about 30°C, is a suitable drug carrier for ophthalmic drug delivery with no need of further manipulation with copolymers. This polymer has the same LA:GA ratio (dl-lactide to glycolide molar ratio in the PLGA block) as ReGel by modifying the block length with minimum impact on molecular weight. Importantly, this PLGA-PEG-PLGA polymer solution can be prepared and administered under room conditions.

This study is the first to examine the versatility of compounds compatible with the PLGA-PEG-PLGA thermogel and the delivering efficiency into eye tissues after subconjunctival injection with model fluorescent hydrophilic or hydrophobic molecules. The safety and biocompatibility of this unique thermogel in ocular tissues after subconjunctival injection was also ascertained. This thermogel enabled sustained release of encapsulated fluorescent molecules to the eye tissues, days for hydrophilic compounds and weeks for hydrophobic compounds. The results garner further studies to examine the therapeutic effects of drug-incorporated PLGA-PEG-PLGA thermogel in treating retina and posterior segment diseases from a single subconjunctival injection.

MATERIALS AND METHODS

Materials

Thermosensitive polymer, PLGA-PEG-PLGA (1800-1500-1800; LA:GA ratio = 3:1), were purchased from SRI Biomaterials Inc. (Riverside, CA, USA). Phosphate-buffered saline (PBS) powder and rhodamine B were obtained from Sigma-Aldrich (St. Louis, MO, USA). Coumarin 6 was procured from Santa Cruz Biotechnology (Dallas, TX, USA). Sulfo-cyanine 7 NHS ester (Cy7) and cyanine 7.5 alkyne (Cy7.5) were supplied by Lumiprobe Corporation (Hunt Valley, MD, USA). Milli Q water with resistivity of 18.2 MΩ·cm at 25°C was freshly dispensed from a Milli Q Integral water purification system of MilliporeSigma (Burlington, MA, USA).

Animals

Female Sprague-Dawley (SD) rats (average weight 200 g ± 20 g; 11–12 weeks of age) were housed in a controlled environment of standard temperature (20–23°C) and humidity (30–70%) with a 12 h light/dark schedule (lights on between 6 am and 6 pm) with food and water *ad libitum*. All procedures were approved by the Animal Experimentation Ethics Committee (AEEC) of The Chinese University of Hong Kong (AEEC No. 14/119/MIS-5-C and 15/024/GRF-5-S). Animals were anesthetized by intramuscular ketamine and xylazine injection (75 mg/kg and 7.5 mg/kg, respectively) prior to operation. The rats were then randomized into groups as described in detail in the following sections for receiving different formulations in the left eyes, which were instilled with a local anesthetic (1% *w/v* tetracaine hydrochloride, Bausch & Lomb) prior to subconjunctival injection. The right eye of each animal was instilled with normal saline (0.9% sodium chloride (NaCl)) to serve as a control.

Biocompatibility with Ocular Tissues

Eye Enucleation Test

Animals were euthanized and the eyes were enucleated from eye cavity with caution in order to keep least contact thus possible damage with the cornea and processed 2 h postmortem (31,32). Enucleated eyes from female SD rats were assigned into groups randomly and stabilized at 34°C in PBS for 30 min prior to treatment. The use of SD rat eyes is to eliminate species difference on the safety evaluation of thermogel on the rats used in the following *in vivo* experiments which only small animals like rats could be accommodated inside the *in vivo* imaging system. Also, rat models were not uncommon in studying novel formulations for subconjunctival drug delivery. A volume of 30 μL thermogel solution (20% *w/v*) was instilled onto the cornea and allowed to retain for 10 s followed by thorough rinsing with normal saline (31) to remove any residual thermogel. Then a moisturized fluorescein sodium ophthalmic strips (Tianjing Jingming New Technological Development Co. Ltd., Tianjing, China) was applied to the surface of the corneas and excess fluorescein was rinsed off with normal saline. When illuminated with blue cobalt light, the dark orange fluorescein on

the corneal surface could reveal spots or region with damage. The fluorescein retention among groups was compared using scoring system from 0 to 3, whereas 0 = no retention, 1 = small amount of cell retention, 2 = individual cells and areas of retention, and 3 = large area of retention (32). Tissue morphology and fluorescein retention in damaged tissues of the cornea were examined with a Nikon stereomicroscope equipped with imaging system (Nikon Instruments Inc., Melville, NY, USA) and evaluated with a scoring system for the corneal opacity (corneal opacity was scored from 0 to 4, whereas 0 = no opacity, 1 = scattered or diffused area, 2 = distinguishable translucency with iris details slightly obscured, 3 = severe opacity with iris details obscured, and 4 = complete opacity with invisible iris) (32).

In vivo Irritation After Topical Instillation

To determine possible acute irritation and inflammatory effects of PLGA-PEG-PLGA thermogel on eye tissues in living animals, a volume of 30 μL of PLGA-PEG-PLGA polymeric solution (20% *w*/*v*) was instilled onto the cornea of SD rats followed by continuous monitoring of cornea for any signs of discomfort in rats. The condition of the corneas was captured using the Nikon stereomicroscope equipped with camera (Nikon Instruments Inc., Melville, NY, USA) and the cornea tissues were sampled after 72 h for histological examination.

In vivo Irritation and Inflammatory Reaction After Subconjunctival Injection

To imitate the administration of the designed ophthalmic delivery system, the PLGA-PEG-PLGA thermogel solution was injected into the subconjunctiva of SD rats and monitored for 28 days. Whole blood samples were collected on days 1, 3, 7, 14, and 28 after the subconjunctival injection and kept at − 80°C freezer until analysis with a commercially available inflammatory biomarker ELISA kit for IL-6 (eBioscience, Affymetrix Inc., San Diego, CA, USA). Eye tissues collected at animal termination were sampled for histological examination.

Histological Analysis

Enucleated eye samples ($n = 6$–8) were immediately fixed in 4% paraformaldehyde solution. Samples were then stored in the fixing agent up to 1 week followed by dehydration and embedding in paraffin. Tissue sections of 4-μm thickness were taken and stained by hematoxylin and eosin (H&E) before imaging with an Eclipse Ti-E fluorescence microscope and imaging system (Nikon Instruments Inc., Melville, NY, USA).

Fluorescent Molecule Loading and Delivery into Ocular Tissues

Preparation of Fluorescent Molecule–Loaded Thermogel

PLGA-PEG-PLGA solution (20% *w*/*v*) was first prepared with Milli Q water and homogenized for 48 h using an orbital shaker operated at 180 rpm at room conditions. Model fluorescent hydrophilic (1, 2, and 4 mg/mL rhodamine B; or 300 μg/mL Cy7) or hydrophobic (50, 100, 200 μg/mL coumarin 6; or 300 μg/mL Cy7.5) dyes were then added to the polymeric solution and shaken for another 48 h with the container wrapped with aluminum foil for light protection. The polymeric dye solutions were filtered with 0.45-μm nylon filter and 0.22-μm sterile filter prior to *in vivo* injections as an ophthalmic product. Samples were analyzed with a CLARIOstar microplate reader (BMG LABTECH, Offenburg, Germany) at corresponding excitation/emission (E_x/E_m) wavelengths (rhodamine B, 540/575 nm; coumarin 6, 466/507 nm) to determine the dye concentration in formulations.

In vitro Release of Molecule from the Thermogel

The *in vitro* release of rhodamine B and coumarin 6 from the thermogel was investigated at 34°C (reference temperature of ocular surface and region around the subconjunctiva (33,34)) incubation under gentle orbital shaking (~ 50 rpm). A volume of 1-mL dye-incorporated (1 mg/mL rhodamine B or 35 μg/mL coumarin 6) thermogel formulations was added into a 4-mL screw-capped glass vial (15 mm × 45 mm) and preincubated at 34°C for 15 min before adding 3 mL PBS with 0.2% Tween 20 as the release medium (18–20,35). Samples with a volume of 3 mL were withdrawn and replaced by 3-mL fresh release medium at designated time points over 30 days. All samples collected were kept at − 80°C until analysis with a CLARIOstar microplate reader (BMG LABTECH, Offenburg, Germany). All experiments were carried out in triplicates ($n = 3$).

Histological Evaluation of Fluorescent Dye Retention in Eye Tissues

Rhodamine B (1 mg/mL)- or coumarin 6 (35 μg/mL)-loaded thermogel solutions and dye solution or suspension (20 μL) were injected subconjunctivally into the left eye of the rats. Animals were sacrificed at designated time points (days 1, 3, 7, 14, 21, and 28 for rhodamine B; and days 1, 7, 14, 21, and 28 for coumarin 6) with eye tissues enucleated and embedded with optimum cutting temperature tissue compound and stored at − 80°C until sectioning at 20 μm using a cryostat equipped with CryoJane transfer system (Leica CM 3050S, Leica Biosystems, Wetzlar, Germany). The fluorescent images of the tissues were captured and analyzed with the Eclipse Ti-E fluorescence microscope and imaging system (Nikon Instruments Inc., Melville, NY, USA). Parameters such as exposure time, analog gain, and beam intensity were kept constant for all image captured of the same dye. A few sample tissue slides after treatment with rhodamine B or coumarin 6 were kept as controls. Fluorescent signals from these controls were captured repeatedly in most imaging experiments to ensure reproducibility of fluorescent signal detection. The retention of the model fluorescent molecule in different position of the eyes (cornea and retina) was used to evaluate the delivery efficiency of thermogel formulations into the eyes.

Continuous Monitoring of Fluorescent Molecule Distribution In vivo

To evaluate the molecule release from the thermogel formulations and the subsequent retention in eye tissues *in vivo*, a pair of model hydrophilic and hydrophobic fluorescent dyes (Cy7 and Cy7.5, respectively) was prepared at 300 μg/mL in 20% (*w*/*v*) PLGA-PEG-PLGA polymeric solution to facilitate whole body imaging in animals. Structurally, similar Cy7 and Cy7.5 representing hydrophilic and hydrophobic compounds respectively were chosen as the model fluorescent dyes because of the restricted spectral specification for the *in vivo* imaging system (the excitation/emission wavelengths of rhodamine B and coumarin 6 were not compatible with the instrument for optimal imaging). An aqueous solution formulation of the same dye (300 μg/mL) was also tested as control. Experimental rats were divided into 2 groups ($n = 5$) randomly for each dye receiving subconjunctival injection of dye solutions or thermogel formulations (20 μL). Anesthetized rats were scanned with a small animal *in vivo* imaging system (Bruker In-Vivo MS FX PRO) at designated time points (0.5 h, 1 h, 2 h, 4 h, 7 h, 24 h, day 2, day 5, and day 6 for Cy7; and day 1, day 5, week 2, week 3, week 4, week 5, and week 6 for Cy7.5) (E_x/E_m, 750 nm/830 nm; exposure time, 30s) to reflect the rate of dye release and localization in the eyes. The image position of each animal was kept consistent by placing the eye of the animal at the central point of the camera with a holding tray, so that the object distance was kept constant. The field of view were overlaid with the white light topology images taken at the same time as the fluorescent image for analysis. Standard solutions of Cy7 and Cy7.5 at 300 μg/mL were used to ensure inter-day signal reproducibility during experiment.

Statistical Analysis

Student's *t* test at a confidence level of 95% was employed to identify any statistically significant differences in the fluorescent intensity signals. A *p* value of < 0.05 was considered statistically significant (denoted as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$).

RESULTS

Biocompatibility of the PLGA-PEG-PLGA Thermogel with Ocular Tissues

Eye Enucleation Test

PLGA-PEG-PLGA (1800-1500-1800, 20% *w*/*v*) thermogel solution was investigated for its possible damage to eye tissues upon topical administration to freshly enucleated eyes. Normal saline, 0.9% *w*/*v* NaCl, and 1 N sodium hydroxide (NaOH) were used as negative and positive controls, respectively. Abnormal tissue with corneal abrasion or invasion of foreign particles was revealed by the retention of the fluorescein dye. Under cobalt blue light illumination, both the 0.9% NaCl and thermogel groups showed smooth spread of fluorescein over the surface without any distinct coloration (Fig. 1), indicating insignificant damage to the eye tissues with a score of 0 in the scoring system. On the other hand, the NaOH group showed significant yellow lamination suggesting the eye tissues were damaged (fluorescein retention score = 3). In terms of morphology, rough surface and irregular lesion patterns were observed in the NaOH group, whereas the eye ball looked normal with shiny and spherical shape in the other two groups. The corneal opacity score of thermogel group (corneal opacity score = 0) was comparable with the NaCl group and significantly lower from the NaOH group (corneal opacity score = 2.3). The results suggested that the PLGA-PEG-PLGA thermogel did not cause notable adverse effect and it was further evaluated *in vivo* in living animals.

Acute Irritation on Eye Tissues After Topical Instillation onto Cornea of Rats

PLGA-PEG-PLGA thermogel solution was further evaluated by an acute *in vivo* inflammation test. No sign of discomfort was noted in rats treated with the thermogel group and the control saline group up to the observation period of 72 h. Similar to the control group, the cornea of rats receiving the thermogel solution remained clear and smooth (Fig. 2). In

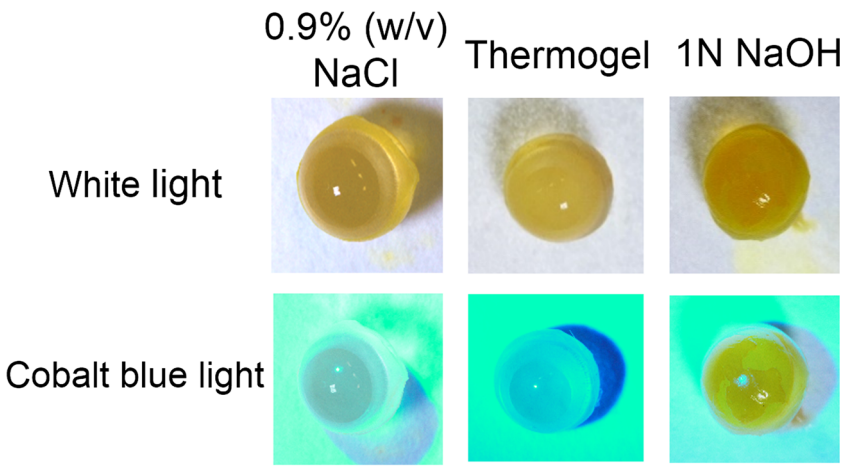

Fig. 1. Representative images from six experimental eyes of each group showing morphology of eye tissues and fluorescein dye retention after instillation of 0.9% (*w*/*v*) NaCl (left), 20% *w*/*v* PLGA-PEG-PLGA thermogel solution (middle), and 1 N NaOH (right) onto the cornea

contrast, obvious sign of damaged cornea was observed in the 1 N NaOH solution group. The cornea became opaque with development of corneal neovascularization. Moreover, remarkable increase in infiltrated inflammatory cells was noted on the cornea in the NaOH group, but not in the normal saline and thermogel groups, by H&E staining (Fig. 2).

Compatibility of the Thermogel After Subconjunctival Injection

While single instillation of thermogel solution revealed no significant irritation on animals, the long-term tolerability (28 days) of the thermogel administered as blank vehicle via subconjunctival injection in rats was also evaluated. A commonly used inflammatory biomarker, interleukin-6 (IL-6), was monitored through blood sampling throughout the experimental period. At all time points, IL-6 level in blood samples was below the detection limit (< 4 pg/mL) for the thermogel and saline groups, suggesting insignificant difference of inflammatory effect induced by thermogel from saline in rats. For the NaOH group, blood level of IL-6 was found to increase from 13.4 ± 8.8 pg/mL on day 3 to 40.6 ± 2.7 pg/mL after 28 days (Fig. 3a) which was significantly higher than both the thermogel and saline groups ($p < 0.001$).

Histopathological analysis of the corneal tissues collected on day 28 revealed no significant difference between the thermogel group and the 0.9% NaCl saline control group (Fig. 3b). Moreover, no observable of infiltrated inflammatory cells (mainly as polymorphonuclear neutrophil, macrophage, and lymphocyte) in the thermogel group by H&E staining showed similar response with saline. In contrast, 1 N NaOH gave rise to remarkable increase of infiltrated inflammatory cells in the eye tissues.

Effect of Dye Loading

Rhodamine B (with an experimental log *p* value of 1.95 (PhysPropNCCT)) and coumarin 6 (with a predicted log *p* value of 4.25 (EPISUITE, NICEATM, ACD/Labs, and OPERA)) were chosen as representative hydrophilic and hydrophobic fluorescent molecule, respectively, for investigation. Rhodamine B is freely soluble in water up to 15 mg/mL, whereas coumarin 6 is poorly soluble in water (water solubility < 0.44 μg/mL). Different amount of fluorescent dyes were added into the thermogel solution (20% *w*/*v*) to prepare formulations at three loading levels. While a 1 mg/mL loading of rhodamine B allowed the 20% *w*/*v* PLGA-PEG-PLGA thermogel solution to form rigid gel at 34°C, thermogel solutions with higher dye loadings (2 and 4 mg/mL) failed to form a rigid gel. It has been reported that the sol–gel transition of thermosensitive polymers is highly dependent on the hydrophilic/hydrophobic balance of the system (36,37). In the case of high rhodamine B dose, the introduction of excessive rhodamine B might interrupt the balance and the interaction of the system thereby prohibiting the formation of gel structure via packing of micelles at the investigated temperature range. The maximum loading of coumarin 6 into the 20% (*w*/*v*) thermogel solution without formation of particulates was 35 μg/mL, as limited by the solubility threshold of coumarin 6 in the thermogel solution, significantly higher than its water solubility (approximately 80-fold). Higher coumarin 6 loadings (100 and 200 μg/mL) resulted in opaque rigid gel with distinct orange solid at the bottom, suggesting the presence of particulates. Therefore, further investigation was carried out on thermogel formulations loaded with 1 mg/mL rhodamine B or 35 μg/mL coumarin 6.

Fluorescent Probes Release *In vitro* and *In vivo*

In vitro Release of Model Fluorescent Molecules

Figure 4 showed the *in vitro* release profiles of the rhodamine B (1 mg/mL)- and coumarin (35 μg/mL)-loaded thermogel formulations over a period of 35 days.

Rhodamine B was released gradually from the thermogel with complete release noted on day 20. Almost no burst effect was

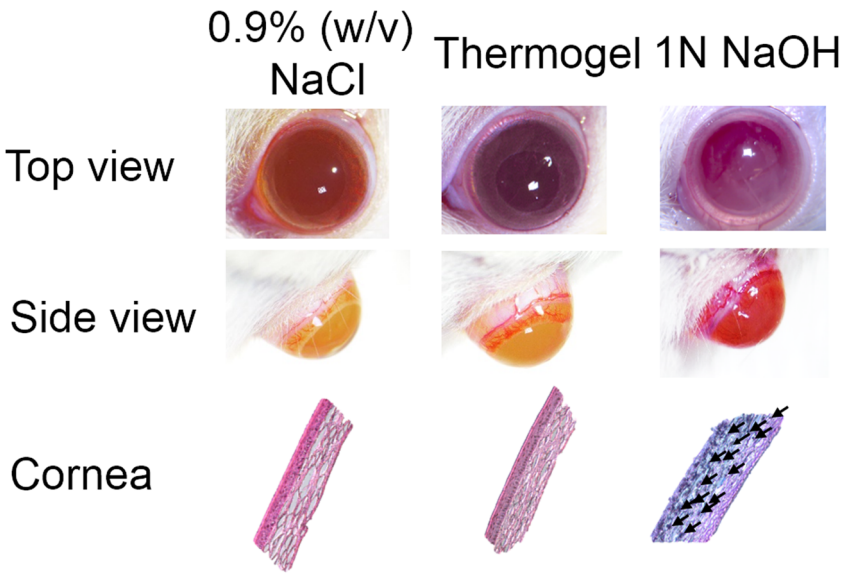

Fig. 2. Morphology of the rat corneas at 72 h (top and side views) after a single topical instillation of thermogel solution and H&E staining of the cornea tissues sectioned in 4 μm thickness (magnification: × 20). NaOH and saline were used as controls for comparison

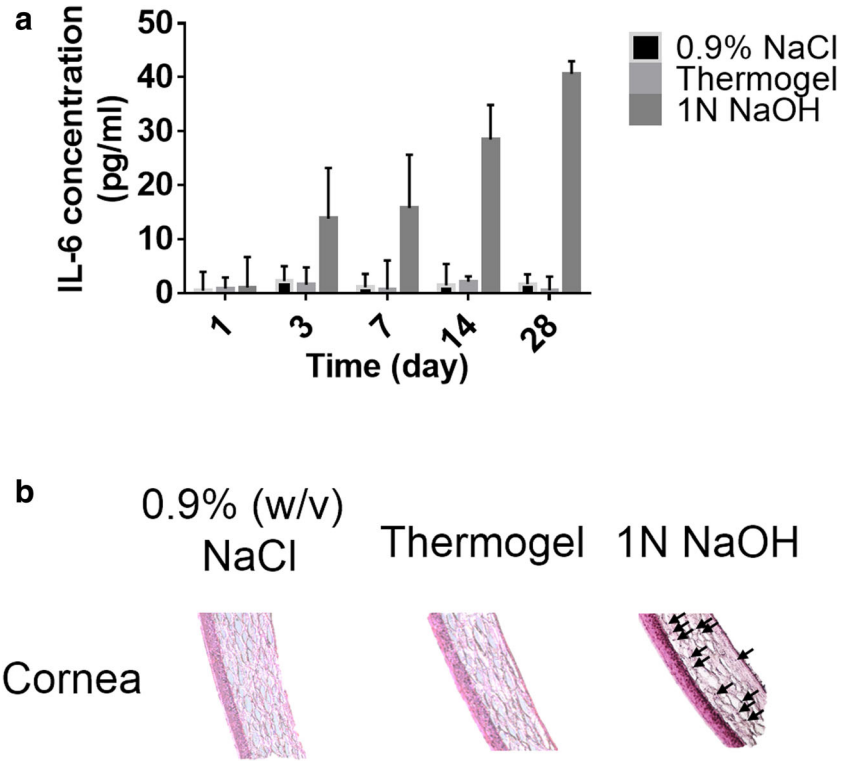

Fig. 3. *In vivo* inflammatory response monitored for 28 days after single subconjunctival injection of thermogel. **a** Graph showing blood concentration of an inflammatory biomarker, interleukin-6 (IL-6). Data were presented as mean ± standard deviation ($n = 6$). **b** H&E staining of the rat corneal tissue (magnification, 20 at 4 μm thickness on day 28 post subconjunctival injection of thermogel solution. Infiltrated into the stroma of the cornea damaged by 1 N NaOH was shown as blue spots (as pointed by black arrows)

observed with less than 15% of rhodamine B released from the thermogel on the first day. The release of rhodamine B continued with a steady rate with around 42% of probe molecule released after 7 days. The release kinetics of rhodamine B from the thermogel was best fitted with Higuchi's model ($R^2 = 0.992$) which was not uncommon for drug released through matrix as a diffusion process based on the Fick's law.

On the other hand, the release of coumarin 6 from the thermogel showed a two-phase kinetics. The initial release was significantly retarded with only 10% cumulative release in the first 2 weeks. Thereafter, in the second stage, 70% of coumarin 6 was released in two week time from day 19 to day 35 at a faster release rate.

In vivo Release of Model Fluorescent Molecules in Eye Tissues (Cornea and Retina)

In vivo release of rhodamine B and coumarin 6 from the thermogel formulations was further investigated in rat eyes after subconjunctival injection by monitoring the fluorescent

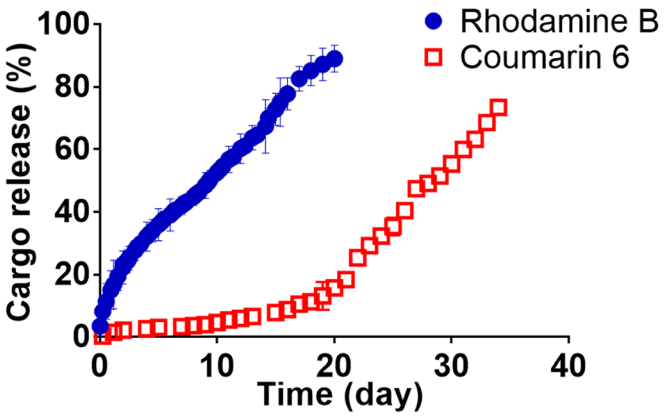

Fig. 4. *In vitro* release of probe fluorescent compounds, rhodamine B (1 mg/mL), and coumarin 6 (35 μg/mL). Data were presented as mean ± standard deviation ($n = 3$)

signal at the corneal and retinal tissues for 28 days. For rhodamine B–loaded thermogel, the fluorescent signal was readily detectable on day 1 in both corneal and retinal tissues with signals decreased over time (Fig. 5a). In general, the fluorescent signals of rhodamine B at the cornea was comparable ($p > 0.05$) for the injected dye solution and the thermogel formulation except for the first day ($0.01 < p < 0.05$) (Fig. 5b) where signals were detectable up to day 3. Contrarily, the signal intensity at the retinal tissue was significantly higher ($p < 0.05$) for the thermogel formulation with detectable signals measured on day 7 while that of the solution group was barely detectable throughout the period of the study. This suggests the thermogel formulation could prolong release of the hydrophilic fluorescent molecule and significantly enhance its retention in retinal tissues since day 1 ($p < 0.01$) with higher significance on days 3 and 7 ($p < 0.05$). (Fig. 5b).

As shown in Fig. 6a, fluorescent signals of coumarin 6 from the thermogel and suspension formulations were readily detectable in the cornea and retina on day 1. While the fluorescent signal from the coumarin 6 thermogel and suspension formulation showed a dropping trend in both the cornea and the retina after day 1 (Fig. 6b), the fluorescent signal from the thermogel formulation exhibited a much slower decline over time. For later time points following day 1, the concentration of coumarin 6 released from thermogel into the corneal tissues was significantly higher than the suspension formulation except day 14 ($p < 0.05$ on day 21 and $p < 0.01$ on days 7 and 28) while that in retinal tissues was significantly higher from day 7 onwards ($p < 0.05$ on day 7; $p < 0.01$ on days 21 and 28; and $p < 0.001$ on day 7).

In vivo Real-Time Molecule Release from Thermogel

Real-time monitoring of the distribution of another pair of model fluorescent molecules (cyanine dyes: Cy7 and Cy7.5) was carried out by *in vivo* imaging system in rats after subconjunctival injection of the corresponding thermogel formulation. To avoid discrepancy in analysis arising from the possible quantum yield difference of the fluorescent probes, the fluorescent signal of cyanine dye in solution and thermogel form (both at 300 μg/mL) were measured under the same setting during our experiments and they were found to be comparable. As illustrated from the whole body scan images (Supplementary 1), both probe molecules (Cy7 and Cy7.5) delivered by thermogel formulations had longer retention in the body compared with the solution counterparts.

Cy 7 released from the thermogel slowly and distributed from the injection site to the body with maximum fluorescent

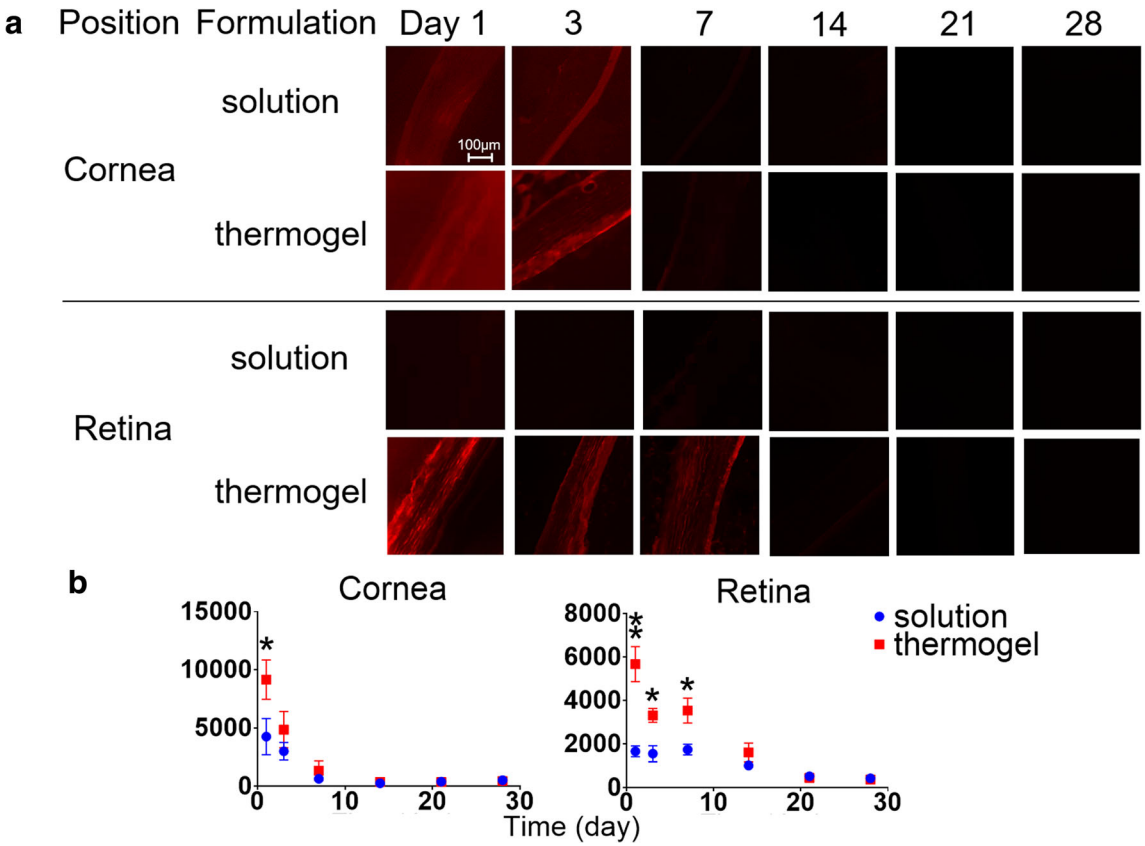

Fig. 5. The retention of rhodamine B was visualized as fluorescent signal detected with the TRITC filter. **a** Representative images of eye tissues sectioned at 10 μm (cornea and retina, magnification ×20) at designated time points after subconjunctival injection of rhodamine B solution or thermogel (1 mg/mL). **b** Rhodamine B signal detected with TRITC filter in corneal and retinal tissue of rat eyes over 28 days. Mean ± standard error of the mean from 6 experimental animals were shown. (*$p < 0.05$; **$p < 0.01$, difference between the solution and thermogel formulation at the same designated time point)

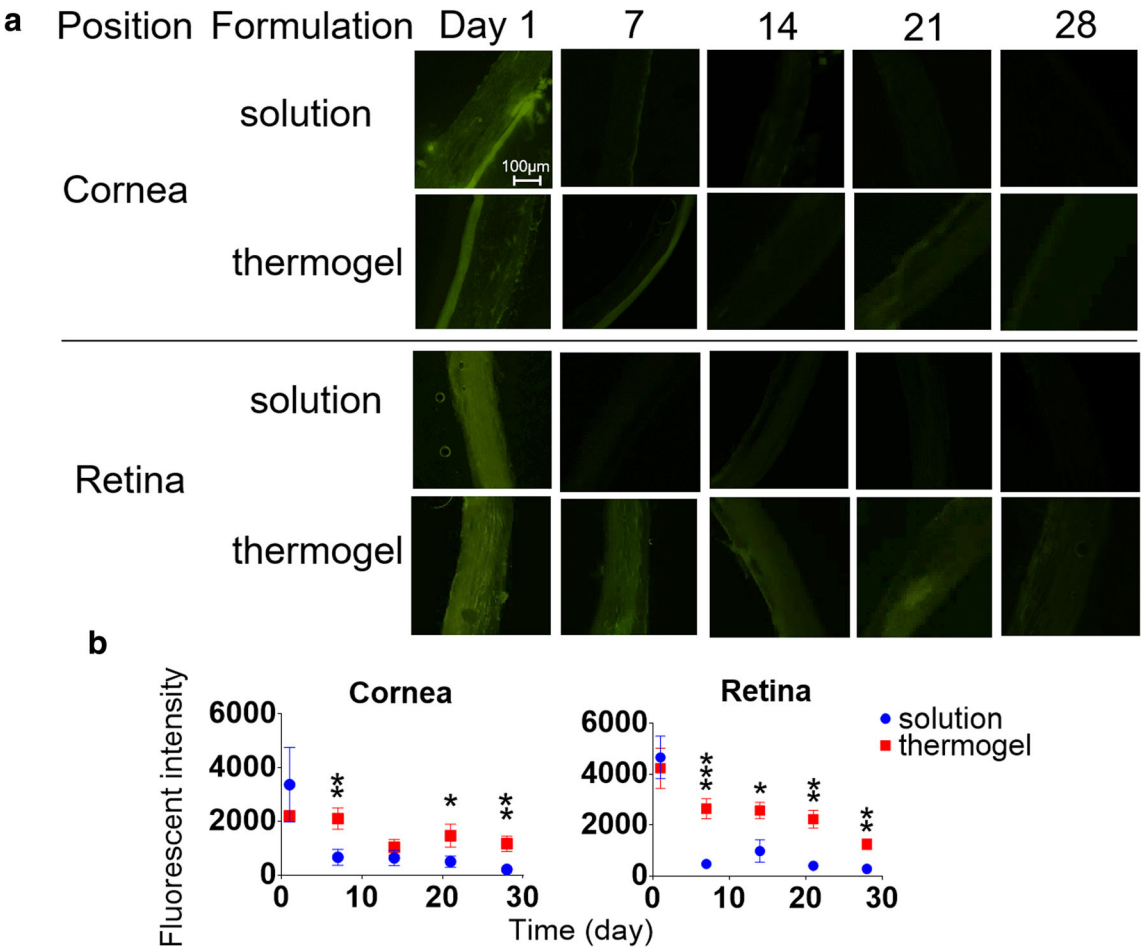

Fig. 6. The retention of coumarin 6 was visualized as fluorescent signal detected with the FITC filter. **a** Representative images of eye tissues sectioned at 10 μm (cornea and retina, magnification × 20) at designated time points after subconjunctival injection of coumarin 6 solution or thermogel (35 μg/mL). **b** Coumarin 6 signal detected with FITC filter in (a) corneal and (b) retinal tissues of rat eyes over 28 days. Mean ± standard error of the mean from 6 experimental animals were shown. (*$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$, difference between the solution and thermogel formulation at the same designated time point)

signal recorded at 4 h and the signal remained detectable in the body until day 4. Comparing with the thermogel formulation, the injected dye solution was rapidly cleared from the body within 24 h (Supplementary 1). At the subconjunctival injection site, the fluorescent intensity of Cy7 from the injected solution remained at a high level in the first 2 h (~ 20,000 a.u.) and then dropped rapidly to undetectable level at 24 h (Fig. 7a). In contrast, the thermogel formulation was able to retain high fluorescent intensity of the Cy7 (~ 20,000 a.u.) at the injection site until 7 h at a statistically significant level compared to solution ($p < 0.01$) before it started to decrease gradually to ~ 2300 a.u. at 24 h ($p < 0.01$) and maintained at ~ 700 a.u. from day 2 to day 6 ($0.001 < p < 0.01$) (Fig. 8b). Beyond 4 h, Cy7-loaded thermogel formulation was able to maintain a remarkably higher fluorescent signal ($0.001 < p < 0.05$) than the dye solution in the subconjunctiva (Fig. 7b).

For the hydrophobic Cy7.5 dye delivered by the thermogel formulation, its fluorescent signal was retained in the injection site for up to week 3 without significant distribution to the circulation (Supplementary 1). This implied the superiority of delivering hydrophobic molecules via the thermogel. Thereafter, the fluorescent molecule remained readily detectable in the whole body up to week 7. However, when the hydrophobic Cy7.5 was delivered as suspension, its fluorescent signal started to distribute to the whole body on day 1 and it faded out gradually after 5 days. In week 7, the fluorescent signal was barely detectable (Supplementary 1).

A prolonged retention of the hydrophobic dye Cy7.5 up to week 6 at the injection site was achieved by both thermogel and suspension formulations, albeit the thermogel group had significantly higher fluorescent intensities in most cases ($0.01 < p < 0.05$) (Fig. 8b). The thermogel structure could be observed physically as a green-colored lump right after injection and remained easily detectable in the subconjunctiva during the first week. Differentiable structure was found when dissecting the tissues at the injection site at day 6 as the terminal time point for Cy7 thermogel. However, at later time points, differentiation of the thermogel was less feasible with faded color because the dye has been released. The lump shrank into a softer structure in the second week and was observed as a thick spread layer on day 21.

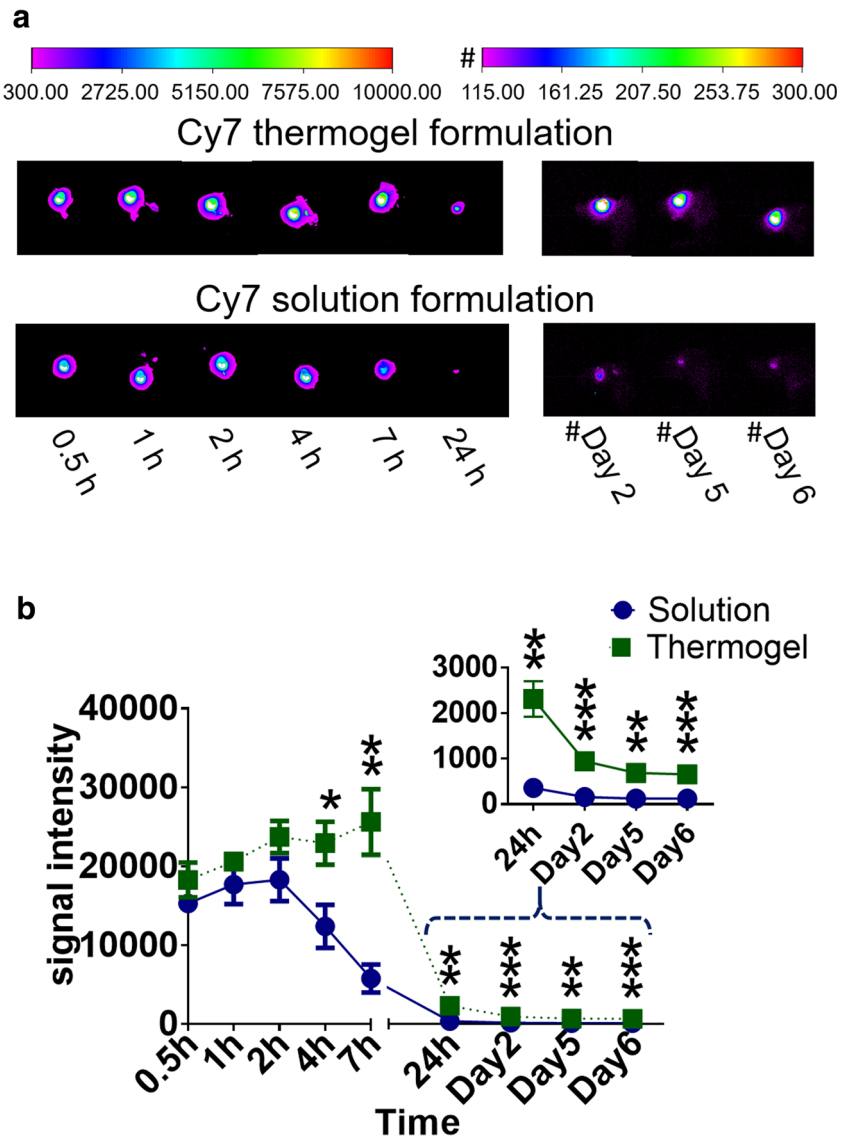

Fig. 7. *In vivo* imaging of rats post subconjunctival injection of Cy7 thermogel or solution formulations. **a** Representative images of the eye area from 5 experimental rats. The signal intensity of all images was calibrated with the scale bars shown at the top (white region representing saturation of signal out of range). A separate scale bar (labeled #), providing narrower range at the low intensity end, is designated for the later time point for clarity (day 2 onwards). **b** Fluorescent intensity of Cy7 monitored by *in vivo* imaging system at the injection site in the subconjunctiva. Data were presented as mean ± standard error of the mean ($n = 5$) (*$p < 0.05$; **$p < 0.01$, and ***$p < 0.001$, difference between the solution and thermogel formulation at the same designated time point)

DISCUSSION

Biocompatibility of PLGA-PEG-PLGA Thermogel

Ophthalmic drug delivery is challenging not only because of the complexity of the eye structure limiting drug delivery into ocular tissues, but also due to the low tolerance of ocular tissues with foreign substances. A biodegradable and thermosensitive PLGA-PEG-PLGA polymer was hypothesized to facilitate efficient and sustained drug delivery to the eyes. In this study, a PLGA-PEG-PLGA (1800-1500-1800, 20% *w*/*v*)-based thermogel formulation was investigated for its biocompatibility with eye tissues and its efficiency to deliver model fluorescent hydrophilic and hydrophobic molecules into rat eyes.

In eye enucleation and acute irritation test, topically instilled thermogel solution caused no apparent damage to the corneal tissues with corneal opacity and fluorescein retention score of 0 which were significantly lower than NaOH group (Figs. 1 and 2). The ex vivo eye enucleation experiment of animals was evaluated to be an alternative in the assessment for eye irritation potential without the use of

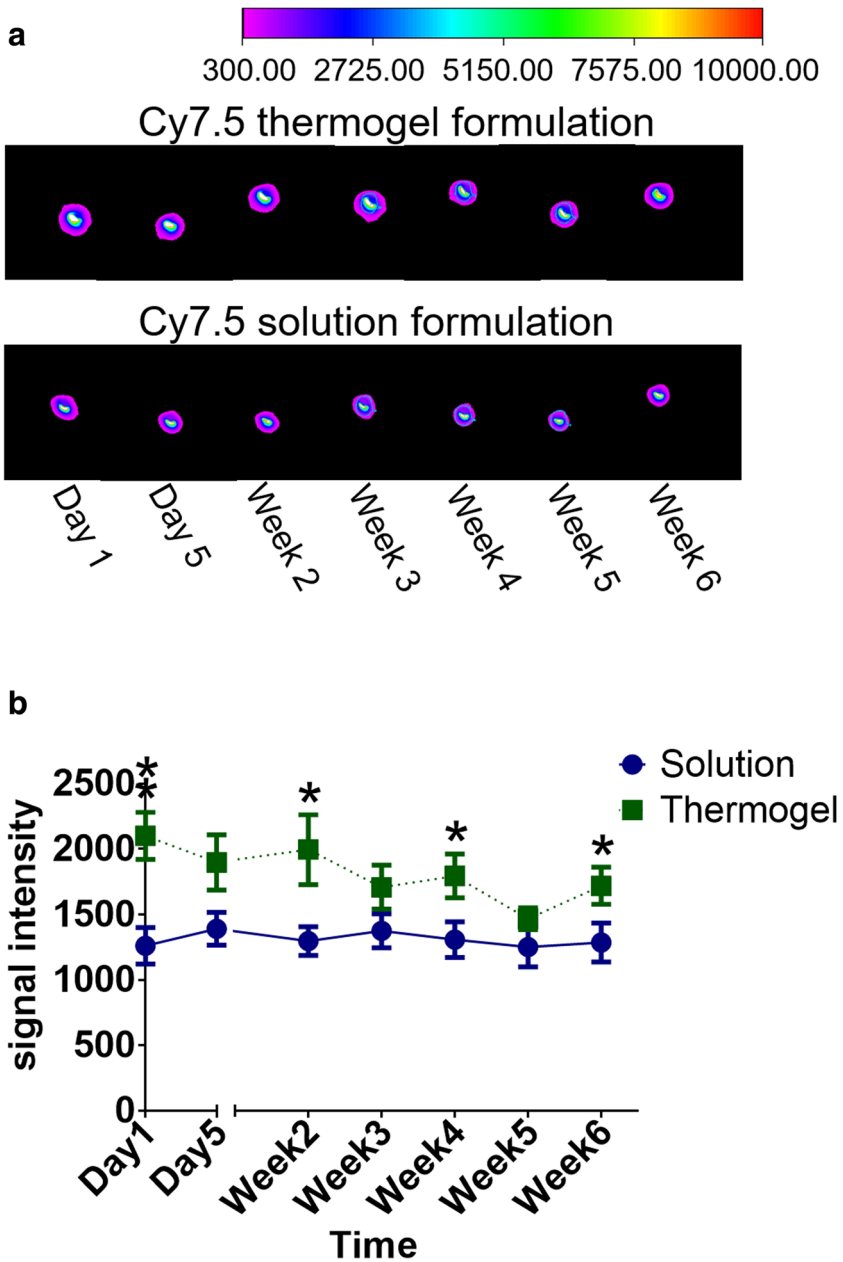

Fig. 8. *In vivo* imaging of rats post subconjunctival injection of Cy7.5 thermogel or solution. **a** Representative images of the eye area from 5 experimental rats. The signal intensity of all images was calibrated with the scale bar shown at the top (white region representing saturation of signal out of range). **b** Fluorescent intensity of Cy7.5 monitored by *in vivo* imaging system at the injection site in the subconjunctiva. Data were presented as mean ± standard error of the mean ($n = 5$) (*$p < 0.05$ and **$p < 0.01$, difference between the solution and thermogel formulation at the same designated time point)

animal and showed good correlation with Drazie test (31). The main purpose of this test in this study was to eliminate any possibility of applying irritating materials in the *in vivo* animal studies for ethical reasons and the *in vivo* behavior was represented with the long-term monitoring post subconjunctival injection which was the intended application of the thermogel. The safety of the thermogel after single subconjunctival injection was also evaluated for 28 days with minimal inflammation reaction in eye tissues (as reflected by the IL-6 inflammation marker, Fig. 3a) observed. These safety data suggest the suitability of the PLGA-PEG-PLGA thermogel for topical instillation and subconjunctival injection to deliver drugs to the anterior and posterior segments of the eyes.

Biocompatibility of PLGA-PEG-PLGA-based thermogels of ophthalmic use was not very clear until recent years after its promising application as *in situ*-gelling system for sustained drug delivery has been reported (21–27). Pratoomsoot *et al.* (24) reported *in vitro* biocompatibility of PLGA-PEG-PLGA with wound healing assay. In most cases, the *in vivo* biocompatibility was only made with observation when investigating the drug delivery via intravitreal and

topical applications. Individual biocompatibility studies showing no local or systemic adverse effect with OCT, ERG, and histology were documented by Xie *et al.* (26), Cuming *et al.* (27), and Zhang *et al.* (25) along with their investigation with drug delivery. The current study confirms the compatibility of PLGA-PEG-PLGA thermogel with ocular tissues with the countable publications found. More importantly, this study strengthened the safety use of PLGA-PEG-PLGA through long-term subconjunctival application as the first report on this free-flowing non-mixture PLGA-PEG-PLGA thermogel at ambient conditions with thorough and well-designed stepwise approach using regular-lab instrumentation. Although further in-depth investigation is warranted, combining the current results with the previous reports, the biocompatibility of PLGA-PEG-PLGA-based thermogels was probably independent of the block length difference and the gelation temperature.

Sustained Release from PLGA-PEG-PLGA Thermogel

After the safety and biocompatibility of the PLGA-PEG-PLGA thermosensitive polymer has been ascertained, its delivery efficiency of fluorescent molecules of distinct hydrophobicity was further investigated *in vitro* and *in vivo*. Our data showed that both model hydrophilic and hydrophobic molecules could be successfully incorporated into the thermogel formulation for sustained ophthalmic drug delivery via the subconjunctival route. The release behavior from the thermogel structure was found to be strongly dependent on the hydrophobicity and water solubility of the loaded molecules. Both *in vitro* and *in vivo* release rates were significantly faster for the hydrophilic compounds. The release of the model hydrophilic rhodamine B from PLGA-PEG-PLGA thermogel was likely mediated by diffusion to allow fast initial molecule release that 50% of molecule was released within 10 days (Fig. 4) and the remaining molecule was released over 2 weeks. The release profile of rhodamine B is consistent with that of other hydrophilic compounds released from PLGA-PEG-PLGA thermogels, though their release was mostly completed in 7 days (19,38). The release profile of coumarin 6 was distinctly different from that of the rhodamine B. The strong hydrophobic interaction between coumarin and the polymer, indicated by the significantly increase drug solubility (~ 80-fold higher than the solubility in water), retarded molecule release from the thermogel. Qiao *et al* (19) suggested that most hydrophobic molecules tend to partition into the hydrophobic PLGA domain of the hydrogel and their release was controlled mainly by hydrogel erosion. This explained the slow release rate of coumarin 6 in the first 2 weeks, followed by a faster release in the second release phase when the thermogel started to break down (Fig. 4).

In vivo retention of both hydrophilic and hydrophobic molecules from subconjunctivally injected thermogel formulations was significantly prolonged compared with the injected dye solution formulations. The sustained release of loaded molecules resulted in enhanced bioavailability in the corneal and retinal tissues (Figs. 5 and 6). The fluorescent signals of hydrophilic rhodamine B decreased gradually with time and vanished completely after 14 days. The discrepancy could be partly attributed to the continuous removal of the released rhodamine B molecules *in vivo* as well as the multi-dimensional contact between the small volume gel structure (20 μL) and the subconjunctival tissues, which resulted in faster dye diffusion rate than the *in vitro* experiment. For the *in vitro* release setup, diffusion can only occur across the fixed surface area between the medium and the thermogel; and the concentration gradient decreased over time. However, the use of the same injection volume (30 μL thermogel) for *in vitro* release was not practical since the release amount was likely to be below the detection limit at the first few time points which makes the evaluation of drug release difficult and unreliable, especially for the hydrophobic compound which was released slowly during the first-stage. The *in vitro* release protocol was based on previous studies for comparing results with the reported drug release from the thermogel system. Interestingly, the data suggested that the thermogel formulation could facilitate a sustained release of the model fluorescent molecules and enhanced bioavailability in the posterior segment of the eyes. Comparing with the rhodamine B–loaded thermogel, the retention of the coumarin 6 thermogel formulations in eye tissues was generally longer which could be explained by the slower release of coumarin 6 from the thermogel as shown in the *in vitro* release profiles (Fig. 4). Similarly, the hydrophilic Cy7-loaded thermogel was able to extend the dye retention compared with the dye solution and the fluorescent signal decreased with time after 7 h (Fig. 7). The lower loading of Cy7 (300 μg/mL) than that of rhodamine B (1 mg/mL) could explain the faster clearance of Cy7 *in vivo*. The statistically significant but low level of Cy7 detected revealed that while applying hydrophilic drugs, drug level might or might not be sufficient for maintaining therapeutic effects after the first few days. Therefore, the thermogel formulation may be more suitable for enhancing the retention of hydrophobic compounds. Although increasing the dose may be a strategic approach to prolong the release, the therapeutic window of the drug molecules and local toxicity raise concerns and thorough investigation must be followed with the use of hydrophilic drug molecules. As a consequence, to facilitate clinical translation, more investigation is needed to establish the relationship between physicochemical properties of drug, its loading efficiency, and drug release behavior of thermogel formulations.

Comparing with the hydrophilic counterparts, thermogel loaded with hydrophobic fluorescent molecules (coumarin 6 and Cy7.5) provided a remarkably prolonged retention, with its fluorescent signal remained readily detectable in the cornea and the retina up to 28 days for coumarin 6 (Fig. 6) and whole body imaging after 7 weeks for Cy7.5 (Fig. 8). Release of hydrophobic fluorescent dyes controlled by erosion of the thermogel might be responsible for their sustained delivery for a period over 4–7 weeks. In general, *in vitro–in vivo* release profiles agreed better for thermogel formulations loaded with hydrophobic compounds. As indicated by data from *in vivo* imaging, clearance of the model dyes (in thermogel) from the eyes and the subsequent distribution to other organs (including liver and kidneys) were also found to occur at a slower rate than the corresponding solution/suspension formulations. Interestingly, retention of coumarin 6 and Cy7.5 fluorescent signal at the subconjunctival injection site was also observed in the solution formulation (Figs. 6 and 8). Indeed, 35 μg/mL

coumarin 6 or 300 μg/mL Cy7.5 was higher than their water solubilities which would result in a dye suspension and the insoluble particulates might be responsible for the slow release resulting in signal at a relatively low intensity over a period of time. Nonetheless, the overall fluorescent intensities from the thermogel systems were higher, suggesting the thermogel offers better control release of incorporated compounds. On the other hand, potential fluorescent probe-tissue binding may also contribute to the retention *in vivo*.

Importantly, our thermogel formulation was found to remarkably increase the solubility of the model hydrophobic fluorescent dye (coumarin 6) than the corresponding aqueous solution by approximately 80-fold. While the enhancement on drug loading could be drug-specific, the extent of enhancement is likely sufficient to allow the effective treatment of eye diseases that otherwise cannot be achieved by aqueous solution. Besides the effect on solubility and drug loading, our thermogel formulation was also shown to allow the incorporated hydrophobic fluorescent dye to retain at a remarkably higher level in both the eye tissues (Fig. 6) and the injection site (Fig. 8) than the corresponding solutions for more than a month. For the hydrophilic compound, the fluorescence signals from the thermogel formulation were also significantly higher in the retina tissue (Fig. 5) and the injection site (Fig. 7) though for a shorter period (~a week). Thus, the thermogel formulation could be beneficial for medications that required high initial drug concentration and prolonged drug release. Overall, our results suggest that the thermogel formulations could allow a high drug concentration to reach the therapeutic range and help reduce the dosing frequency. In the clinic, this will be translated into higher efficacy and better patient compliance with subconjunctival injection. The thermogel studied is specifically valuable for long-term ophthalmic therapies such as injection following surgery for prevention of inflammatory complication or corneal neovascularization which normally lasts for months with monthly frequency.

CONCLUSIONS

The excellent safety profile and biocompatibility of the thermosensitive PLGA-PEG-PLGA (1800-1500-1800; LA:GA = 3:1) polymer after topical instillation to the cornea and subconjunctival injection were demonstrated. In addition, both model hydrophilic and hydrophobic fluorescent molecules could be incorporated into the thermogel formulation to achieve localized and sustained release to the eye tissues through single subconjunctival injection. While the *in vitro* and *in vivo* release of loaded molecules depended strongly on their hydrophobicity, a remarkably prolonged release with high concentration of molecules available in the posterior segment for a duration of 4–7 weeks was achieved for thermogel system loaded with hydrophobic compounds and suggested that the injectable thermosensitive PLGA-PEG-PLGA polymer has high potential to be further developed as new ophthalmic drug delivery system to formulate poorly soluble drug for sustained delivery to the eyes. This versatile thermogel-based ocular drug delivery system has high impact in the clinical setting by enhancing both drug bioavailability and patient compliance.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of Interest The authors declare that they have no conflict of interest.

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