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Sustained intravitreal delivery of dexamethasone using an injectable and biodegradable thermogel

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ABSTRACT

Delivery of therapeutic agents to posterior segment of the eyes is challenging due to the anatomy and physiology of ocular barriers and thus long-acting implantable formulations are much desired. In this study, a thermogelling system composed of two poly(lactic acid-*co*-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) triblock copolymers was developed as an injectable matrix for intravitreal drug delivery. The thermogel was prepared by mixing a sol and a precipitate of PLGA–PEG–PLGA triblock copolymers with different block ratios, among which a hydrophobic glucocorticoid, dexamethasone (DEX), was incorporated. The DEX-loaded thermogel was a low-viscous liquid at low temperature and formed a non-flowing gel at body temperature. The *in vitro* release rate of DEX from the thermogel could be conveniently modulated by varying the mixing ratio of the two copolymers. The long-lasting intraocular residence of the thermogel was demonstrated by intravitreal injection of a fluorescence-labeled thermogel to rabbits. Compared with a DEX suspension, the intravitreal retention time of DEX increased from a dozen hours to over 1 week when being loaded in the thermogel. Additionally, intravitreal administration of the thermogel did not impair the morphology of retina and cornea. This study reveals that the injectable PLGA–PEG–PLGA thermogel is a biocompatible carrier for sustained delivery of bioactive agents into the eyes, and provides an alternative approach for treatment of posterior segment diseases.

1. Introduction

Eye is a relatively independent organ with high sensitivity and multiple protective mechanisms like corneal, scleral, and blood–retinal barriers, and thus it is very challenging to design and evaluate the drug delivery systems for ocular diseases [1,2]. Generally speaking, the ocular bioavailability of topically applied eyedrops is less than 7% [3]. It is undoubtedly more difficult to treat the posterior segment ocular diseases, such as chronic uveitis, because of the longer diffusional distance from ocular surface to posterior segment which retards significantly bioactive agents to reach the therapeutic concentration [4,5]. Therefore, development of novel intraocular delivery systems represents the trend of ophthalmic remedies, and a corresponding study is very meaningful.

Intravitreal injection is regarded as an important and effective approach to treat posterior segment ocular diseases, which not only delivers directly the bioactive agents into the eye but also avoids the potential side effects of systemic administration [6,7]. However, some issues associated with this administration route, such as repeated injections, the poor patient compliance, and the risks of serious complications [8], limited its application. To overcome these shortcomings, novel drug delivery systems are devised to reduce the frequency of intraocular injection, and thus ameliorate the suffering of patients and decrease the complications of repeated intravitreal administration [9,10]. To date, a few of long-acting formulations have been successfully utilized in ophthalmology clinic. For instance, the Surodex delivery system, a biodegradable depot composed of poly(lactic-*co*-glycolic acid) (PLGA), has been designed to put in the eye after cataract surgery, and a sustained release of dexamethasone (DEX) over a period of 7 days has been achieved [11]. Such implants also include Ozurdex, Retisert, and so on. However, a surgical procedure or a special device is required to implant these products into eyes

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[12]. To increase the convenience of administration and improve patient compliance, development of injectable intravitreal drug delivery systems using a conventional syringe is much desired.

Hydrogel is a kind of polymeric network containing plenty of water, which is physiologically similar to the vitreous, so it is suitable as a promising carrier for intraocular delivery of bioactive agents [13–15]. Recently, biodegradable thermogelling hydrogels which undergo a reversible sol–gel transition upon heating to body temperature have gained increased attention as a minimally invasive depot system for drug delivery, tissue engineering, etc. [16–22]. Chemical drugs, bioactive macromolecules, and even cells can be incorporated into the polymer aqueous solution at low or room temperature. The corresponding formulation can be injected into the target tissue, and then rapidly turns into a gel due to contacting with physiological heat at 37 °C to act as a slow releasing depot of drugs or a cell growing matrix. The use of organic solvents is thoroughly avoided during the whole process of application. So far, thermogelling PEG/polyester block copolymers [22–27], PEG/polypeptide block copolymers [28,29] and poly(phosphazenes) [30,31] have been reported.

As a typical example of thermogel, the poly(lactic acid-*co*-glycolic acid)–poly(ethylene glycol)–poly(lactic acid-*co*-glycolic acid) (PLGA–PEG–PLGA) triblock copolymers have been investigated extensively due to their many advantages such as facile synthesis, adjustable gel performance, controllable biodegradation rate, versatile drug delivery, etc. [16,32–36]. However, the composition window of PLGA–PEG–PLGA triblock copolymers to form a thermogel is quite narrow [37,38]. Otherwise, the polymers are just soluble or precipitate in aqueous medium. Recently, our group has exploited a convenient approach to obtain a thermogelling system by simply mixing an aqueous sol of a PLGA–PEG–PLGA triblock copolymer and a precipitate of another triblock copolymer with different PEG/PLGA ratios. Thus, the applicable window of pertinent polymers has been broadened to a large extent [39,40]. Furthermore, the biodegradation and biocompatibility of these mixture hydrogels have been confirmed [40].

The initial attempt of the thermogel containing single PLGA–PEG–PLGA copolymer for intraocular application was to modulate the release of ganciclovir-loaded PLGA microspheres by dispersing the microspheres in the thermogel matrix [41]. Afterward, the thermoreversible PLGA–PEG–PLGA hydrogel was used for a topical administration of DEX acetate, a water-soluble form of DEX [42]. When the thermogel was instilled in the conjunctival sac, significantly increased bioavailability was observed compared with the normal eye drop. Nevertheless, the drug was rapidly eliminated from the anterior chamber in 8 h. Theoretically, the more advantages of the thermogel composed of PEG/PLGA copolymers, such as its biodegradability and the capacity of sustained drug delivery, will be well utilized when being injected directly in the vitreous. Unfortunately, whether the aqueous environment would affect the *in situ* formation of the PEG/PLGA hydrogel in the eye and how the incorporated bioactive agents should be released is still unknown. Herein, an injectable thermogel will be constructed based on the mixture of PEG/PLGA copolymers and be used as an intravitreal implant to perform prolonged drug delivery. Two PLGA–PEG–PLGA triblock copolymers with similar compositions but different block ratios were synthesized, in which one is soluble while the other precipitates in water. Their corresponding mixture thermogel was prepared, as presented in Fig. 1a and tried as an injectable depot for intravitreal delivery of DEX, a mainstay of uveitis treatment, as illustrated in Fig. 1b. Different from its water-soluble acetate and phosphate, DEX as a hydrophobic drug was used in the present study. The *in vivo* performance and biocompatibility of the mixture thermogel was evaluated after intravitreal injection. Finally, the intraocular pharmacokinetics of the DEX-loaded thermogel was examined, and correlation of the *in vivo* and *in vitro* DEX release from the thermogel was also discussed.

2. Materials and methods

2.1. Materials and animals

Poly(ethylene glycol) (PEG, molecular weight (MW): 1000 and 1500), stannous octoate ($Sn(Oct)_2$, 95%) and DEX (CAS: 50-02-2) were acquired from Sigma–Aldrich. D,L-Lactide (LA) and glycolide (GA) were purchased from Purac. Fluorescein sodium was supplied by Solarbio Corporation. Purified deionized water was prepared by the Milli-Q plus system from Millipore (Bedford, USA). All other chemicals used in this research were of analytical grade.

Male albino rabbits, weighing 1.5–2.0 kg, were provided by the Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and maintained individually in standard cages in a light-controlled room (12 h light and 12 h dark cycles) at 20–24 °C and 30–75% relative humidity, with access to food or water *ad libitum*. All animal experiments were performed in accordance with protocols evaluated and approved by the ethics committee of Fudan University.

2.2. Synthesis and characterization of PLGA–PEG–PLGA copolymers

PLGA–PEG–PLGA triblock copolymers were prepared by ring-opening copolymerization of LA and GA using PEG as the macroinitiator in the presence of $Sn(Oct)_2$. The detailed synthesis process has been described in previous publications concerning both ours and others' work [39,43]. In this study, two triblock copolymers with different PEG blocks (MW 1000 and 1500) but a fixed LA/GA ratio (4/1) were synthesized. For example, to synthesize Copolymer-1, 10 g of PEG 1000 was put into a three-neck flask and heated under vacuum at 130 °C for 3 h to remove the residual moisture of polymers. Next, LA (24.14 g) and GA (4.86 g) were added and heated under reduced pressure at 100 °C for 30 min. Then, $Sn(Oct)_2$ was transferred into the mixtures, and the reaction system was heated with continuous stirring under an argon atmosphere at 150 °C for 12 h. Then, the crude products were purified by washing with 80 °C water 4 times to remove soluble and low MW by-products. The residual water in the polymer was eliminated via freeze-drying and the final products were kept at −20 °C until further use. Another triblock polymer with PEG 1500 was synthesized using a similar procedure.

2.2.1. ^1H NMR measurements

A 500-MHz NMR spectrometer (Bruker, DMX500 spectrometer) was used for ^1H NMR measurements to investigate the chemical structure and composition of the PLGA–PEG–PLGA triblock copolymers. $CDCl_3$ and tetramethylsilane (TMS) were used as the solvent and the internal standard, respectively.

2.2.2. Gel permeation chromatography (GPC) characterization

The gel permeation chromatography system (Agilent 1100) with a differential refractometer was used to determine the MWs and their distributions of the PLGA–PEG–PLGA triblock copolymers. The measurements were performed at 35 °C and tetrahydrofuran was used as the eluent at a flow rate of 1.0 mL/min. Monodispersed polystyrene was used as the standard for MW calculation.

2.3. Characterization of sol–gel transition

2.3.1. Determination of sol–gel transition temperature

The sol–gel transition of the aqueous polymer solutions was determined via the test tube inverting method with an increase

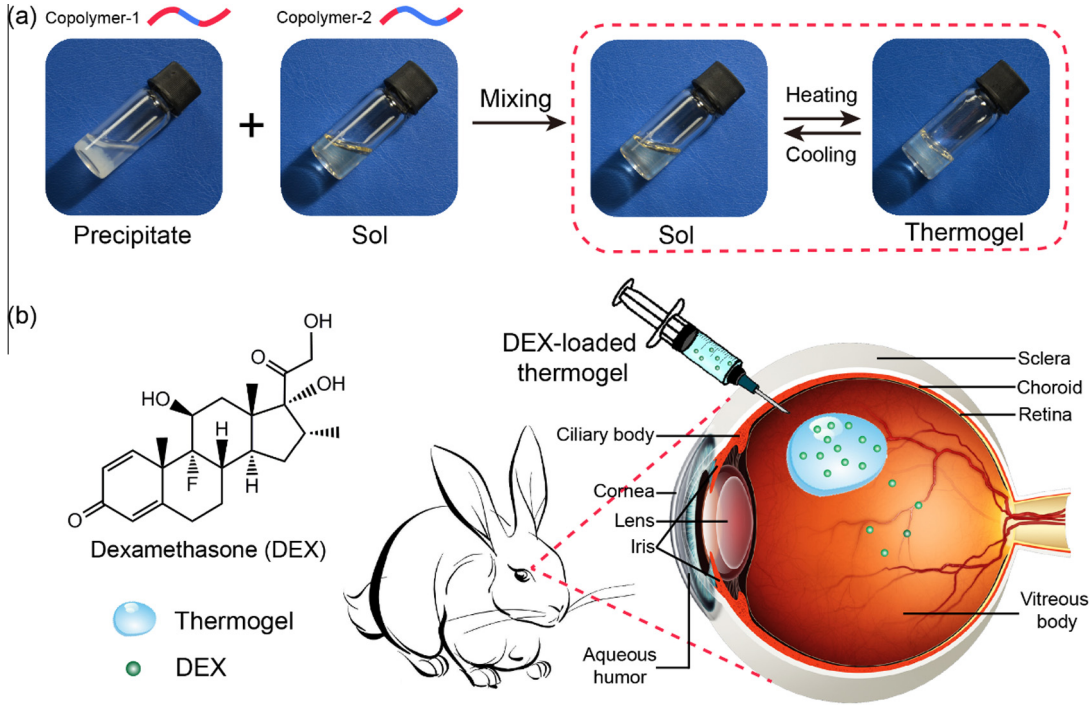

Fig. 1. (a) Schematic diagram showing a mixing approach to prepare a thermogel system; (b) schematic diagram showing the molecular structure of DEX and intravitreal delivery of DEX using the mixture thermogel.

of 1 °C per step [44,45]. Polymer solutions with given concentrations were prepared by dissolving polymers in normal saline solution (NS) and equilibrating for 24 h at 4 °C. The vials containing 0.5 mL solutions were immersed in a water bath at each temperature for 10 min before measurements. The sol–gel transition temperature was determined via inverting the vials 180°. If there was no visible flow within 30 s, the sample was considered as a "gel".

2.3.2. Rheological analysis

The sol–gel transition of the aqueous polymer solutions was also investigated using an Advanced Rheometric Expanding System (ARES, Rheolometric Scientific) with a Couette cell (Couette diameter, 34 mm; bob diameter, 32 mm; bob height, 33.3 mm; bob gap, 2 mm). Polymer solutions (25 wt.% in NS) were injected into the Couette cell and then a thin layer of silicone oil was added carefully to prevent evaporation of water. The measurements as a function of temperature were performed at a fixed oscillatory frequency of 10 rad/s with a 0.5 °C/min temperature ramp from 10 °C to 45 °C. Temperature was controlled using an environment controller with an accuracy of ±0.05 °C (Neslab, RTε-130).

2.4. In vitro drug release

Polymer aqueous solution (25 wt.%) containing 1 mg/mL DEX was prepared by directly mixing polymer solution with drug and stirring at room temperature. 2 mg/mL and 4 mg/mL DEX-loaded polymer solutions were obtained via another approach. Namely, DEX was first dissolved in a minimized amount of alcohol and then was added into the polymer solutions. The residual alcohol in polymer solutions was removed by evaporation with continuously stirring. Next, 0.5 g of DEX-loaded polymer solution was injected into a porous dialysis cassette (3500 MWCO, Thermo Scientific, Slide A Lyzer, 0.1–0.5 mL) and a weighing bottle (60 × 30 mm) containing the dialysis cassette was incubated in a water bath at 37 °C. After 10 min, the *in situ* formed hydrogel was achieved with the dimensions of 12 × 12 × 3.5 mm and 20 mL of phosphate buffered saline solution (PBS, pH 7.4) containing 0.025 wt.% NaN_3 was added into the weighing bottle as the release medium. The bottles were sealed to minimize water evaporation and shaken at 50 rpm. At given intervals, the release mediums were totally replaced with the same amount of fresh medium. Samples were collected at 3, 12, 24, 36, and 60 h and thereafter at 24 h intervals.

The amount of released DEX was determined by a high-performance liquid chromatography (HPLC, Waters e2695) equipped with a C18 column and a UV detector (Waters 2489). The mobile phase was acetonitrile–water with a ratio of 50:50 (v/v) and its flow rate was 1.0 mL/min at 25 °C. UV–Vis detection at 249 nm was used for the analysis of DEX. The accumulated release amount was calculated by the calibration curve.

The release data were assessed via model-dependent methods. First-order release profile [46–48] could be described as the following equation:

$$\ln(1 - M_t/M_\infty) = -Kt \quad (1)$$

Here, M_t means the cumulative release amount at time t, and M_∞ is the final release amount at infinite time. The corresponding release rate constant K is related to diffusivity, hydrogel chemical structure and some geometric parameters.

2.5. Intraocular retention and biocompatibility evaluation

Intraocular retention and biocompatibility evaluation of the mixture thermogel were performed on healthy rabbits. Ophthalmological examination on rabbit eyes was conducted before the experiment to make sure that no ocular abnormalities were observed. The rabbits were randomly divided into three groups (three eyes in both the thermogel group and solution group, and two eyes in the control group). The rabbits were firstly systemically anesthetized with pentobarbital (30 mg/kg) by injection via the ear marginal vein, and then procaine hydrochloride eye drop

(0.4 wt.%) was applied for topical anesthesia. Punctures were performed with a 26-gauge needle and 1-mL syringe 5 mm posterior to the corneoscleral limbus, where the needle was visible in the pupil area. For the thermogel group, 200 μL vitreous humor was gently aspirated to reduce intraocular pressure before injection. Then an aliquot of 100 μL Mixture-1 solution containing 0.1 wt.% fluorescein sodium was injected into the vitreous of rabbit eyes. The syringe was held for about 30 s to allow the solution to form a semi-solid gel and make sure no vitreous humor bleeding occurred during the procedure. For the solution group and the control group, 0.1 wt.% fluorescein sodium-dissolved NS solution and blank NS were injected into the vitreous respectively. All the formulations were sterilized by filtration before administration. Ciprofloxacin eye drops were topically used twice daily before and after intravitreal injection to protect the eyes from inflammation.

Tropicamide eye drops were instilled in the rabbit eyes for mydriasis before each examination. Thirty minutes later, a slit lamp microscope (Model YZ5G, 66 Vision-Tech Co., Ltd.) equipped with a digital camera was used to record the fluorescence images of the rabbit eye under conscious condition at given time intervals (0, 1, 2, 4, and 8 h after administration, and then every day).

On the 21st day, the rabbits were euthanatized, then the eyes were enucleated and immersed in Davidson's solution for 4 h. After dehydration, the eyeballs were transversely opened to remove the lens. The remaining anterior and posterior segments were sectioned and histologically processed using hematoxylin–eosin (HE) stains for examination. The morphology of ocular tissues was assessed using an automated upright microscope system (Leica DM4000B).

2.6. Ocular pharmacokinetics

Six healthy rabbits were randomly divided into two groups. After conduction of systemic and topical anesthesia, the DEX-loaded thermogel and a DEX suspension in NS were administered respectively, following the procedure as described above. For the thermogel group, Mixture-1 aqueous solution containing 1 mg/mL DEX was sterilized using 0.45-μm filter before use and then 100 μL of the solution was injected into the vitreous. For the DEX suspension group, the formulation was prepared by dispersing 1 mg/mL DEX in sterilized NS under ultrasonic and then immediately injected into the vitreous. The dose of the DEX suspension is equal to that of the thermogel formulation.

At predetermined time points after administration of the thermogel (1, 6, 12 h, and then 1, 3, 6, 9 day), 200 μL vitreous humor was carefully collected under anesthesia, and immediately stored at −80 °C until use. The vitreous humor in the DEX suspension treated group was also collected at 0.5, 1, 3, 6, 9, 12, and 24 h for comparison. At the end of the experiment, the rabbits were euthanatized by injection of a lethal dose of pentobarbital.

The amount of DEX released *in vivo* was measured by HPLC (Agilent 1100). The measurements were carried out using a C18 reverse-phase column with the mobile phase of water-acetonitrile at a ratio of 45:55 at a constant flow rate of 1.0 mL/min. An ultraviolet detector was used at a wavelength of 254 nm. The samples were mixed with methanol at a volume ratio of 1:3 by vortex, and then centrifuged at 12,000 rpm for 10 min to denature and remove the proteins in the vitreous humor. An aliquot of 20 μL supernatant was injected into the HPLC system to determine the concentration of DEX.

2.7. In vitro/in vivo correlation of DEX release

The deconvolution method was used to elevate the *in vivo* release profile [32]. This method is based on the convolution integral transform, as shown in Eq. (2):

$$R(t) = \int_0^t I(\tau)W(t-\tau)d\tau = I(t) * W(t) \quad (2)$$

Here, the response function $R(t)$ means the transient plasma drug concentration at time t for a formulation; the input function $I(t)$ is the *in vivo* drug release rate, i.e., dA/dt (A is the release amount); and the weighting function $W(t)$ is a so-called unit impulse response, which corresponds to the instantaneous plasma drug concentration at time t after administration of a unit amount drug; "*" means convolution. Since both $R(t)$ and $W(t)$ could be obtained by *in vivo* drug release experiments, the input function $I(t)$ can be transformed by the deconvolution method, and furthermore the *in vivo* cumulative release was calculated according to the following relation:

$$(in\ vivo\ \text{release})\% = \int_0^t I(\tau)d\tau \Big/ \int_0^\infty I(\tau)d\tau \quad (3)$$

It is needed to point out that the above relations are based upon the superposition principle, which abides by linear assumptions of dose proportionality and time invariance of the system investigated.

2.8. Statistical analysis

Data were analyzed using a one-way ANOVA or Student's *t*-test when appropriate. Differences were considered to be significant at a level of $P < 0.05$.

3. Results

3.1. Synthesis and characterization of PLGA–PEG–PLGA triblock copolymers

Two PLGA–PEG–PLGA triblock copolymers, Copolymer-1 and Copolymer-2, were synthesized via the ring-opening copolymerization of LA and GA in the presence of PEG as the macroinitiator and $Sn(Oct)_2$ as the catalyst. The molecular composition of PLGA–PEG–PLGA triblock copolymer was determined by ^{1}H NMR measurements, as shown in Fig. 2. The characteristic peaks at 5.20, 4.80, 3.60 and 1.55 ppm were assigned to the methine proton of the LA units, the methylene protons of the GA units, the methylene protons of PEG and the methyl protons of the LA units, respectively. The methylene protons of PEG segment neighboring the PLGA blocks appeared at 4.31 ppm. The number-average MW (M_n), LA/GA molar ratio and PEG/PLGA molar ratio were calculated via the integral of the peaks at 5.20, 4.80, and 3.60 ppm, as described in the previous publication [38]. The results confirmed that the two copolymers had similar total MWs (approximately 4000) but different block ratios of PEG/PLGA. The GPC traces of the two specimens exhibited a unimodal distribution with dispersity less than 1.25, indicating that the polymerization was well-controlled and the desired polymers were obtained. Interestingly, their mixtures with different ratios (2/1, 1/1, and 1/2) also showed unimodal and their dispersities just located between the initial two polymers with the results presented in Fig. 3. This feature was attributed to the very close MWs of the two polymers synthesized by us. Molecular parameters of the two triblock copolymers and their mixtures with the indicated mixing ratios determined by ^{1}H NMR and GPC are listed in Table 1.

3.2. Sol–gel transition of the aqueous solutions of the PLGA–PEG–PLGA triblock copolymer mixture

Although Copolymer-1 and Copolymer-2 had similar total MWs, their aqueous behaviors were entirely different. Copolymer-1 was too hydrophobic to dissolve in water, while Copolymer-2 was soluble in water but could not form a physical

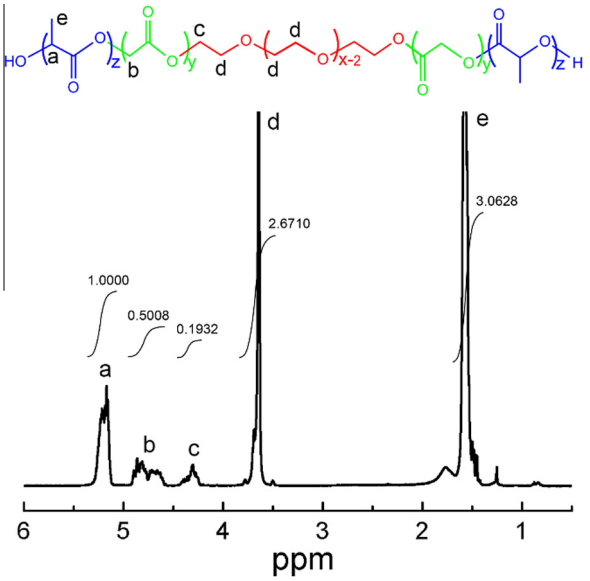

Fig. 2. ^{1}H NMR spectrum of a PLGA–PEG–PLGA triblock copolymer (Copolymer-1) in $CDCl_3$.

hydrogel at any concentration or temperature. Fortunately, their mixture with an appropriate mixing ratio underwent a reversible sol–gel transition triggered by an increase in the temperature. The phase diagrams of the indicated mixtures are shown in Fig. 4, which were determined via the test tube inverting method. All of the three mixtures formed free-flowing sols in water below or at room temperature and turned into semi-solid gels as the temperature increased. Further increase in the temperature resulted in another transition from gel to turbid suspension of the polymer in water. The mixing ratios of the two copolymers had a significant influence on their phase transition behaviors. The mixture/water system with a higher component of Copolymer-1 presented a lower sol–gel transition temperature, a wider gel window and a smaller critical gel concentration (CGC).

Dynamic rheological measurements were further performed to estimate the changes in modulus (G') and viscosity (η) during the sol–gel transition of aqueous polymer solutions. The G' values of the mixture/water systems were very low below or at room

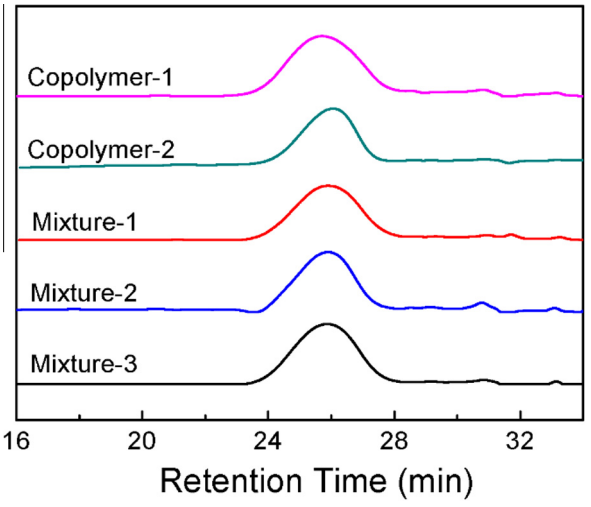

Fig. 3. GPC traces of PLGA–PEG–PLGA triblock copolymers and their mixtures with the fixed mixing ratios as indicated in Table 1.

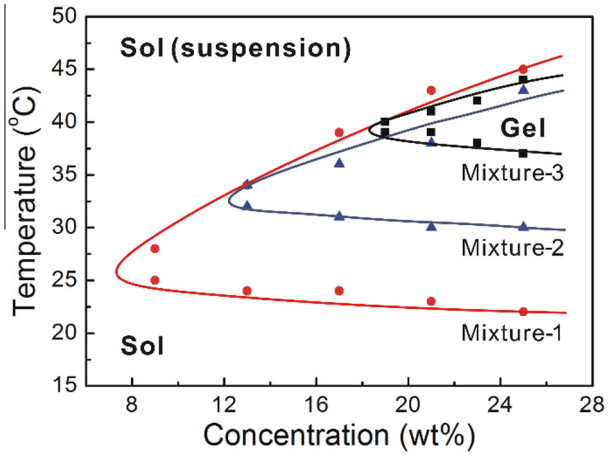

Fig. 4. Phase diagrams of PLGA–PEG–PLGA triblock copolymer mixtures in NS with the indicated mixing ratios. The mixing ratios were presented in Table 1.

temperature (Fig. 5a), suggesting that they were low-viscous liquids and had good injectability. As the temperature increased, the G' of the samples rapidly increased over 4 orders of magnitude. The abrupt increase in G' reflects the formation of *in situ* thermogel [23]. As the mixing ratio of Copolymer-1 and Copolymer-2 varied from 2/1 to 1/2, the sol–gel transition temperature increased from 22 °C to 37 °C while the corresponding maximum of G' decreased. The change in viscosity as a function of temperature presented a similar manner (Fig. 5b). Considering that the gel window of Mixture 3 was too narrow for biomedical applications, our follow-up experiments mainly focused on Mixture-1 and Mixture-2.

3.3. In vitro drug release

Although the solubility of DEX in water is quite low (0.1 mg/mL) [49], we found surprisingly that 1 mg/mL of DEX could be dissolved in the polymer aqueous solution just by stirring. For the polymer solution containing a higher drug concentration, DEX was first dissolved in a minimized amount of alcohol and then added into the polymer solution to ensure the homogeneous distribution of drugs. After stirring in an open system, the residual alcohol was removed due to evaporation and a translucent polymer solution with well dispersed DEX was obtained. There was almost no loss of drug occurred during the whole preparation process, and the drug loading efficiency of the DEX-contained thermogels was approximately 100%.

Table 1
Characterization of PLGA–PEG–PLGA triblock copolymers and their mixtures.

Sample	Mixing ratio[c]	PEG M_n[d]	M_n[d]	LA/GA (mol/mol)[d]	M_n[e]	(M_w/M_n)[e]
Copolymer-1[a]	/	1000	1510–1000–1510	4.0	5210	1.21
Copolymer-2[b]	/	1500	1270–1500–1270	4.0	5030	1.13
Mixture-1	2/1	/	/	4.0	5210	1.19
Mixture-2	1/1	/	/	4.0	5190	1.15
Mixture-3	1/2	/	/	4.0	5120	1.16

[a] Insoluble in water.
[b] Dissolved in water as a sol in a broad temperature region.
[c] Mixing ratio by weight of Copolymer-1 and Copolymer-2 and the obtained mixtures exhibited a thermoreversible sol–gel transition in water upon increasing temperature.
[d] Number-averaged MW, M_n of the central block PEG was provided by Aldrich. Molar ratio of lactide/glycolide (LA/GA) and M_n of each PLGA block were calculated by ^{1}H NMR.
[e] Measured by GPC.

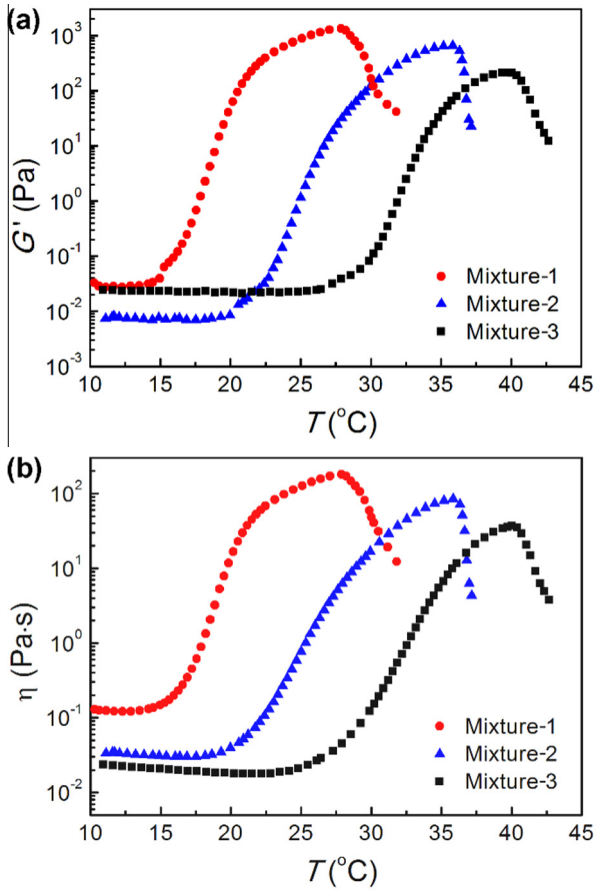

Fig. 5. (a) Storage modulus (G') and (b) viscosity (η) of copolymer mixtures with the indicated mixing ratios in NS (25 wt.%) as a function of temperature. Heating rate: 0.5 °C/min; oscillatory frequency: 10 rad/s.

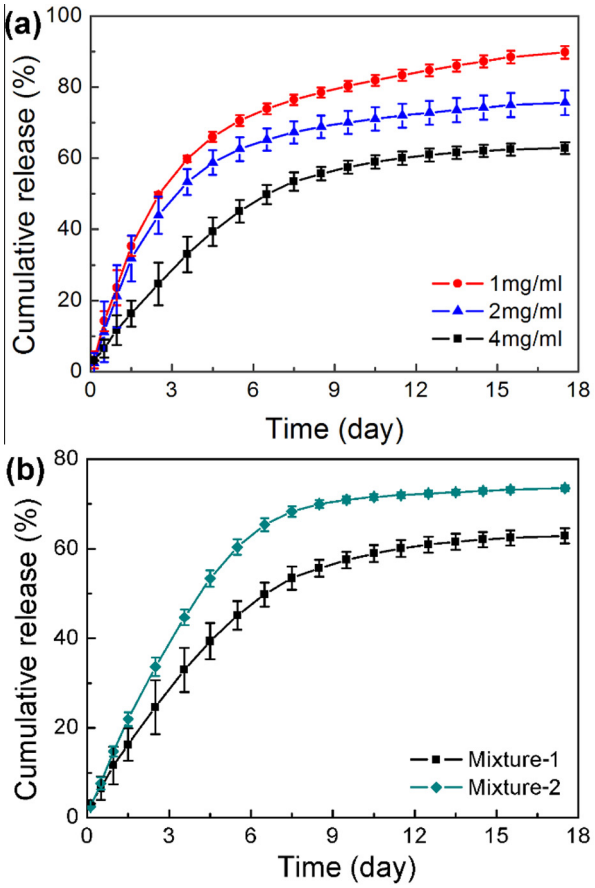

Fig. 6. (a) Cumulative release profiles of DEX from Mixture-1 thermogel with the indicated concentrations in PBS at 37 °C. Polymer concentration was 25 wt.%. (b) Cumulative release profiles of DEX from mixture thermogels with the indicated mixing ratios of Copolymer-1/Copolymer-2 in PBS at 37 °C. Polymer concentration was 25 wt.% and DEX concentration was 4 mg/mL. Error bars represent the standard deviation (n = 3).

The release profiles of DEX from Mixture-1 thermogel at different drug concentrations are shown in Fig. 6a. The drug exhibited a sustained release for more than 10 days. As DEX concentration increased from 1 mg/mL to 4 mg/mL, not only the initial burst on the first day reduced from 24% to 12%, but also the totally cumulative release decreased from 90% to 60%. This finding indicated that both burst and cumulative release significantly relied on DEX concentration. The influence of copolymer mixing ratio on release profile was also investigated, as presented in Fig. 6b. With the decrease of the Copolymer-1 component in the hydrogel matrix, DEX exhibited a faster release rate and a higher cumulative release amount.

The release data were further fitted by the first-order release equation, and the results listed in Table 2 show that all of the profiles were well consistent with the equation and good correlation coefficients R were obtained ($R^2 > 0.99$). This feature indicates that drug diffusion combined with gel erosion governed the release of DEX from the hydrogel depots.

Table 2
First-order kinetic assessment of the *in vitro* release data.

Sample	DEX concentration (mg/mL)	K	M_∞	R^2
Mixture-1	1	0.321	0.864	0.995
	2	0.351	0.738	0.998
	4	0.206	0.659	0.998
Mixture-2	4	0.264	0.759	0.994

3.4. Intraocular retention and biocompatibility of the mixture thermogel

A fluorescein sodium-loaded thermogel was injected into the vitreous of rabbit eyes and then a series of fluorescence images were recorded at predetermined time points to noninvasively monitor the intraocular retention of the *in situ* formed hydrogel. No fluorescence was observed in the eyes receiving NS treatment (images not shown). After administration of a conventional solution, dispersive yellow fluorescence was seen in the rabbit eyes through the pupil, however, it quickly faded away within 8 h. The next day, no appreciable fluorescence could be observed (Fig. 7a), and fluorescein sodium might be rapidly cleared from the posterior segments. In contrast, in the thermogel-treated group, fluorescence was still obviously visible in the eyes even on the 11th day (Fig. 7b), indicating the long retention of the thermogel in the posterior segment.

To evaluate the biocompatibility of the implanted thermogel, the rabbits were kept for an additional week in order to allow the hydrogel matrix to totally degrade, and then the eyes were harvested for histological observation. As shown in Fig. 8, the tissues exposed to the hydrogel exhibited the regular morphology compared with the normal ones, and no obvious damage or change was found in the retina and cornea 3 weeks post-administration of the fluorescein sodium-loaded Mixture-1 hydrogel. In addition, no sign of inflammation, tissue necrosis or cornea endothelium cell loss was observed in any of the examined eyes. Therefore, the thermogel matrix had good biocompatibility and great potential for ophthalmic drug delivery.

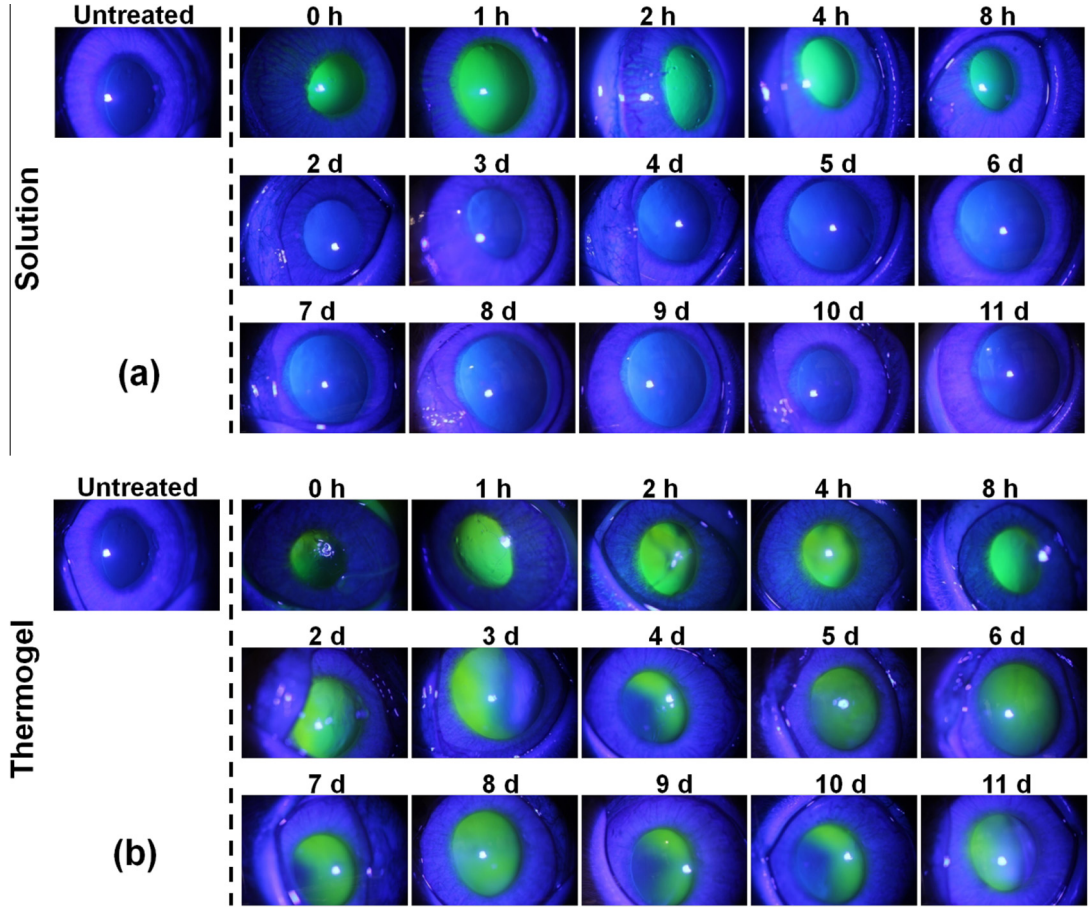

Fig. 7. Fluorescence images of the rabbit eyes under a cobalt-blue exciting light of the slit lamp after injection 100 μL fluorescein sodium solution (0.1 wt.%) (a) or fluorescein sodium-loaded Mixture-1 thermogel (0.1 wt.%) (b) into the vitreous. Polymer concentration was 25 wt.%.

3.5. Intraocular pharmacokinetics of the DEX-loaded thermogel

The intraocular pharmacokinetics of the DEX-loaded Mixture-1 thermogel and the DEX suspension was determined by HPLC. DEX concentrations in the vitreous were plotted against time as shown in Fig. 9. After the suspension was administered, DEX rapidly reached the peak concentration and then was quickly eliminated from the vitreous. DEX concentration subsequently decreased below the limit of quantitation (LOQ, approximately 52 ng/mL) of the assay method, and could not be detected 24 h post-injection. In contrast, the pharmacokinetics of DEX in the thermogel-treated group was much different, which exhibited a low burst release and a very slow elimination rate. The concentration of DEX in the vitreous peaked at 9.34 μg/mL in the first 6 h, which was only 1/18 of the peak concentration of DEX suspension, and then remained steady above the minimal effective concentration of DEX [50] for at least 9 days.

Intraocular pharmacokinetic parameters of the DEX-loaded thermogel and the DEX suspension could be calculated according to the obtained data. It should be noted that both of the formulations possessed similar bioavailability due to the approximate area under curve ($AUC_{(0-\infty)}$) as shown in Table 3, however, the mean residence time ($MRT_{(0-\infty)}$) of the DEX-loaded thermogel was much longer than that of the DEX suspension.

3.6. In vitro/in vivo correlation of the DEX-loaded thermogel

Furthermore, a deconvolution method was employed to calculate the input function $I(t)$, which corresponded to the *in vivo* drug release rate. Here, the transient DEX concentration in the vitreous for the DEX-loaded thermogel was defined as the response function $R(t)$, and the data for the group of DEX suspension divided by the total drug dose (100 μg) as the weighting function $W(t)$. Following the input function $I(t)$ determined based on Eq. (2), the cumulative drug release *in vivo* was finally achieved via Eq. (3). As shown in Fig. 10, the *in vivo* release profile also exhibited a sustained release manner. Although the *in vivo* cumulative release was faster compared with the *in vitro* experiment results, the *in vivo* pharmacokinetics was essentially consistent with the *in vitro* release profile.

4. Discussion

In the present study, a thermoreversible hydrogel composed of copolymers with diverse properties was developed as an injectable implant for intravitreal drug delivery. Two PLGA–PEG–PLGA triblock copolymers with similar total MWs but different PEG/PLGA ratios were synthesized, among which one is soluble while the other precipitates in water. At appropriate mixing ratios, their mixtures underwent a reversible sol–gel transition as the temperature increased. The sol–gel transition temperature, width of gelation window, CGC and gel modulus of the mixture hydrogels could be well-tuned via varying the mixing ratio of these two copolymers (Figs. 4 and 5). For example, the sol–gel transition temperature was conveniently adjusted in a physiologically important temperature range (20–45 °C) by simply changing the mixing ratio from 2/1 to 1/2. Meanwhile, increasing hydrophobic Copolymer-1 component in the thermogel system led to a decrease in the sol–gel

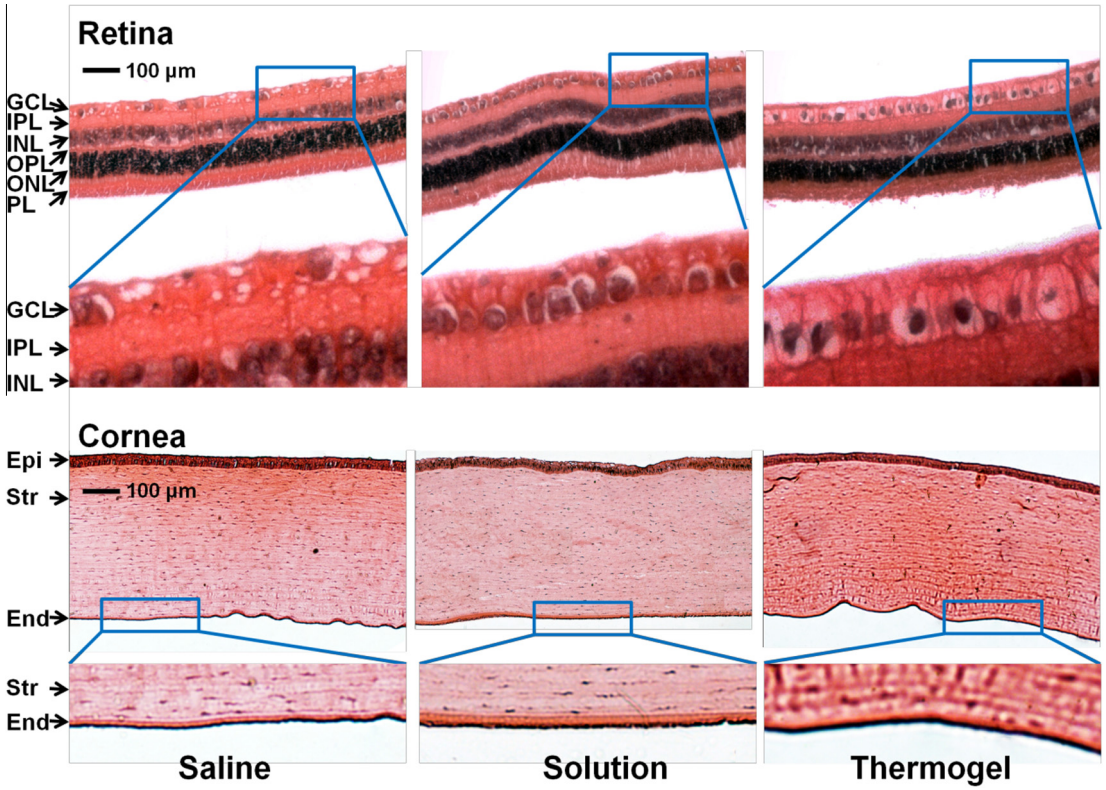

Fig. 8. Representative histological microphotographs of retina (up) and cornea (down) after being exposed to NS, fluorescein sodium solution and fluorescein sodium-loaded Mixture-1 thermogel for 21 days, at magnification 100×. From the inner to the outer part of retina, cellular layers are organized as follows: ganglion cell layer (GCL), inner plexiform layer (IPL), inner nuclear layer (INL), outer plexiform layer (OPL), outer nuclear layer (ONL), and photoreceptor layer (PL). From the outer to the inner part of cornea, cellular layers are organized as follows: epithelium (Epi), stroma (Str), and endothelium (End).

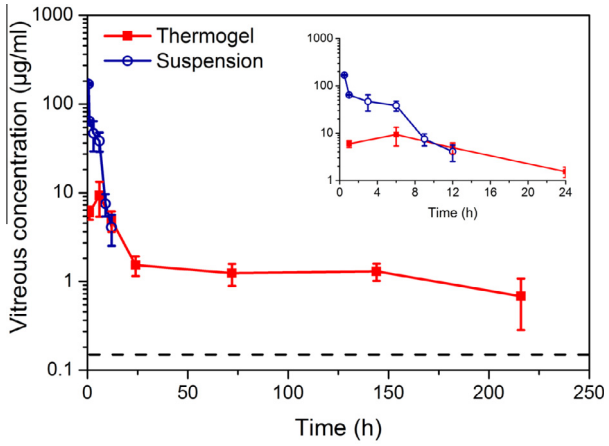

Fig. 9. Vitreous DEX concentrations in rabbits treated with the DEX-loaded thermogel or DEX suspension. Mixture-1 was chosen as the thermogel matrix, and polymer concentration was 25 wt.%. DEX concentration was 1 mg/mL and the injection volume was 100 μL for both the thermogel and the suspension ($n = 3$). The dash line represents the minimal effective concentration of DEX. The inserted graph shows the initial release of DEX on the first day after administration.

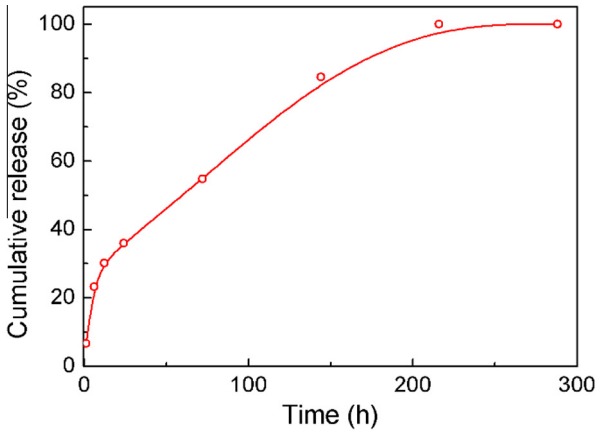

Fig. 10. *In vivo* cumulative release of DEX from the thermogel calculated according to the deconvolution method based on the response and weighting functions via Eqs. (2) and (3). Mixture-1 was used as the thermogel matrix, polymer concentration was 25 wt.% and drug concentration was 1 mg/mL.

Table 3
A summary of intraocular pharmacokinetic parameters of the DEX-loaded thermogel and the DEX suspension ($n = 3$).

Parameters	DEX-loaded thermogel	DEX suspension
$MRT_{(0-\infty)}$ (h)	115.8 ± 68.3*	4.1 ± 0.3
$AUC_{(0-\infty)}$ (mg/L h)	608.0 ± 50.9	522.7 ± 90.1

* Statistical difference at $P < 0.05$ compared with that of the DEX suspension, calculated using Student's *t*-test.

transition temperature but an enhancement in both modulus and viscosity of the gel.

The injectable DEX-loaded thermogel formulation was conveniently prepared by simply adding DEX into the aqueous polymer solution at low temperatures and then stirring to achieve a homogeneous system. The solubility of DEX in the polymer solution was increased to 1 mg/mL compared with its low solubility (0.1 mg/mL) in water. In fact, amphiphilic PLGA–PEG–PLGA triblock copolymers easily self-assemble into core–shell micelles in water and the spontaneous gelation of concentrated PLGA–PEG–PLGA solution (>CGC)

occurs upon the formation of a percolation micelle network as the temperature increases [38,39]. An important property of polymeric micelle is able to increase the solubility of hydrophobic drugs, which can be physically incorporated within the hydrophobic cores of micelles and solubilized in the polymer solution. Consequently, it is obvious that the increase of DEX solubility in the aqueous polymer solution was attributed to the solubilization of micelles.

The *in vitro* release of DEX from the thermogel matrix lasted over 10 days in a sustained manner, and both the initial burst and the accumulative release decreased with the increased drug loading (Fig. 6a). It is worth of pointing out that some DEX was just located at the hydrophilic shells or between micelles after being entrapped into the thermogel depot due to a certain solubility of DEX in water (0.1 mg/mL), leading to some burst release at the initial stage. Moreover, the mixing ratio of the two copolymers has a significant effect on the release profile as well (Fig. 6b). A higher ratio of hydrophobic Copolymer-1 component in the formulation resulted in a slower release, and the total release amount simultaneously decreased. This result might be attributed to the different compositions, mechanical properties and degradation rates of the mixture hydrogels.

Recently, a few of other thermogels containing single polymers have been tried as intraocular implants. For example, a biodegradable *in situ* gelling system based on a graft copolymer of gelatin-*g*-poly(*N*-isopropylacrylamide) was designed for intracameral administration of pilocarpine to the anterior segment [51]. Another thermosensitive hydrogel composed of poly(trimethylene carbonate)$_{15}$–Pluronic F127–poly(trimethylene carbonate)$_{15}$ copolymer was prepared and then the mitomycin C-loaded hydrogel was injected into the subconjunctival space to inhibit postoperative fibrosis in a rabbit glaucoma filtration surgery model. However, this formulation exhibited a significant burst release *in vitro* and approximately 57% of the incorporated drug was released within the first day [52]. Also, Hwang and Hsiue [53] proposed a thermoreversible hydrogel composed of an amphiphilic copolymer containing poly(2-ethyl-2-oxazoline) and poly(ε-caprolactone) blocks as a sustained carrier for intraocular delivery of bevacizumab. The biocompatibility of the hydrogel to neuroretina was confirmed and the *in vitro* release of monoclonal antibody from hydrogel depot lasted for about 10 days. In another work, amphiphilic block copolymers of methoxy–PEG–PLGA were linked using 2,2-bis (2-oxazoline) as the coupling agent, and then used as the thermogel matrix for intravitreal injection. Released bevacizumab *in vitro* from the hydrogel showed the bioactivity to inhibit angiogenesis [54]. Nevertheless, the duration of *in vivo* drug release was not demonstrated in these two studies. Compared with these single polymer-based hydrogels, our mixture hydrogel is more flexible in adjustment of the thermogelling property and release rate just by varying the mixing ratio of the two polymer components to meet the requirements of a given therapeutic agent and the demands of a specific clinical situation.

Slit lamp microscope is a powerful tool in clinical ophthalmology, which has been utilized to observe the hydrogels implanted in the rabbit anterior chamber [55,56] and to examine the fundus after intravitreal injection of nanoparticles [57]. It is difficult to legibly observe an almost translucent hydrogel that has been administered to the posterior segment of eye. Therefore, in the current study, fluorescein sodium was introduced as a fluorescence probe to noninvasively monitor the intraocular fate of the thermogel. Slit lamp photographs of the rabbit eyes under a cobalt-blue light show that fluorescein was completely eliminated from the vitreous within 24 h after intravitreal administration of the solution (Fig. 7a), indicating that daily injection is required to maintain effective therapeutic concentration for a normal liquid dosage form. The quick clearance of fluorescein is primarily due to intraocular convection from vitreous humor to aqueous humor [10]. Compared with the solution group, the preservation of fluorescence in the thermogel-treated eyes was significantly prolonged over 10 days (Fig. 7b), which was attributed to the sustained release of fluorescein from the hydrogel matrix. This feature provides a strong proof for the long retention of the thermogel in eyes, and also suggests the thermogel has great potential as a long-acting intraocular delivery system and thus can decrease the risks of frequent injections.

Biocompatibility, especially the tolerance of posterior segment tissues, is a crucial issue that should be carefully taken into consideration for an intraocular implanted formulation. Retina is a spatial information processor built upon a mosaic of rod and cone photoreceptors, which are the physical basis of vision [58]. Corneal endothelium is constituted of monolayer cells that regulate stromal hydration and maintain corneal clarity, and would be exposed to the degraded fragments of the thermogel via the intraocular fluid flow. Once damaged, the corneal endothelial cells would cause an irreversible decrease in cell density due to their limited regenerative capacity [59]. Therefore, the histological evaluation of the retina and cornea was performed on the thermogel-treated eyes. Both soluble PEG and biodegradable PLGA blocks have low cytotoxicity, and have been approved by FDA to use in clinic [60–62]. Moreover, some marketed intravitreal implants are also constructed based on PLGA [11]. As expected, neither decrease in cell density of corneal endothelium, nor retinal necrosis and infiltration of inflammatory cells were observed in these tissues exposed to the thermogel for 21 days (Fig. 8), as compared to the normal eyes. These results demonstrate that the PLGA–PEG–PLGA mixture thermogel was nontoxic to corneal and retinal tissues, confirming its good biocompatibility as an intraocular delivery vehicle.

The ocular pharmacokinetics after intravitreal injection of DEX-loaded thermogel and DEX suspension was further evaluated via paracentesis. After the vitreous humor is withdrawn, the aqueous humor, driven by intraocular pressure, will supplement the vacant space [63], which will slightly accelerate the convection of intraocular fluid and the elimination of drugs. Nevertheless, convection has a relatively minor influence for small molecule drugs and high diffusivity drugs [64]. The DEX concentration–time profiles in Fig. 9 reflect the remarkable differences between the two formulations. In the vitreous, DEX was quickly cleared away within 24 h after administration of the suspension. In contrast, DEX remained detectable for at least 9 days after a single injection of the DEX-loaded thermogel. This finding is well consistent with the fluorescence tracing as illustrated in Fig. 7. Diverse solubility of DEX and fluorescein resulted in similar elimination behaviors, implying that *in vivo* erosion of the *in situ* formed hydrogel matrix plays an important role in the intraocular retention of the embedded molecules. Nevertheless, the concentration of DEX in aqueous humor was too low to be detected by HPLC (data not shown) after intravitreal injection. The same phenomenon was also observed by other group [57]. The retention time of DEX suspension in the vitreous was too short to treat chronic conditions of the eye. Fortunately, our thermogel significantly prolonged the intravitreal release of DEX, and the $MRT_{(0-\infty)}$ of DEX exhibited a 28-fold increase compared with that of the suspension (from 4.1 h to 115.8 h). More importantly, the initial concentration of DEX delivered by the thermogel is well-controlled below 10 μg/mL in the vitreous, which is almost two orders of magnitude lower than the peak concentration of the suspension. This feature reveals that the aqueous copolymer solution rapidly transformed into a semi-solid gel in the physiological environment of the eye once administered, and thus the initial burst release was inhibited significantly. According to the previous study, the effective level of DEX to suppress inflammation is 0.15–4.00 μg/mL [50]. After a single injection, our thermogel provided constantly intravitreal

DEX concentrations ranging from 0.67 μg/mL to 9.34 μg/mL for 9 days, during which the released amount of DEX met the therapeutic dosage. Thus, fluctuation of DEX concentration associated with multiple doses is able to be avoided by application of the thermogel. Meanwhile, not only patient compliance would be improved, but also the risk of complications should be lowered because of the reduced injection frequency. In addition, the *in vivo* cumulative release profile calculated from the pharmacokinetic data via the deconvolution method is basically correlated with the *in vitro* release curve, indicating that the *in vitro* evaluation could be used for prediction of the intraocular performance.

5. Conclusion

The present study developed an injectable intravitreal implant using thermogel for sustained delivery of DEX to the posterior segment of the eye. PLGA–PEG–PLGA copolymers with different block ratios were synthesized as the thermogel matrix, and the micelles formed by the copolymer mixture facilitate solubilization of hydrophobic DEX molecules in an aqueous medium. Gelation performance of the thermogel and release rate of DEX are easily adjusted via varying the mixing ratio of the two copolymers, which is more flexible in the aspect of formulation design. Both *in vitro* and *in vivo* evaluations confirmed the sustained release of DEX from the hydrogel matrix. Compared with other injections like suspension, the ocular retention time of DEX was lengthened from several hours to more than 1 week after a single intravitreal injection of the DEX-loaded thermogel. Moreover, histological examination demonstrated excellent biocompatibility of the mixture thermogel in eyes. Taken together, the injectable mixture thermogel is a promising drug delivery vehicle to remit frequent intraocular injections, therefore brings an alternative approach for treatment of posterior segment diseases.

Acknowledgments

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Appendix A. Figures with essential colour discrimination

Certain figures in this article, particularly Figs. 1–10, are difficult to interpret in black and white. The full colour images can be found in the on-line version, at doi: http://dx.doi.org/10.1016/j.actbio.2015.05.005.

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