Sustained subconjunctival delivery of cyclosporine A using thermogelling polymers for glaucoma filtration surgery†

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Filtration surgery is currently being acknowledged as the most effective therapeutic modality for glaucoma; however, excessive scarring of a filtering bleb often leads to surgical failure. Subconjunctival administration of drug delivery systems provides a localized and sustained delivery of antifibrotic drugs to inhibit post-operative scarring. In this study, a long-acting delivery system of Cyclosporine A (CsA) was developed using thermogelling poly(e-caprolactone-co-glycolic acid–poly(ethylene glycol)–poly(e-caprolactone-co-glycolic acid) (PLGA–PEG–PLGA) triblock copolymers as the carrier. The drug could be easily entrapped into the polymer aqueous solution by simply mixing them at room temperature, and the prepared formulation was spontaneously transformed into an in situ thermogel loaded with drugs at body temperature. The formulation exhibited a sustained in vitro release of CsA from the thermogel over 2 months. The in vivo efficacy of subconjunctival administration of the CsA-loaded hydrogel system in inhibiting filtering bleb scarring was evaluated on a rabbit model of filtration surgery. No ocular tissue abnormalities and damage were observed. Compared with a local administration of the popular Mitomycin C solution at the surgery site, the CsA-loaded thermogel system remarkably inhibited scar formation, maintained stable low intraocular pressure and promoted filtering bleb survival for more than 10 weeks. This feature was attributed to both the anti-adhesion nature of the thermogel matrix and the antifibrotic effect of the sustainedly released CsA. These results suggest that the injectable PLGA–PEG–PLGA thermogel is a biocompatible carrier for sustained subconjunctival drug delivery, and the CsA-loaded hydrogel formulation has great potential for ocular antifibrotic therapy after glaucoma filtration surgery and others.

1. Introduction

Glaucoma is the most common cause of irreversible blindness worldwide and is usually associated with increased intraocular pressure (IOP). In order to lower IOP, glaucoma filtration surgery is performed by creating a fistula between the subconjunctival space and the anterior chamber to allow the drainage of aqueous humor out of eyes. Unfortunately, the failure rate of surgery is as high as 30–40% for patients receiving glaucoma filtration surgery because of filtering bleb scarring through fibroblast proliferation and collagen deposition at the surgery site, which further leads to a fistula block.1

As is well-known, scarring is an inevitable process to all wound healing, yet glaucoma filtration surgery seeks to achieve an incomplete healing. Its success is linked to the interruption of wound-healing response in order to maintain patency of the new filtration pathway and allow aqueous humor to escape the eye.2 Therefore, effective modulation of wound healing is critical to ensure successful surgery. Numerous ophthalmic drugs, such as anti-inflammatory agents,3 antifibrotic agents,4 antiproliferative agents5 and growth factor inhibitors,6 have been employed to modulate the wound healing process and thus to prevent or reduce scar formation.

Among the antifibrotic agents, Mitomycin C (MMC) and 5-fluorouracil (5-FU) are the most popular agents and are often

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††Electronic supplementary information (ESI) available: The optical microscope image of the silicon GDD, the 1H NMR spectrum of the PLGA–PEG–PLGA triblock copolymer, the GPC curve of the synthesized copolymer, and the TEM image of copolymer micelles. See DOI: 10.1039/c7tb01556a
used to inhibit scar formation after glaucoma surgery.\textsuperscript{1,7–9} However, the two drugs suffer from some serious complications, such as chronic hypotony, bleb leaks, ruptured blebs, corneal epithelial and endothelial toxicity, and conjunctival wound.\textsuperscript{2,10} Cyclosporine A (CsA) is a highly effective immunosuppressive agent and has less toxicity compared with MMC and 5-FU.\textsuperscript{11,12} To date, CsA has been widely utilized in clinics for prevention of allograft rejection after cornea transplantation, immunosuppression in high-risk Keratoplasty, and treatment of ocular inflammation.\textsuperscript{13–15} Meanwhile, CsA has been employed to inhibit scar formation after ocular surgeries.\textsuperscript{16,17}

Actually, CsA has also some drawbacks, such as large molecular weight (MW, 1202 Da), poor penetration and low solubility in water (27.67 $\mu$g mL$^{-1}$, 25 °C),\textsuperscript{18} which lead to a low and irregular bioavailability. In view of the clinical importance of CsA, many delivery systems of CsA, such as liposomes,\textsuperscript{19} nanoparticles,\textsuperscript{20,21} microemulsions/emulgels,\textsuperscript{22,23} and polymeric micelles\textsuperscript{24,25} have been exploited to increase its solubility and improve its bioavailability. Nevertheless, these delivery systems exhibited a short drug release period, which limited their application during glaucoma surgery unless with repeated administrations. Frequent intraocular administrations not only drastically reduce the compliance of patients, but also significantly increase the risk of complications. Therefore, development of long-term delivery systems of CsA is much desirable.

Recently, thermogelling polymers have gained increased attention as minimally invasive depot systems for drug delivery.\textsuperscript{8,26–33} Tissue repair,\textsuperscript{34–36} post-operative anti-adhesion,\textsuperscript{37–40} and polymer/water system undergoes a sol–gel transition upon heating, so it is a flow-free sol phase at ambient temperature and can spontaneously transform into a gel with an increase in temperature. Fragile drugs and bioactive molecules are easily incorporated into the polymer aqueous solution by simply mixing them at low temperature, and the prepared formulation can be spontaneously transformed into a gel with an increase in heating, so it is a free-flowing sol phase at ambient temperature as the carrier was subconjunctivally administrated to inhibit post-operative scar formation and thus maintain filtering bleb survival, as schematically presented in Fig. 1. An intraoperative 5 minute application of 0.4 mg mL$^{-1}$ MMC solution in rabbit eyes was selected as the control. The morphology of the filtering bleb, lowering effect of IOP, patency of the drainage pathway, and histological analysis as the judgment indexes of therapeutic efficacy were examined and compared.

2. Experimental

2.1. Materials

PEG with MW 1500, stannous octoate of purity 95%, 1,1'-dioctadecyl-3,3',3'-tetramethylindocarbocyanine perchlorate (Dil) and Dulbecco's modified Eagle's medium/Nutrient Mixture F-12 Ham (DMEM-F12, D9800) were purchased from Sigma-Aldrich (Shanghai, China). $\beta_1$-Lactide (LA) and glycolide (GA) were obtained from Purac and used as received. An injection of glycerol (10 wt%) was obtained from Cisen Pharmaceutical Co., Ltd (Shanghai, China). Cyclosporin A (CsA, 99%) was purchased from J&K Scientific Ltd (Shanghai, China). All other chemicals used were of analytical grade and were used without further purification. The liquid medical grade silicone elastomer (MED-4244) was obtained from Nusil Silicone technology (Carpinteria, USA). The medical grade silicone tube was purchased from Guangzhou Tianling silicone Co., Ltd. Other drugs included xylazine hydrochloride (Jilin Province TAT Animal Pharmaceutical Co., Ltd, Jilin, China), diazepam injection (Shanghai Xudong Haipu Pharmaceutical Co., Ltd, Shanghai, China), tobramycin and dexamethasone eye ointment (S.A. Alcon Couvreur N.V., Belgium), ofloxacin eye ointment (Shenyang Sinqi Pharmaceutical Co., Ltd, Shenyang, China), oxybuprocaine hydrochloride eye drops (Santen Pharmaceutical Co., Ltd, Shanghai, China), phosphate-buffered saline (PBS; Thermo Scientific, Shanghai, China), paraformaldehyde (PFA; Thermo Fisher Scientific, Shanghai, China), penicillin-streptomycin (15140-122, Gibco, USA) and fetal bovine serum (FBS; AXB30114, Hyclone, USA).

2.2. Animals

Male New Zealand white rabbits free of any ocular damage and weighing 2.0–2.5 kg were obtained from the Yin'gen Rabbits' house (Shanghai, China). The rabbits were housed under standard conditions (25 °C, relative humidity 50%) in the animal facilities of the Eye & ENT Hospital (Fudan University) with free access to food and water. All experimental protocols, including experiments, transportation and care of the animals complied with the Association for Research in Vision and Ophthalmology Statement for the Use of Animals in Ophthalmic and Vision Research and the guidelines of the Animal Care and Use Committee of Fudan University (Shanghai, China).

2.3. Synthesis and characterization of the copolymer

The triblock copolymer, PLGA–PEG–PLGA, was synthesized through ring-opening polymerization of LA and GA in the presence of PEG (MW 1500) and the catalyst stannous octoate, as described in our previous publications.\textsuperscript{44,56} Briefly, PEG (15 g)
was added into a three-necked flask and dried under vacuum with stirring at 120 °C for 4 h to eliminate the residual moisture of the polymer. Then, a given amount of LA, GA and the initiator, stannous octoate, were added. The reaction system was further heated at 150 °C for 12 h under an argon atmosphere. After completion of the reaction, the system temperature was reduced to 120 °C, and unreacted monomers were removed under vacuum for 60 min. Crude polymer products were washed with 80 °C deionized water three times to remove water-soluble low-MW polymers, and the residual water in the polymer was removed by lyophilization. The final products were stored at /°C0 20 °C until use.

The chemical structure and composition of PLGA–PEG–PLGA copolymers were confirmed using a 400 MHz nuclear magnetic resonance (NMR) spectrometer (AVANCE III HD, Bruker). CDCl3 and tetramethylsilane (TMS) were used as the solvent and the internal standard, respectively. The MW and MW distribution of the copolymer were determined using a gel permeation chromatography system (GPC, Agilent 1260) equipped with a refractive index detector and a differential refractometer. The measurements were performed at 35 °C using tetrahydrofuran as the eluent at a flow rate of 1.0 mL min⁻¹. A series of monodispersed polystyrene standards were used for MW calculation. The PLGA–PEG–PLGA copolymer solution (20 μL, 0.5 wt%) was placed on a copper grid coated with a superthin carbon film and dried under an infrared lamp. The microscopic images were obtained by transmission electron microscopy (TEM, JEOL, JEM-2100F, Japan) with an accelerating voltage of 240 kV. The polymer aqueous solutions used for in vitro and in vivo experiments were sterilized through a 0.22 μm filter at 4 °C and CsA was sterilized under UV light (254 nm) for 30 min.

2.4. Dynamic mechanical analysis

Moduli of the PLGA–PEG–PLGA copolymer aqueous solutions with or without drugs were detected using a stress-controlled rheometer (Kinexus Pro, Malvern) equipped with a Peltier plate (1° steel cone, 60 mm diameter with a solvent trap). The polymer aqueous solution (25 wt% in normal saline (NS)) was placed between plates with a bob gap of 0.3 mm, and then a thin layer of silicone oil was added carefully to the edge of the upper plate to prevent evaporation of water. The plates were equilibrated to the starting temperature of 10 °C, and then temperature sweep tests were carried out at a fixed oscillatory frequency of 10 rad s⁻¹ within the temperature range of 10–45 °C at a heating rate of 0.5 °C min⁻¹. Rheological test parameters, storage modulus (G’), and shear loss or viscous modulus (G’’) were reported as a function of temperature.

2.5. In vitro drug release

In vitro drug release experiments were carried out according to the previous protocol⁵⁷,⁵⁸ and some details have been improved. The PLGA–PEG–PLGA copolymers (1.0 g) were dissolved in
NS (3.0 mL) to obtain 25 wt% polymer aqueous solution. Then, a given amount of CsA [1, 2 or 4 mg] was added to the copolymer solution (1 mL (~1 g)) and magnetically stirred at 600 rpm and 4 °C for 3 d to form a homogeneous and transparent solution. Subsequently, the drug-loaded polymer solution (0.2 g) was injected into a 15 mL vial and the vials containing samples were incubated in a water bath at 37 °C for 10 min. After the formation of a hydrogel, 10 mL phosphate buffered saline (PBS, pH 7.4, 37 °C) containing 0.5% Tween 80, which is used to improve the solubility of CsA in PBS,18 was added into the vial as the release medium. The shaking rate of the water bath was set at 50 rpm. 5 mL of the release medium were taken out and collected at predetermined time intervals (namely 1/48, 1/24, 1/6, 1, 2, 3, 5, 7, 10, 14, 18, 22, 27, 34, 41, 48, 55 and 62 d) and then the same amount of fresh PBS was refilled to maintain the sink conditions. The withdrawn release medium was filtered through a 0.45 μm filter and the drug amount released into the release medium was quantified using high-performance liquid chromatography (HPLC, Waters Separation Module e2695 and Waters UV/visible detector 2486, USA) equipped with a C18 reverse phase column. The mobile phase was composed of 80% acetonitrile and 20% water at a flow rate of 1 mL min⁻¹. The detection wavelength was 210 nm and the column temperature was fixed at 60 °C. The concentration of drug released into the release medium was calculated from a linear calibration curve plotted from known concentrations of CsA and then the cumulative release amount of CsA was obtained (n = 3).

2.6. In vitro cytotoxicity

The in vitro cytotoxicity of the thermogel and CsA@Thermogel with a given amount of CsA (1, 2 or 4 mg mL⁻¹) against human tenon’s fibroblasts (HTFs) was evaluated by the cell counting kit-8 (CCK-8, Sigma-Aldrich) assay. Polymer aqueous solution (Thermogel) or drug-loaded polymer aqueous solution (CsA@Thermogel) (0.1 mL, 25 wt%) was transferred into a transwell chamber (matching with 12-well cell culture plates) and then incubated at 37 °C for 10 min to form an in situ thermogel. HTFs were seeded into a 12-well cell culture plate with a density of 10 000 cells per well and cultured in DMEM-F12 containing 1% penicillin–streptomycin and 5% fetal FBS at 37 °C with 5% carbon dioxide and 95% relative humidity. After incubation for 24 h, the cells were washed with PBS and then the transwell chambers containing the Thermogel or CsA@Thermogel were added into the culture well. After 48 h of incubation, all the cells were washed with PBS, and the CCK-8 method was used to measure cell viability. The cells without any treatment were cultured under the same condition as the blank control. The relative cell viability in the Thermogel or CsA@Thermogel groups (n = 4) was assessed using the cell viability in the blank group as 100%.

2.7. In vivo application and evaluation of post-operative anti-scarring efficacy after glaucoma filtration surgery

To facilitate long-lasting intraocular monitoring of the Thermogel and CsA@Thermogel, Dil, a lipophilic orange-red fluorescent dye, was mixed into the polymer aqueous systems (0.5 wt% of total weight).59,60 A glaucoma drainage device (GDD) fabricated by us, as presented in Fig. S1 in the ESI† was used to construct an animal model of filtration surgery. A total of 24 rabbits were randomly divided into 4 groups (six rabbits per group): GDD group, GDD/MMC (0.4 mg mL⁻¹) group, GDD/Thermogel group, and GDD/CsA@Thermogel group. All the right eyes were set as treated groups and all the left eyes as the blank control group, as shown in Table 1.

<table>
<thead>
<tr>
<th>Groups</th>
<th>n</th>
<th>Treatment in the filtration surgery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blank</td>
<td>24</td>
<td>No operation</td>
</tr>
<tr>
<td>GDD</td>
<td>6</td>
<td>Filtration surgery with only GDD</td>
</tr>
<tr>
<td>GDD/MMC</td>
<td>6</td>
<td>Filtration surgery with GDD and MMC (0.4 mg mL⁻¹, 5 min)</td>
</tr>
<tr>
<td>GDD/Thermogel</td>
<td>6</td>
<td>Filtration surgery with GDD and 0.1 mL Thermogel (25%)</td>
</tr>
<tr>
<td>GDD/CsA@Thermogel</td>
<td>6</td>
<td>Filtration surgery with GDD and 0.1 mL CsA@Thermogel (25%, 2 mg mL⁻¹)</td>
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† The number of left eyes of rabbits.

At designated time points (7, 14, 21, 28, 35, 42, 49, 56, 63 and 70 d) post-operation, eyes of all the rabbits were observed under a slit lamp to evaluate surgery complications, such as general health, corneal edema, corneal epithelial defects, anterior chamber depth, anterior chamber reaction, intraocular inflammation or hemorrhage, and subconjunctival hemorrhage. Bleb condition, tube placement, implant extrusion, and suture exposure were...
also noted and recorded at each postoperative examination up to 10 weeks, which was thought to be a sufficient duration to compare differences in tissue response. IOP was also measured by gently and vertically touching the corneal centre using an Appplanation Tonometer (Tono-Pen Avia, Reichert, Inc., Depew, NY) under topical anesthesia, and each measurement was performed at 10–11 a.m. by the same technician. Baseline IOP was obtained after anesthesia but before operation. IOP measurements were taken again immediately after surgery, and at each sampling time point thereafter. Six data per eye were measured and averaged (36 data per group).

Optical coherence tomography (OCT, AS-OCT CASIA, Tomey, Japan) was introduced to observe the structure of filtering bleb. In general, an ideal bleb was diffused, bulged, diaphanous and well healed, while bleb failure was defined as the appearance of a broken bleb or a localized, flat, vascularized and scarred bleb associated with a deep anterior chamber.61

2.8. Anterior chamber fluorescence imaging
At ten weeks after implantation surgeries, each eye underwent anterior chamber fluorescence imaging to determine whether the drainage pathway was open or blocked.62 After the rabbits were generally anesthetized, 100 μL of fluorescein sodium solution (1 wt%) was injected into the anterior chamber through cornea using a 30 G needle. Digital videos were recorded with the assistance of a slit lamp microscope and a digital camera. Then, the largest area marked by fluorescein sodium in each eye was recorded; or, the largest area reached by fluorescein sodium in 60 s was recorded when the fluorescein sodium solution didn’t enter the blebs swimmingly.

2.9. Histologic analysis
Animals were euthanized with an overdose of anaesthesia at ten weeks after implantation surgeries, and rabbit eyes were taken out for histologic analysis. The eyes were enucleated and fixed in 10% paraformaldehyde solution. The samples were dehydrated in a graded ethanol series and then embedded in paraffin and sectioned at 5 μm thickness. Following deparaffinization, sections were stained with hematoxylin and eosin (H&E) to assess tissue morphology. The fibrous capsule was defined as the innermost, discrete band of collagen fibrils adjacent to the implant cavity.

2.10. Statistical analysis
Data analysis and statistical comparison between various groups were performed by the Origin Pro 8 software and a one-way ANOVA or Student’s t test was used when appropriate. Statistical differences were considered to be significant at a level of P value <0.05. The results are expressed as mean ± standard deviation (SD).

3. Results
3.1. Synthesis and characterization of the PLGA–PEG–PLGA triblock copolymer
The PLGA–PEG–PLGA triblock copolymer was synthesized by ring-opening polymerization of LA and GA with PEG containing two hydroxy end groups. The 1H-NMR spectrum of PLGA–PEG–PLGA with its chemical structure is presented in Fig. S2 in the ESI.† The peaks appearing at 5.20, 4.80 and 3.65 ppm were used to calculate the number-average MW (Mn) and LA/GA molar ratio of the resultant polymer.63 The calculation results showed that the Mn of PLGA–PEG–PLGA reached 1835–1500–1835 and the LA/GA molar ratio was 3/1. The weight-average MW (Mw) and MW distribution (Mw/Mn) of the polymer obtained from GPC analysis relative to polystyrene standards were 8160 and 1.23, respectively, as shown in Fig. S3 in the ESI.† A relatively narrow MW distribution reflected the successful synthesis of the desired product.

3.2. Temperature-induced sol–gel transition
The PLGA–PEG–PLGA triblock copolymers were dissolved in NS at room temperature and at a concentration of 25 wt%. The prepared polymer/water system exhibited a thermoreversible sol–gel transition with an increase in temperature. Fig. 2 displays the changes in the storage modulus (G’), loss modulus (G’’), and loss tangent (G’’/G’) of the polymer aqueous system as a function of temperature. In general, G’ represents the elastic behaviour or the energy stored in the sample during the deformation, whereas G’’ denotes the viscous character. At low or room temperature, the G’ was lower than the G’’, indicating its free-flowing sol state and good injectability. As the temperature increased, both G’ and G’’ increased and the sol–gel transition was observed at approximately 32 °C at which G’ was equal to G’’.64 In the gel state, G’ exceeded G’’, and both G’ and G’’ reached their peak values (1380 Pa and 1120 Pa, respectively) near body temperature. This feature is beneficial for the in vivo biomedical application of such a thermogel system. Meanwhile, the introduction of CsA did not obviously affect the rheological properties of the polymer aqueous system, indicating that the CsA@Thermogel is suitable as an injectable drug delivery system.
3.3. In vitro drug release

The in vitro drug release profiles of CsA@Thermogel systems were checked and the results are presented in Fig. 3. All the three CsA@Thermogel formulations underwent a burst release in the initial 48 h and more than 20–30 wt% drugs were released out of the thermogel matrix, which provided a sufficient drug dosage for the inhibition of inflammation at the beginning stage of wound healing at which the fibroblastic response is the most active. Subsequently, the release of CsA maintained a relatively slow and consistent rate up to two months. Interestingly, in the first 7 days, the release profile of 2 mg mL\(^{-1}\) CsA@Thermogel was almost as the same as that of 4 mg mL\(^{-1}\). Nevertheless, the cumulative release percent of drug was dependent on the drug loading amount during the whole examined period. The higher the drug loading amount, the more the cumulative release amount, but the lower the cumulative release percent. This finding coincides well with the release profiles of other hydrophobic drug-loaded thermogel systems.\(^{42,64,65}\)

3.4. In vitro cytotoxicity

The in vitro cytotoxicity of both thermogel itself and CsA@Thermogel with various drug concentrations against HTFs was evaluated and the results are shown in Fig. 4. The HTFs’ viability was maintained around 100% with the treatment of the thermogel, indicating that the thermogel itself had good biocompatibility. With CsA concentration increasing from 1 to 4 mg mL\(^{-1}\) in the CsA@Thermogel systems, the viability of HTFs decreased from ~100% to ~80%. This decreased trend was attributed to the inhibitory action of released CsA against HTFs, which was supported by our previous research.\(^{66}\) CsA could effectively inhibit the cell growth of rabbit Tenon’s fibroblast (RTFs), and the inhibiting efficacy was dependent on drug concentration.\(^{66}\) The present outcome provided direct evidence that the released drug from the CsA@Thermogel system with proper dosage has the capacity of inhibiting the growth of HTFs and thus possesses the potential for preventing or reducing the scar formation after glaucoma filtration surgery.

3.5. Significant improvement of post-operative anti-scarring efficacy and filtering bleb survival after glaucoma filtration surgery

Considering the in vitro drug release profile, drug dosage required to inhibit scar formation and the in vitro cytotoxicity of CsA@Thermogel formulations, the thermogel system containing 2 mg mL\(^{-1}\) CsA was selected as an optimal option to evaluate the intraocular anti-scarring efficacy and filtering bleb survival after glaucoma filtration surgery. A total of 24 implantation surgeries in the right eyes of rabbits were successfully performed. Photographs of filtering blebs were taken with slit lamps at the predetermined time points post-operation, as shown in Fig. 5. During the whole examined period of 10 weeks, no serious side effects were detected in all the four groups.

Neoangiogenesis was obvious in the first week after implantation surgeries in all the experiment groups, especially in the GDD and GDD/Thermogel groups (Fig. 5b1 and d1). Relatively low angiogenesis was observed in the GDD/MMC and GDD/CsA@Thermogel groups, indicating that the local treatment of MMC in the conjunctival flap and the released CsA from the GDD/CsA@Thermogel effectively regulated the wound healing process of the surgery site and thus reduced angiogenesis (Fig. 5c1 and e1). From the fourth week, no obvious neoangiogenesis was seen in all the experiment groups, suggesting that the extracorporeal GDD was gradually accepted by the eye tissue of rabbits.

The morphology and lifting height of filtering blebs in each group can be used to roughly estimate their status and function. In the GDD group, shrunken and flat filtering blebs were observed (Fig. 5b4, b7 and b10), suggesting that the drainage of aqueous humor by the GDD tube might be limited. In sharp contrast, the rabbits receiving the treatment of the GDD/Thermogel (Fig. 5d4, d7 and d10) or GDD/CsA@Thermogel (Fig. 5e4, e7 and e10)
maintained a bulged morphology and high lifting of filtering blebs during the whole examined period. It is obvious that such a bulged morphology and high lifting of the filtering blebs in the GDD/Thermogel and GDD/CsA@Thermogel groups were mainly attributed to the filling of the thermogel matrix at the initial stage. Interestingly, the morphology and high lifting of the filtering blebs were well maintained at the later stage even after the degradation of the thermogel, which could be explained by the filling of aqueous humor flowing from the anterior chamber through the drainage channel. In fact, in comparison with the red fluorescence color (different from tissue color) of the hydrophobic DiI probe in Fig. 5e4, no obvious red DiI was observed in Fig. 5e7, which revealed that the thermogel was completely degraded at 7 w after surgery. Such a degradation period \textit{in vivo} was consistent with that after subcutaneous injection.\textsuperscript{39} This feature also indicated that the filtering bleb survival was still maintained even after the complete degradation of the thermogel. In addition, a thin-walled and avascular filtering bleb was observed in the GDD/MMC group, as described in the previous report.\textsuperscript{4}

For the GDD/CsA@Thermogel group, the internal structures of filtering blebs were further observed by OCT and the representative images are shown in Fig. 6. The functional filtering bleb exhibited a striking lifting with a bulged morphology, as presented in Fig. 6a and d. The longitudinal and horizontal profiles of the filtering bleb in the A–A and B–B directions are displayed in Fig. 6b and c, respectively. The visualized observation evidently demonstrated that the upper surface of the implanted GDD was not directly attached to the inner wall of the filtering bleb and there was an effective drainage channel of aqueous humor between the filtering bleb and the anterior chamber. This finding also indicated that the present filtering bleb was filled with aqueous humor.

In the current research, IOP was also periodically measured during the whole experimental period and the results are shown in Fig. 7. It is worth noting that the change in IOP was just used as a rough reference index in the present normotensive IOP model. The normal rabbit eyes had a stable IOP of \textasciitilde11 mmHg in the blank group. In the other four groups, IOP decreased immediately
by more than 5 mmHg just after surgery and then gradually rebounded. The rabbit eyes treated with MMC solution exhibited the lowest mean IOP and the slowest increasing rate of IOP in the initial several weeks postoperatively. This was mainly attributed to the excessive antifibrotic action of MMC, resulting in poor wound healing and the overdrainage of the filtering channel. This finding also revealed that there was a high risk of hypotony with the treatment of MMC.7 In the follow-up stage, the mean IOP in the GDD/MMC group continually increased and was very close to that in the GDD/CsA@Thermogel group 6 weeks after surgery. The IOP evolution in the other three groups exhibited a similar trend: decreased immediately post-surgery and then increased in the next one week to a relatively stable platform level, which was still lower than the mean IOP in the blank group. However, the mean IOP in the GDD group was higher than that in the GDD/CsA@Thermogel group 6 weeks post-surgery. This feature might be due to the scar formation of the filtering bleb around the GDD implant. The GDD/CsA@Thermogel group exhibited the lowest terminal IOP level, suggesting that the filtration surgery combined with the treatment of the CsA@Thermogel system could effectively control IOP over 10 weeks. In addition, the eyes that received the treatment of the thermogel alone presented the lower terminal IOP level. We speculated that this was mainly because the in situ-formed thermogel in the filtering bleb played the role of an anti-adhesion barrier which could resist fibroblast adhesion and reduce the scar formation to a certain degree.

The main goal of the filtration surgery is to drain aqueous humor from the anterior chamber to filtering blebs through the GDD tube and ultimately make aqueous humor enter the systemic circulation. Although the morphology of the filtering bleb could reflect its function to a certain extent, it is difficult to use its morphology to determine whether the drainage pathway was unobstructed and whether the filtration surgery was successful. The fluorescence imaging could provide the most direct and powerful evidence that the filtration pathway was open or blocked. Generally speaking, the results of fluorescence imaging could be divided into three kinds of situations: obstructed, limited and diffused, as shown in Fig. 8. Once the filtration pathway was thoroughly obstructed, the fluorescein solution did not diffuse into the filtering bleb (Fig. 8a). In sharp contrast, when the filtration pathway was diffused, the fluorescein solution could rapidly diffuse and fill with the whole filtering bleb (Fig. 8c). When the situation was somewhere in between, the fluorescein could just partially diffuse into the filtering bleb, which was defined as “limited” (Fig. 8b).

According to the above classification standard, the rabbit eye percentage of each classification in every group is shown in Fig. 8d. In the GDD group, half of the filtration pathways were obstructed or limited after implantation surgery of GDD for 10 weeks. The usage of MMC avoided the obstruction of filtration pathway post-operation, while 50% of rabbit eyes still developed the limited filtration pathway. In the case of the GDD/Thermogel group, the administration of the thermogel into the filtering bleb increased the proportion of the diffused filtration pathway. This finding was due to the anti-adhesion efficacy of the thermogel itself which could resist fibroblast adhesion and scar formation.37,63,67,68 As for the GDD/CsA@Thermogel group, the highest ratio of diffused filtration pathway was observed and

![Fig. 6](image-url) The internal structure of filtering blebs in the GDD/CsA@Thermogel group at 8 weeks post-surgery. (a) Photograph of a filtering bleb; (b) OCT image in the A–A direction; (c) OCT image in the B–B direction; (d) 3D reconstruction of the filtering bleb by the OCT software. The red thin lines in (b) and (c) show the outline of the implanted GDD.

![Fig. 7](image-url) IOP lowering effects with the indicated treatments. Preop: before operation; postop: just after operation. #1, #2, #3 and #4 were used to mark significant differences from blank, GDD, GDD/MMC and GDD/Thermogel groups respectively.
only one case suffered from the limited filtration pathway, which was attributed to the combined effect of CsA as an antifibrotic agent and the thermogel as an anti-adhesion barrier.

### 3.6. Histologic analysis

At 10 weeks post-operation, the rabbits were euthanized and their eyeballs were harvested for pathological examination. The results are shown in Fig. 9. In the GDD group, there were very dense collagen fibers wrapped around the GDD implant (Fig. 9b and g), which obstructed or limited the outflow of aqueous humor. Compared with the fibrous capsule that formed in the GDD group, the density of collagen fibers in the GDD/MMC and GDD/Thermogel groups was highly reduced (Fig. 9c, e, h and j). The loose reticular fiber was mainly ascribed to the inhibiting effect of CsA or MMC against fibroblasts, and thus modulating wound healing by preventing or reducing scar formation. As for the GDD/Thermogel group, although a fibrous capsule wrapped around the GDD implant was observed (Fig. 9d), there was a relatively loose reticular fiber structure (Fig. 9i) which could support aqueous humor flow. Meanwhile, the infiltration areas of blebs in the GDD/Thermogel group seemed to be larger than those in the GDD/MMC group, indicating a greater drainage capability. Obviously, the GDD/CsA@Thermogel group presented the loose fiber structure, the biggest infiltration areas of bleb and the best drainage effect, due to the combination of the sustained release of CsA as an antifibrotic agent, and the carrier thermogel itself as an anti-adhesion barrier.

### 4. Discussion

Excessive scarring at the surgery site is acknowledged as the most important cause of failure in glaucoma filtration surgery. The intraoperative local treatment with MMC solution (0.4 mg mL$^{-1}$, 5 min) has been widely used to improve the success rate of filtration surgery in clinics; however, this therapy produces various ocular discomfort and complications, including ruptured blebs, chronic hypotony, maculopathy, wound leakage and epithelial toxicity due to the uncontrolled drug residues.$^2$ Recently, CsA has been considered to be a less toxic alternative to MMC in glaucoma surgery.$^{12}$ Considering that the post-operative process of scar formation often lasts for several weeks,$^3$ development of long-acting delivery systems of CsA is very meaningful and desirable. Meanwhile, subconjunctival administration of such a system during glaucoma surgery is a minimally invasive...
therapy with enhanced therapeutic efficacy, reduced side-effects, and good patient compliance. However, such a subconjunctival delivery system of CsA for preventing or reducing scar formation after glaucoma filtration surgery is very scarce.

In this study, we developed an injectable thermogel made up of biodegradable and biocompatible PLGA–PEG–PLGA triblock copolymers to subconjunctivally deliver CsA for a long-term period. As shown in Fig. S4 in the ESI, the amphiphilic PLGA–PEG–PLGA polymers easily self-assembled into micelles with hydrophilic PEG segments as coronas and hydrophobic PLGA blocks as cores, making them water soluble at room temperature. Also, as presented in Fig. 2, the concentrated polymer aqueous solution exhibited a sol–gel transition with increasing temperature due to the formation of a percolated micelle network via micellar aggregation and such a sol–gel transition could be fulfilled within half a minute at body temperature. The CsA@Thermogel system was conveniently fabricated by physically mixing the polymer aqueous solution with CsA and then continuously stirring them to become a homogeneous and transparent system. The solubility of hydrophobic CsA in the polymer aqueous solution was drastically increased up to 4 mg mL\(^{-1}\). This feature was attributed to the solubilization effect of polymeric micelles with the corona–core structure. Meanwhile, the introduction of CsA into the thermogel had no significant effect on its injectability and thermally induced gelation properties (Fig. 2).

The *in vitro* sustained release of CsA from the CsA@Thermogel systems was maintained over 2 months after a burst release in the first two days (Fig. 3). Due to a certain dissolution of CsA in water (27.67 µg mL\(^{-1}\), 25 °C), a number of drugs just entered the hydrophilic coronas or lay between micelles, which was easily diffused out of the thermogel matrix, resulting in the initial burst effect. Nevertheless, such an initial burst could well satisfy the requirement of suppressing the active fibroblastic response at the initial stage after surgery. Meanwhile, while the *in vitro* cytotoxicity results demonstrated that the thermogel itself had good biocompatibility, the released CsA with proper dosage from the thermogel depot indeed effectively inhibited the growth of HTFs (Fig. 4).

A glaucoma filtration surgery was performed via the subconjunctival implantation of the GDD in the rabbit model of normotensive IOP. The CsA-loaded polymer solution was then administrated using a conventional syringe around the GDD. After being exposed to body heat, the polymer solution rapidly turned into an *in situ* thermogel loaded with CsA to cover the irregular surface of the GDD. Subsequently, the sustained release of drug significantly prolonged the subconjunctival retention of CsA. No obvious inflammation, tissue necrosis or other adverse reactions were observed post-surgically in the GDD/CsA@Thermogel or GDD/Thermogel groups. Compared with a 5 minute application of 0.4 mg mL\(^{-1}\) MMC, the administration of the CsA@Thermogel system exhibited a better morphology and higher lifting of filtering blebs (Fig. 5e vs. Fig. 5c), a wider drainage channel (Fig. 9c vs. Fig. 9e) and a more stable lowering effect of IOP (Fig. 7) during the whole 10-week study period. In particular, after the complete degradation of the thermogel and the thorough release of CsA, such a bulged morphology and high lifting of the filtering bleb were still maintained in the GDD/CsA@Thermogel group (Fig. 5e10 and 6) owing to the filling of aqueous humor. In general, the *in vivo* degradation of PLGA–PEG–PLGA thermogel lasted 4–6 weeks after subcutaneous injection and their final degradation products were lactic acid, glycolic acid and nondegradable PEG, which are nontoxic and easily cleared from the body. In the current study, considering the continuous drainage of aqueous humor from the anterior chamber to filtering blebs through the GDD tube, these degradation products were easily taken away by aqueous humor and subsequently cleared in the systemic circulation. Also, the *in vivo* degradation rate of the PLGA–PEG–PLGA thermogel was remarkably faster than the *in vitro* one due to the complicated biological environment in the body.

As is well known, the fibroblastic response is greatest in the initial days to weeks following filtration surgery, and the present release period of CsA from the thermogel covered the most dangerous period of scar formation after glaucoma filtration surgery. As a result, the GDD/CsA@Thermogel group exhibited the highest proportion of unobstructed filtration pathway and formed a loose fiber structure around the GDD at 10 weeks after surgery (Fig. 8d), indicating that the overall success rate of glaucoma filtration surgery was greatly improved with the administration of CsA@Thermogel systems.

Interestingly, the use of the thermogel alone also inhibited scar formation and maintained filtering bleb survival to a certain degree (Fig. 5d, 7 and 9d). This feature might be attributed to the anti-adhesion nature of the thermogel. It is well-known that PEG has a strong capacity to resist adhesion of proteins and cells including fibroblasts, and cell adhesion plays a key role in tissue adhesion. Also, the formation of the PLGA–PEG–PLGA thermogel used here was due to the aggregation of micelles with the corona–core structure. Consequently, the surface of the thermogel is evidently rich in PEG coronas, resulting in good efficacy in preventing tissue adhesion. In fact, the previous studies have demonstrated that such a thermogel has great potential as a physical barrier for prevention of post-operative intestinal adhesion and epidual adhesion. Although the GDD/CsA@Thermogel group did not show significant differences in the IOP measurement and the morphology of filtering blebs (Fig. 5) compared with the GDD/Thermogel group, it presented a higher ratio of unobstructed filtration pathway (Fig. 8d), especially a looser collagen fiber structure around the GDD (Fig. 9i vs. Fig. 9j), at 10 weeks post-operation due to the sustained release of CsA and its inhibitory effect against fibroblast activity, which is helpful in constructing a physiological and functional filtering bleb after glaucoma filtration surgery. Therefore, the combination of the inhibitory effect of released CsA and the anti-adhesion property of the carrier itself offered the best therapeutic efficacy, and the GDD/CsA@Thermogel system was the optimal option for glaucoma filtration surgery in the present study. In addition, we believe that the superiority of CsA@Thermogel system over the pure thermogel should be further displayed using a larger number of animals and prolonging the examination time.
5. Conclusions

In this study, we successfully developed a subconjunctival delivery system of CsA using an injectable PLGA–PEG–PLGA thermogel as the carrier to inhibit post-surgical scar formation after glaucoma filtration surgery. The amphiphilic polymers effectively solubilized CsA in water due to the formation of polymeric micelles, and the concentrated polymer aqueous solution containing CsA exhibited a sol–gel transition upon heating with the transition temperature below the body temperature. The prepared CsA@Thermogel systems presented a sustained drug release in vitro over two months and the drug release profile was able to be modulated by the drug loading amount. Both in vitro evaluation of cytotoxicity and in vivo histological analysis confirmed the good biocompatibility of the thermogel itself. In vivo results further demonstrated that the use of the CsA@Thermogel system promoted bleb survival, maintained stable low IOP, and reduced complications, indicating that such a system is more effective than the local treatment with MMC solution. Therefore, this novel GDD/CsA@Thermogel system has great potential for glaucoma filtration surgery, especially for those patients with a high risk of surgery failure.

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