



Thermosensitive glycol chitosan-based hydrogel as a topical ocular drug delivery system for enhanced ocular bioavailability



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ABSTRACT

In the present study, we developed and evaluated an *in situ* gelling system based on hexanoyl glycol chitosan (H-GCS) for enhanced ocular bioavailability. An aqueous solution of H-GCS exhibited a typical sol-gel transition at 32 °C. The formed H-GCS hydrogel was characterized by rheology and scanning electron microscopy (SEM). H-GCS had minimal *in vitro* cytotoxicity against L-929 and HCEC cells over a concentration range of 0–0.8 mg/mL. Additionally, the H-GCS hydrogel exhibited good ocular tolerance and biocompatibility after a single instillation. Moreover, H-GCS hydrogel significantly prolonged the precorneal retention of fluorescein sodium compared with its aqueous solution. An *in vivo* pharmacokinetic study demonstrated that the levofloxacin-loaded H-GCS hydrogel could provide a significantly higher C_{max} and AUC_{0-12h} compared with the levofloxacin aqueous solution, thus increasing ocular bioavailability. Overall, the proposed H-GCS hydrogel acts as an *in situ* gelling system that might represent a promising vehicle for topical ocular drug delivery.

1. Introduction

Ophthalmic drug delivery remains a great challenge for pharmaceutical scientists due to the inherent physiology and natural barriers of eyes. Conventional ophthalmic formulations in the form of eye drops (e.g., solution, suspension) and ointments are preferred to deliver therapeutic agents to the ocular surface and anterior segment. However, these conventional formulations have distinct advantages, such as non-invasive administration, low production cost and great patient compliance, but suffer from a very short retention time at the corneal surface site owing to the rapid clearance of tears and nasolacrimal drainage, resulting in poor ocular bioavailability (< 5%) (Lang, 1995; Le Broulais et al., 1998; Li et al., 2013). Given these disadvantages of ophthalmic conventional formulations, solid and semi-solid drug delivery systems, including micro/nanoparticles, inserts, and preformed gels, have been proposed (Reimondez-Troitiño et al., 2015; Zhang et al., 2016; Zhang et al., 2018). With these formulations, the incorporated drug can be sustainably released to some extent and provide a relatively longer duration at the corneal surface, thus enhancing its bioavailability. However, given the blurred vision and uncomfortable nature of preformed gels, microparticles and inserts, the administration of such formulations has not been fully accepted and favored.

To overcome these shortcomings of existing ophthalmic

formulations, a number of studies have been focused on the use of *in situ* gelling delivery systems as potential ophthalmic dosage forms (Del Amo and Urtili, 2008; Byeongmoon et al., 2012; Almeida et al., 2014; Al-Kinani et al., 2018). The *in situ* gelling system is in liquid form during instillation into the eye and then quickly transforms into viscoelastic gels at the conjunctiva sac of the eye in response to various stimulus (e.g., pH, temperature, ionic strength) (Souza et al., 2014; Calles et al., 2015; Kirchoff et al., 2015; Koetting et al., 2015; Cho et al., 2016). Consequently, the residence time of gel formed *in situ* will be significantly extended, and the encapsulated drugs are sustainably released in a period of time, thus providing increased bioavailability and reducing administration frequency. To date, there are numerous *in situ* gelling systems, including temperature-, pH- and ionic-activated systems, which have been proposed as novel ophthalmic dosage forms to improve the treatment of anterior disorders (e.g., glaucoma, cataract, dry eye, inflammation) (Koetting et al., 2015; Wang and Han, 2017). Some *in situ* gelling ophthalmic formulations (e.g., Poloxamers, Carbopol, Cellulose derivatives) have already been commercially launched or are in clinical trials (Yellepeddi and Palakurthi, 2016).

Given the great biocompatibility, low toxicity, mucoadhesiveness and excellent ocular tolerance, chitosan, which is produced by the exhaustive deacetylation of chitin, has been considered a promising candidate as *in situ* gelling polymer for topical ophthalmic drug delivery (José and Alejandro, 2003; Sheetu et al., 2009; Giri et al., 2012; Meng-

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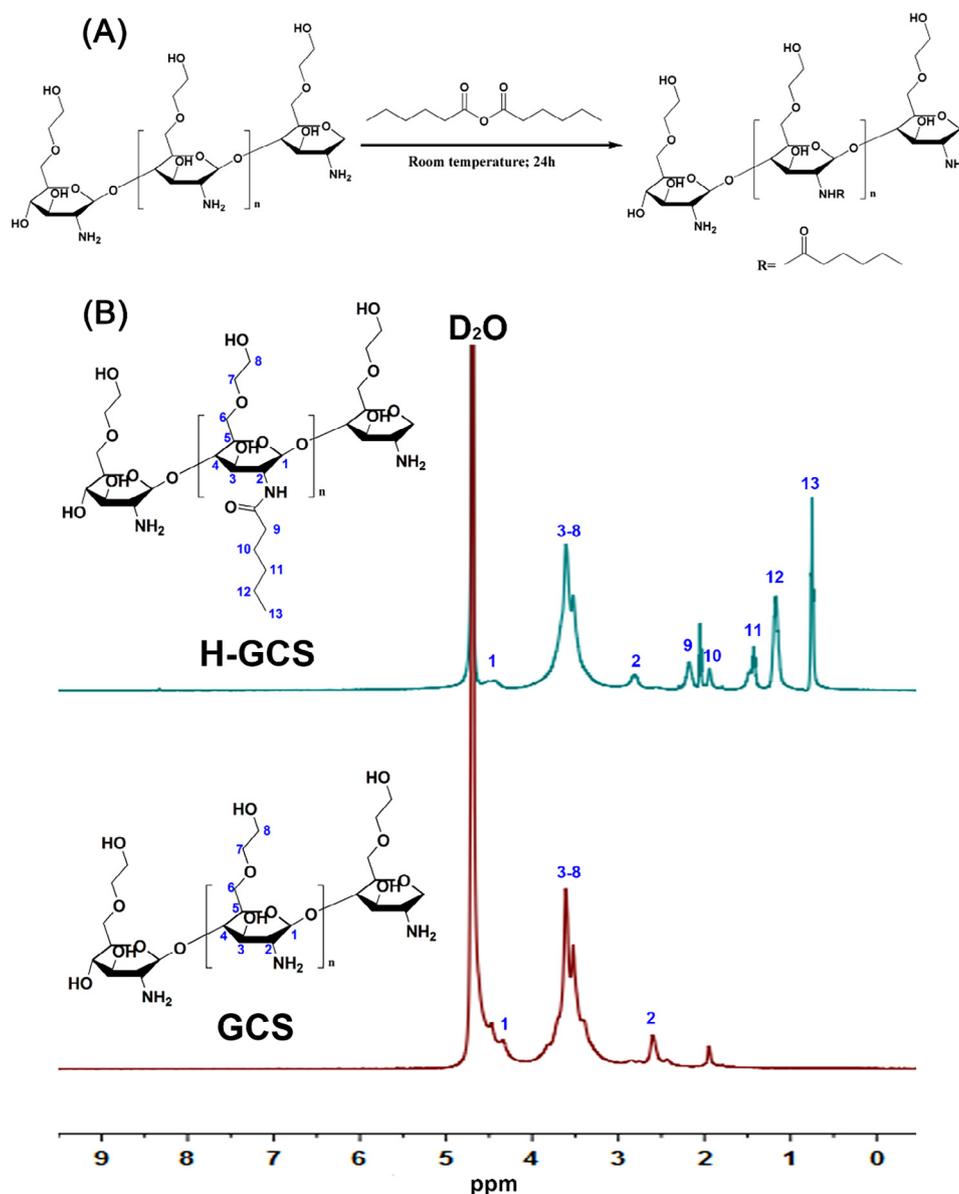


Fig. 1. (A) Synthetic scheme of H-GCS and (B) ¹H NMR spectra of H-GCS and GCS.

Hsuan et al., 2014). For instance, chitosan has been conjugated with poly(N-isopropylacrylamide) (PNIPAAm) to confer a thermosensitive gelling system for topical ocular delivery of timolol maleate. Compared with conventional eye drops, the proposed system exhibited two-fold increased C_{\max} and superior efficacy to reduce intraocular pressure up to 12 h (Cao et al., 2007). More recently, an injectable chitosan/gelatin hydrogel was proposed through double cross-linking for sustained intraocular drug delivery. The formed hydrogel exhibited great ocular tolerance and could remarkably extend the drug duration at corneal surface, thus leading to a two-fold increase in the IOP-lowering duration compared with conventional eye drops (Song et al., 2018). Although a number of chitosan-based *in situ* gelling systems have been applied for ophthalmic drug delivery, further development of a facile chitosan-based *in situ* gelling systems for topical ocular drug delivery is still urgently required to achieve better bioavailability and therapeutic efficacy.

To assess the potential application of the thermosensitive chitosan derivative as an ocular delivery system, a thermosensitive hydrogel based on hexanoyl glycol chitosan (H-GCS) was developed under mild condition. Unlike the previously reported chitosan based hydrogel, the

proposed H-GCS thermosensitive hydrogel formed in phosphate buffered saline (PBS, pH = 7.4) without involve of any other additives. The formed H-GCS hydrogel was thoroughly characterized by rheology and scanning electron microscopy (SEM). The feasibility of using the proposed H-GCS hydrogel as topical eye drop formulation containing levofloxacin was investigated in a rabbit model.

2. Materials and methods

2.1. Materials

Glycol chitosan (GCS, Degree of Polymerization ≥ 400 , Purity $\geq 60\%$) and hexanoic anhydride (97%) were provided by Sigma-Aldrich (USA). Levofloxacin was purchased from Dalian Meilun Biotechnology Co., LTD (China). All other used agents were analytical grade.

2.2. Synthesis and characterization of hexanoyl glycol chitosan (H-GCS)

According to a previous study (Cho et al., 2016), hexanoyl glycol

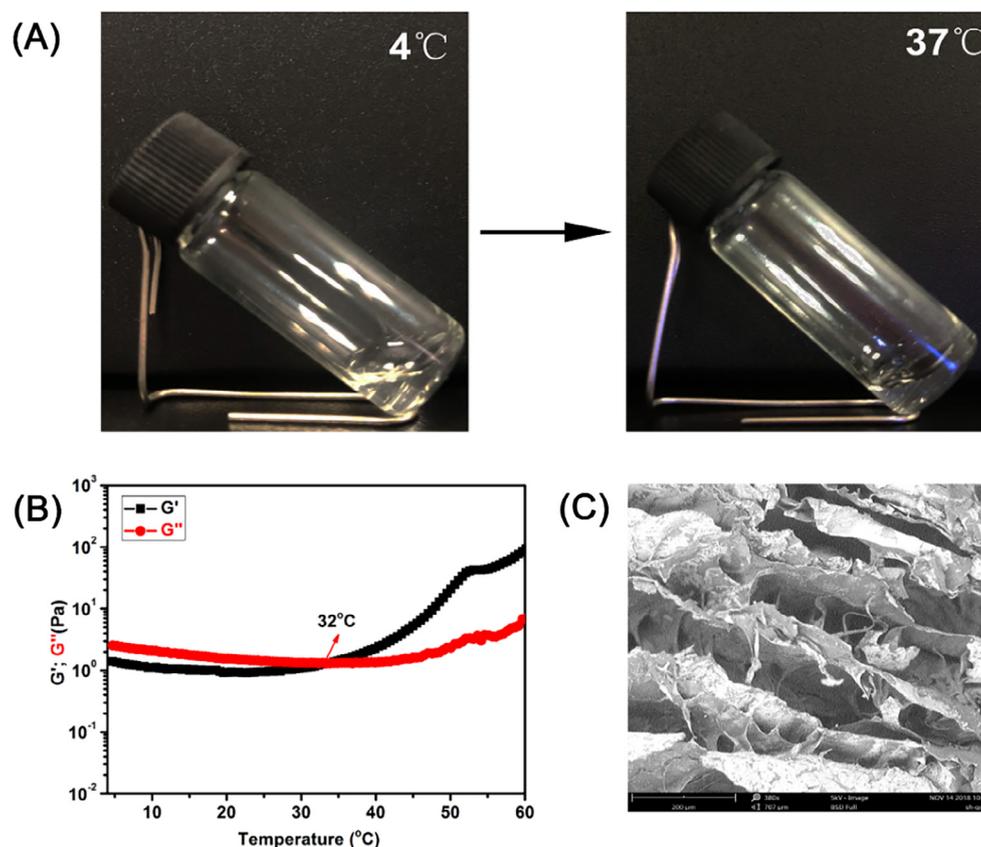


Fig. 2. (A) Sol-gel transition of the 2 wt% H-GCS aqueous solution; (B) temperature-dependent rheological property of the 2 wt% H-GCS aqueous solution and (C) SEM image of the 2 wt% H-GCS hydrogel.

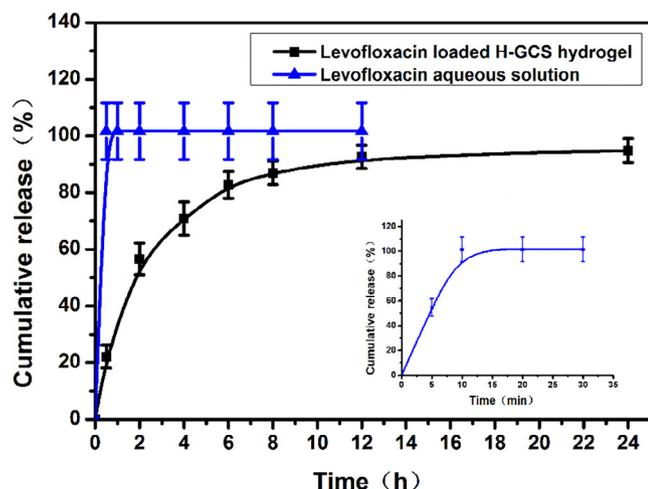


Fig. 3. In vitro release profiles of levofloxacin from the levofloxacin aqueous solution (3 mg/mL levofloxacin) and levofloxacin-loaded 2 wt% H-GCS hydrogel (3 mg/mL levofloxacin) in phosphate buffered saline (PBS, pH = 7.4) at 37 °C.

chitosan (H-GCS) was synthesized by the N-acylation of GCS under mild conditions. In brief, 1 g of GCS was dissolved into 100 mL of distilled water followed by the addition of 100 mL of methanol. After complete dissolution, a calculated amount of hexanoic anhydride (0.3 M equivalent to GCS) was added to GCS solution and allowed to react at room temperature for 48 h. Thereafter, the resulting solution was dialyzed against distilled water solution for 3 days using a dialysis membrane (molecular weight cut-off: 3500) and then lyophilized at -80°C for 96 h in a freeze-dryer (FD-1D-50, Biocool®, China). The lyophilized

powders were finally obtained and characterized by ^1H NMR.

2.3. Formation of H-GCS hydrogel and levofloxacin-loaded H-GCS hydrogel

A specific amount of H-GCS lyophilized powder was completely dissolved into phosphate buffered saline (PBS, pH = 7.4) at 4°C for 1 day. Subsequently, the solution was placed in a water bath (37°C) for gelation. For levofloxacin encapsulation, levofloxacin was co-dissolved into PBS at 4°C for 1 day followed by incubating at 37°C for gelation to afford a levofloxacin-loaded H-GCS hydrogel.

2.4. Rheology

The rheological property of hydrogel was measured with an AR2000 rheometer (TA, USA) using a parallel-plate 40 mm in diameter. The storage modulus (G') and loss modulus (G'') of hydrogel in response to the change of temperature were monitored at a constant strain of 1% and a frequency of 1 Hz.

2.5. Scanning electron microscopy (SEM)

The morphology of hydrogel was observed by scanning electron microscopy (Phenom ProX, Phenom-World B.V.). The lyophilized hydrogel sample was immersed in liquid nitrogen and cut into thin sections for SEM observation.

2.6. In vitro drug release study

The *in vitro* release profile of levofloxacin from levofloxacin-loaded H-GCS hydrogel and levofloxacin aqueous solution was measured in phosphate buffered saline (PBS, pH = 7.4) at 37°C . In brief, 200 μL of

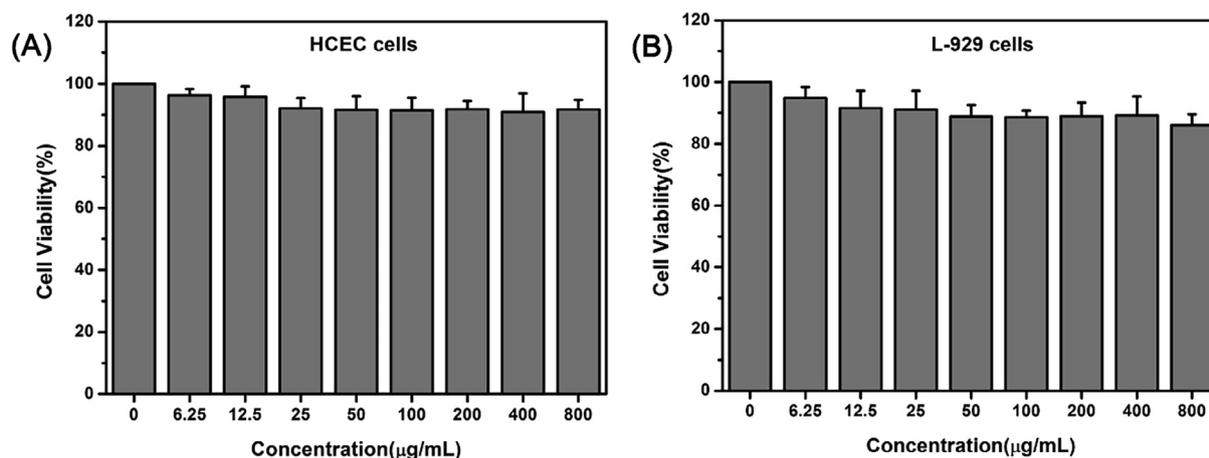


Fig. 4. Viability of (A) HCEC cells and (B) L-929 cells at different H-GCS concentrations (n = 3).

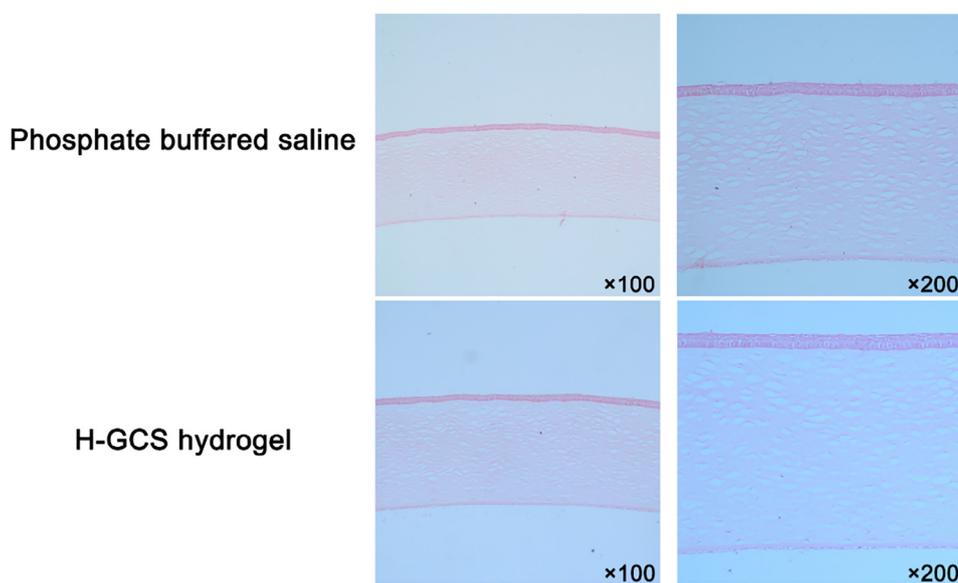


Fig. 5. H&E sections of cornea from eyes treated with phosphate buffered saline (PBS, pH = 7.4) and the 2 wt% H-GCS hydrogel at 24 h.

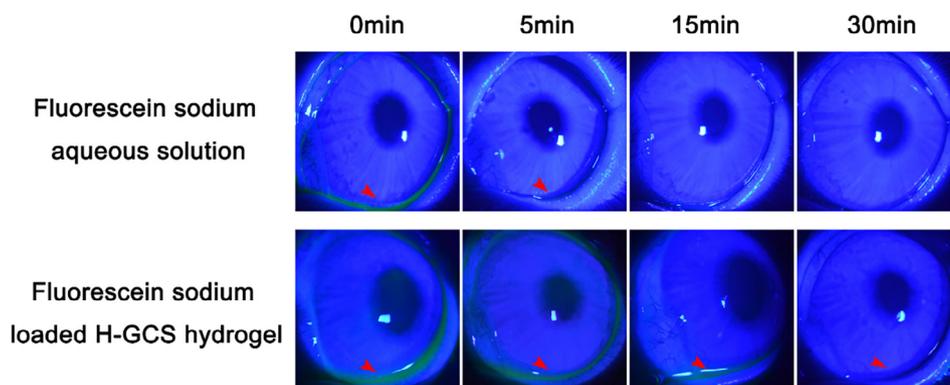


Fig. 6. Precorneal retention of the fluorescein sodium aqueous solution and fluorescein sodium loaded 2 wt% H-GCS hydrogel in rabbit eyes.

levofloxacin-loaded H-GCS hydrogel was prepared in a 5 mL Eppendorf (EP) tube to yield a final drug concentration of 3 mg/mL, while 200 µL of levofloxacin aqueous solution (3 mg/mL levofloxacin) was sealed into a dialysis membrane (molecular weight cut-off: 3500). Then, 2 mL of PBS was added as a release medium for a period for the *in vitro* release study. At specific time points, 1 mL of release medium was collected, and an additional 1 mL freshly prepared PBS was added at

each time interval. The released levofloxacin was quantified by high-performance liquid chromatography (HPLC, ZORBAX Eclipse XDB-C18, 150 mm × 4.6 mm i.d., 5 µm, Agilent) as reported previously (Sedova et al., 2014; Lei et al., 2018). The mobile phase used for HPLC analysis was acetonitrile and ammonium acetate perchlorate solution (20/80; v/v). The eluate was detected at 294 nm using a DAD detector.

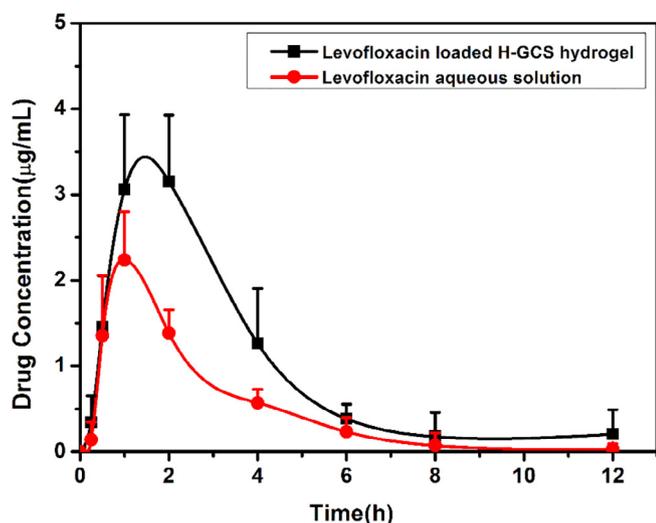


Fig. 7. In vivo pharmacokinetic data of the levofloxacin aqueous solution (3 mg/mL levofloxacin) and levofloxacin-loaded 2 wt% H-GCS hydrogel (3 mg/mL levofloxacin) after topical instillation in rabbit eyes (n = 3).

Table 1

Pharmacokinetic parameters of a single instillation of the levofloxacin aqueous solution (3 mg/mL levofloxacin) and levofloxacin-loaded 2 wt% H-GCS hydrogel (3 mg/mL levofloxacin) in rabbit eyes. *P < 0.05, comparing the C_{max} and AUC_{0-12h} of the levofloxacin loaded 2 wt% H-GCS hydrogel with those of the levofloxacin aqueous solution.

Parameters	levofloxacin aqueous solution	levofloxacin-loaded 2 wt% H-GCS hydrogel
C_{max} (µg/mL)	2.24 ± 0.28	3.50 ± 0.30*
AUC_{0-12h} (µg/mLh)	6.18 ± 1.94	11.89 ± 1.46*

2.7. In vitro cytotoxicity test

In vitro cytotoxicity of H-GCS against L-929 cells and human corneal epithelial cells (HCEC) was assessed using the cell-counting kit-8 (CCK-8) assay. In brief, cells were seeded in a 96-well plates at a density of 1×10^4 cells/well. After 24-h incubation in a CO₂ incubator, 0.1 mL of H-GCS with concentration in a range of 0–800 µg/mL was added for co-incubation. After 24 h of incubation, 20 µL of CCK-8 solution was added to each well for an additional 4-h incubation. Then, the absorbance of each sample was measured at 450 nm using a micro-plate reader (Bio Tek, Synergy NEO2, USA). Additionally, untreated cells were used as control. Cell viability was calculated using the following formula: Cell viability (%) = absorbance of tested sample/absorbance of the control sample × 100. All experiments were performed in triplicate (n = 3).

2.8. Animal experiment

2.8.1. Eye irritation test

The Draize rabbit eye test is a widely used assay for the measurement of irritation of substances to eyes (Abraham et al., 2003). Six healthy Japanese big-eared white rabbits (2.0–2.5 kg) were used in this study. This animal experiment complied with the Guide for the Care and Use of Laboratory Animals, Institute of Laboratory Animal Resources and was approved by the Institutional Animal Care and Use Committee of Wenzhou Medical University. In brief, 50 µL of 2 wt% H-GCS aqueous solution was instilled under the conjunctiva sac of left eyes, while an equal volume of PBS (pH = 7.4) was topically instilled into the under conjunctiva sac of right eyes. Subsequently, treated eyes were carefully monitored using a slit-lamp by an experienced doctor during the entire study period. Twenty-four hours post medication, the

rabbits were sacrificed, and the eyeballs were harvested. After the enucleation, the eyeballs were fixed in 4% paraformaldehyde, embedded into paraffin and cut into 5-µm sections. Finally, the sections were stained with hematoxylin-eosin (H&E) for histopathological observation.

2.8.2. Precorneal retention test

To investigate the precorneal retention of formulations, we compared the precorneal retention of fluorescein sodium aqueous solution with that of fluorescein sodium-loaded 2 wt% H-GCS hydrogel in rabbit. In brief, 50 µL of fluorescein sodium aqueous solution or fluorescein sodium-loaded 2 wt% H-GCS hydrogel was topically instilled into the under conjunctiva sac of eyes. At predetermined time points, the precorneal retention of these two formulations were visualized using a slit lamp (SLM-4ER, KANGHUA, ChongQing) by an experienced doctor.

2.8.3. In vivo pharmacokinetic study

An in vivo pharmacokinetic study was performed by measuring the drug concentration of the aqueous humor over time after topical instillation. In brief, 50 µL of levofloxacin aqueous solution or levofloxacin-loaded 2 wt% H-GCS hydrogel (levofloxacin: 3 mg/mL) were instilled into the under conjunctiva sac of eyes. At predetermined time points, 20 µL of aqueous humor solution from each eye was collected using an insulin syringe (29-G needle) followed by the mixture with 80 µL of mobile phase. After centrifuging at 13000 rpm for 10 min, the supernatant was obtained, and the drug concentration was quantified by a HPLC assay as described above.

2.9. Statistical analysis

Data were presented as the mean and standard deviation (SD) and analyzed by one-way analysis of variance (ANOVA) using SPSS version 22 (IBM). A p-value less than 0.05 was considered statistically significant.

3. Results and discussion

3.1. Preparation and characterization of H-GCS

H-GCS was successfully synthesized by the covalent conjugate of GCS with hexanoic anhydride under mild conditions and characterized by ¹H NMR. As shown in Fig. 1B, the D₂O peak was used as a reference peak at 4.7 ppm. The peaks at 4.3 ppm and 2.65 ppm were attributed to H1 and H2 of the gluconic ring, respectively. The overlapped peaks at 3.5–3.8 ppm were ascribed to H₃₋₈ of the gluconic ring. In terms of H-GCS spectrum, the new peaks at 0.8, 1.2, 1.4, 1.8 and 2.1 ppm were assigned to the protons of hexanoic groups. Based on this result, the hexanoic group was successfully covalently bound with GCS to generate H-GCS with yield of 85%.

3.2. Formation and characterization of H-GCS hydrogel

After dissolving the H-GCS into phosphate buffered saline (PBS, pH = 7.4) at 4 °C, sol-gel transition occurred at 37 °C to afford a transparent hydrogel (Fig. 2A). The mechanism of sol-gel transition for H-GCS might be explained by following features. At low temperature, hydrogen bonds between GCS chain and water molecules predominated in system, leading to the dissolution of H-GCS. As the temperature increased, the hydrophobic interactions between hexanoic groups prevailed, leading to the association of the GCS chain and the reduction of the mobility of hexanoic groups. Similarly, a pilot study assessed thermosensitive hydrogel based on PEG-g-chitosan at an optimum balance between hydrophilic and hydrophobic moieties (Bhattarai et al., 2005). Rheological test (Fig. 2B) indicated that 2 wt% H-GCS is a viscous liquid below 32 °C, corresponding to the loss moduli (G'') value over storage moduli (G') value. As the temperature reached to 32 °C, the

sol-gel transition of 2 wt% H-GCS occurred with the crossover of G' and G'' . Further increasing the temperature to 60 °C, a stable gel formed with G' value much greater than the G'' value. The ocular surface temperature ranges from 32 to 35 °C (Klamann et al., 2012). Therefore, it is reasonable to believe that the H-GCS aqueous solution as an eye drop formulation could quickly transform into a gel state at the corneal surface after topical instillation. Additionally, morphological observation (Fig. 2C) indicated that the formed H-GCS hydrogel displayed a typical interconnected porous structure with a pore size in a range of 50–200 μm .

3.3. *In vitro* release study

The percentage of cumulative drug released from various formulations as a function of time is presented in Fig. 3. Almost all levofloxacin was released from its aqueous solution within 20 min. Conversely, H-GCS hydrogel provided a sustain-release of levofloxacin over 12 h, which is a time scale of interest for ophthalmic drug delivery. After 12 h of incubation, H-GCS hydrogel completely dissolved into release medium. Generally, drug release from this type hydrogel (e.g., Poloxamer) occurs through a combined diffusion/dissolution mechanism. Similar results were reported by Mayol et al. who developed a poloxamer/hyaluronic acid hydrogel that was able to prolong and control acyclovir release over 6 h (Mayol et al., 2008).

3.4. *In vitro* cytotoxicity

In vitro cytotoxicity of H-GCS was assessed by an CCK-8 test using L-929 cells and human corneal epithelial cells (HCEC). As shown in Fig. 4, H-GCS caused minimal cytotoxicity (cell viability > 80%) at various concentrations (0–0.8 mg/mL) for L-929 and HCEC cells after 24 h of incubation. This preliminary result seems to suggest that H-GCS exhibits good biocompatibility *in vitro* and might act as safety vehicle for ophthalmic drug delivery.

3.5. Ocular irritation and biocompatibility

To develop optimal ocular formulations, it is very important to evaluate any potential irritation of formulation components on ocular tissues. One of the most common methods used for assessing the ocular irritation potential is the Draize test on rabbit eyes given the rabbit eye which is more susceptible than human eyes (Luo et al., 2016; Zhang et al., 2016). After single topical instillation of H-GCS hydrogel and phosphate buffered saline (PBS, pH = 7.4), no signs of discomfort appeared in both groups in the period of 24-h study. According to the Draize test criterion (Wilhelmus, 2016), the scores were zero for both group within 24 h, suggested that H-GCS hydrogel did not irritate eyes. Histopathological observation was further adopted to evaluate the influence of formulations on cell structure and tissue integrity. As shown in Fig. 5, the corneal tissue from both the PBS and H-GCS hydrogel groups exhibited normal morphology without epithelial defects and inflammatory cell infiltration. Based on these results, we conclude that H-GCS hydrogel possesses good ocular biocompatibility.

3.6. Precorneal retention study

To examine the *in vivo* precorneal retention, we compared the fluorescence intensity of the fluorescein sodium aqueous solution with fluorescein sodium-loaded H-GCS hydrogel after single instillation in rabbit eyes at different time intervals. As shown in Fig. 6, the fluorescein sodium aqueous solution exhibited a rapid clearance after single topical instillation, and green fluorescence in the conjunctival sac almost disappeared at 5 min. This result was consistent with the early report that eye drops in solution were quickly cleared by tear flushing and drainage within 5 min (Nagarwal et al., 2009; Yoeruek et al., 2010). Interestingly, the precorneal retention of fluorescein sodium was

significantly extended to 30 min after topical instillation of fluorescein sodium-loaded H-GCS hydrogel. This result seems to suggest that the gelation of H-GCS was able to effectively resist the effect of tear clearance and drainage, thus providing relatively long-term precorneal retention. Unlike the *in situ* gelling system reported previously (Luo et al., 2016; Al-Kinani et al., 2018), the mechanical strength of H-GCS hydrogel was very weak with a G' value of approximately 10 Pa, which is expected to minimize blurred vision.

3.7. *In vivo* pharmacokinetic study

The *in vivo* pharmacokinetics in the aqueous humor following topical instillation of levofloxacin aqueous solution (3 mg/mL levofloxacin) and levofloxacin-loaded 2 wt% H-GCS hydrogel (3 mg/mL levofloxacin) were investigated in rabbits. The aqueous humor drug concentration-time profiles and the pharmacokinetic parameters of these two formulations are presented in Fig. 7 and Table 1. The mean C_{max} values for levofloxacin aqueous solution and levofloxacin-loaded H-GCS hydrogel were 2.24 ± 0.28 and 3.50 ± 0.30 $\mu\text{g/mL}$, respectively. The difference between these two formulations was statistically significant ($P < 0.05$). Generally, drug bioavailability was judged by the area under the concentration-time curve (AUC). $\text{AUC}_{0-12\text{h}}$ of levofloxacin-loaded H-GCS hydrogel was 1.92-fold greater than levofloxacin aqueous solution, which indicated enhanced ocular bioavailability. The enhanced ocular bioavailability of levofloxacin-loaded H-GCS hydrogel might be related to its ability to significantly increase precorneal retention of drug, thus providing better transcorneal drug permeability to the aqueous humor.

4. Conclusions

The current study successfully developed an *in situ* gelling system based on H-GCS for topical ocular delivery of levofloxacin. The aqueous solution of H-GCS exhibited a typical sol-gel transition at 32 °C, and encapsulation of levofloxacin did not apparently influence its gelation behavior. H-GCS at concentrations below 0.8 mg/mL caused minimal cytotoxicity against L-929 and HCEC cells after a 24-h incubation. In addition, 2 wt% H-GCS hydrogel did not irritate rabbit eyes after a single topical instillation. Compared with an aqueous solution formulation, topical instillation of 2 wt% H-GCS hydrogel significantly prolonged the precorneal retention of drugs, thus providing increased ocular bioavailability.

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Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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