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In situ formation of injectable chitosan-gelatin hydrogels through double crosslinking for sustained intraocular drug delivery

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ABSTRACT

Rapid clearance and low ocular bioavailability are drawbacks of conventional ophthalmic eye drops. To increase the ocular drug resistance time and improve efficacy, an *in situ* forming and thermosensitive chitosan-gelatin hydrogel was developed. The feasibility of using this hydrogel as a topical eye drop formulation for sustained release of timolol maleate was evaluated. The flexible hydrogel that was co-crosslinked with β-glycerophosphate disodium salt hydrate (β-GD) and genipin showed a fast gel formation at 37 °C. The swelling properties and *in vitro* biodegradation characteristics showed a strong relationship with the initial genipin concentration. *In vitro* release profiles demonstrated that crosslinking with genipin reduced the release rate of entrapped model drugs and timolol maleate. *In vitro* cytotoxicity tests showed that the hydrogel was non-toxic to Chinese hamster fibroblast V79 cells. The hydrogel was further applied as eye drop formulations for sustained release of timolol maleate to reduce intraocular pressure (IOP). A fast gel formation was observed after instilling the chitosan-gelatin solution into the lower conjunctival sac of the rabbit eyes, and the *in situ* formed hydrogels protected the drugs from clearance by tears, and released the drugs in a sustained manner. Furthermore, administration of timolol maleate containing chitosan-gelatin hydrogels showed a long-lasting and effective IOP lowering efficacy for up to 24 h compared with the conventional eye drops. These results suggested that β-GD and genipin co-crosslinked chitosan-gelatin hydrogels could be a useful ocular drug delivery platform with enhanced therapeutic effects and reduced side effects.

1. Introduction

Topical delivery of drugs into the lower cul-de-sac using eye drops is a widely accepted and commonly used administration approach for treatment and diagnosis of ocular diseases, due to its usability, convenience, and good patient compliance [1–3]. However, the instilled drug formulation is immediately diluted by the tear fluid, and then drained away from the pre-corneal cavity because of the constant lacrimal secretion and lacrimo-nasal drainage, resulting in few drugs (< 5%) reach the intraocular tissues [4,5]. To reach the desired therapeutic effect, frequent eye drop administration is needed to maintain the effective therapeutic drug concentration, while this is associated with a higher risk of side effects and lower patient compliance [6–8].

Over the past decade, efforts have been made to enhance the efficacy of eye drops. A number of novel ocular drug delivery systems, including nanoparticles, nanoemulsions, and hydrogels, have been developed to improve the *in vivo* bioavailability and prolong pre-corneal drug retention time [9–11]. Among these strategies, *in situ* forming, stimuli-responsive hydrogels have gained considerable attention. Hydrogels are three-dimensional (3D) cross-linked polymeric networks, and have been widely used for drug delivery and tissue engineering [12–19]. Reports have demonstrated that topical administration of drug loaded hydrogels could prevent the rapid clearance of the instilled drug from the nasolacrimal system of the orbit, thus, effectively prolong the drug retention time [20–22].

Thermosensitive polymers behave as liquid state when the ambient temperature below low critical solution temperature (LCST), and gelation when the environmental temperature is above the LCST. Sasaki et al. demonstrated that application of thermosensitive *in situ* gel-forming solution could effectively enhance the ocular absorption of the ophthalmic drug Tilisolol [23]. Chitosan-based formulations have been widely used as topical ophthalmic drug delivery systems and have shown an effective drug residence time [24–26]. Cheng et al. [26] and Chen et al. [27] have successfully fabricated glycerol phosphate

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crosslinked chitosan-gelatin hydrogels based ocular drug delivery systems. These drugs exhibited the desired high drug-loading capacity, lower cytotoxicity, sustained release of drugs, and prolonged pre-corneal retention time, but the effective period of the released drug lasted for only few hours. Thus, further modification of the hydrogels is still needed to reach a longer action time.

Genipin is a natural crosslinking reagent obtained from geniposide, and has been widely used to crosslink polymers and proteins containing primary amine groups, particularly gelatin and soy protein isolates [28]. Genipin has been widely used as a crosslinking agent of chitosan for drug delivery [29–32], and as a gelatin conduit for peripheral nerve regeneration [33]. Compared with other crosslinking reagents such as glutaraldehyde, formaldehyde, and epoxy compounds, genipin demonstrated higher biocompatibility and lower cytotoxicity [34–37].

To investigate the potential application of the new thermosensitive polymer as an ocular delivery system, a muco-adhesive ophthalmic drug delivery system was developed using chitosan-gelatin that co-crosslinked with β-GD and genipin. The feasibility of using this newly developed thermosensitive chitosan-based hydrogel as a topical eye drop formulation containing timolol maleate as a model drug was evaluated. Timolol maleate is a non-selective beta adrenergic receptor blocking agent that is widely used to treat increased pressure inside the eye, such as in ocular hypertension and glaucoma, both as an ophthalmic drop and gel-forming formulation [38,39]. The effects of genipin concentration on the gelation time, the swelling behavior, *in vitro* biodegradability, and the cytotoxicity of the hydrogels were evaluated. The intraocular pressure lowering effect of the timolol maleate-loaded hydrogel was evaluated using rabbits. Herein, we report that our chitosan-gelatin hydrogel formulation could be a useful ocular drug delivery platform with enhanced therapeutic effects and reduced side effects.

2. Materials and methods

2.1. Materials

Chitosan (500 kDa, degree of deacetylation 100%) was provided by Dainichiseika Color & Chemicals (Tokyo, Japan). Type A gelatin (Bloom 300), lysozyme, and β-glycerophosphate disodium salt hydrate (β-GD) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Phosphate-buffered saline (PBS), sodium fluorescein, and genipin were purchased from Wako (Tokyo, Japan). Dulbecco's Modified Eagle's Medium (DMEM), fetal bovine serum (FBS), and L-glutamine were purchased from Gibco (Gaithersburg, MD, USA). Timolol maleate was purchased from Tokyo Chemical Industry (Tokyo, Japan). The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) were obtained from Promega (Sunnyvale, CA, USA).

2.2. Preparation of in-situ crosslinking chitosan-gelatin hydrogels

Chitosan-gelatin solution (CS-G solution) was prepared by dissolving 2.5 g chitosan and different amounts of type A gelatin in 100 mL of 1% acetic acid to form 2.5% CS-X% gelatin. A 0.8 (w/v) β-GD solution was prepared by dissolving 0.8 g of β-GD in 1 mL of distilled water and sterilizing the solution by filtration through a 0.22 μm syringe filter. The 10 mg/mL genipin solution was prepared by dissolving 10 mg of genipin in 1 mL of distilled water and sterilizing by filtration through a 0.22 μm syringe filter.

To prepare the β-GD crosslinked CS-G hydrogels, 1 mL of chilled β-GD was added dropwise into the 1.5 mL CS-G solution with different gelatin concentrations, while stirring on ice.

To prepare β-GD and genipin co-crosslinked CS-G hydrogels, 0.8 mL of chilled β-GD was added dropwise into the 2.5% CS-1% gelatin solution while stirring on ice, and then, different amounts of genipin were added to the CS-G/β-GD solution to reach a final genipin concentration of 0, 10, 25, 50, or 100 μg/mL. The mixture was stored at 4 °C until further use. To prepare drug (sodium fluorescein or timolol maleate) loaded hydrogels, model drug was first added to the CS-G solution under stirring for 30 min, and then β-GD and genipin was added in turn into the CS-G solution while stirring on ice. The hydrogel was formed by incubating at 37 °C for 24 h.

2.3. Gelation formation time determination

The gelation formation was assessed using an inverted tube test [40,41]. It has been reported that a sample having yield stress (gel) will not flow when tilting the solution-containing tubes, whereas a viscous but inelastic sample (sol) will show appreciable flow. Briefly, 1 mL of CS-G solution (kept at 4 °C or 25 °C) that was crosslinked with β-GD or β-GD and genipin was maintained in 5 mL vials, and then the vial was incubated in a water bath at 37 °C. The sol-gel transition time was determined by inverting the vial horizontally every 5 s after the initial 30 s incubation. The time at which the gel did not flow was defined as the gelation formation time.

2.4. Elastic modulus measurement

To measure the elastic properties of the hydrogels with different genipin content, the developed hydrogel (250 μL) was placed in a 48-well plate and incubated at 37 °C for 24 h. The samples were then placed on a holder for force measurements (EMX-1000 N; ZTS-5 N; Imada, Aichi, Japan) and the elastic modulus was measured.

2.5. Fourier transform infrared spectroscopy

The Fourier transform infrared (FTIR) spectroscopy was employed to investigate the effect of different crosslinkers on the production of CS-G hydrogels. The hydrogels (250 μL) were placed in the polydimethylsiloxane (PDMS) mold and incubated at 37 °C for 24 h, the samples were air-dried under room temperature for 3 days to produce thin films, and then the films were used to measure the FTIR spectra using the FT/IR-3600 Japan.

2.6. Swelling measurement

Swelling tests were performed on the β-GD and genipin crosslinked CS/G hydrogels (~700 μL) with different chemical crosslinker loads at 37 °C. After gel formation at 37 °C for 24 h, the gels were further incubated in PBS at 37 °C for 6 h to reach their equilibrium swelling state. Then, the excess PBS was removed, and the surface water on the hydrogels was blotted gently with a filter paper. The gels were subsequently weighed (W_e). Afterward, the hydrogels were frozen at −80 °C and then freeze dried with the lyophilizer (FDU1200; EYELA, Tokyo Rikakikai, Tokyo, Japan) at −80 °C for 2 days and then weighed (W_d). All of the experiments were performed in triplicate.

The swelling ratio was calculated using Eq. (1)

$$\text{Swelling ratio} = (W_e - W_d)/W_d \quad (1)$$

2.7. Scanning electron microscopy

The morphology of the hydrogel was observed using a field-emission scanning electron microscope (SEM; FESEM, Model JSM-7600F; JEOL Ltd., Tokyo, Japan). After the lyophilization process, the samples were fixed on a metal stub and then coated with osmium under vacuum by an ion sputter (JFC-1200; JEOL Ltd. Tokyo, Japan).

2.8. In vitro drug release study

The *in vitro* release behavior of sodium fluorescein and timolol maleate from the hydrogels was studied in PBS (pH 7.4) at 37 °C. In