Abstract. In the present study, a temperature-sensitive gel composed of chitosan, carboxymethyl chitosan and glycerophosphate was prepared and loaded with chitosan microspheres encapsulating levofloxacin. The bioavailability of levofloxacin and the safety of this novel ophthalmic drug delivery formulation were evaluated. Levofloxacin chitosan microspheres were prepared using the ionic gelation method, and the particle size and entrapment rate were determined. The morphology of the microspheres was observed by scanning electron microscopy. The pH and zeta potential were measured. The in vitro release of levofloxacin by the chitosan temperature-sensitive gel loaded with drug microspheres was determined using spectrophotometry. The eye retention time of the chitosan temperature-sensitive gel was calculated using a fluorescein sodium test. To assess the bioavailability and safety of the chitosan temperature-sensitive gel, a cell compatibility test, a cytotoxicity test and skin irritation test were performed. The entrapment rate of levofloxacin in the chitosan microspheres was determined to be 26.5%. The levofloxacin chitosan microspheres that were formed by chitosan and sodium tripolyphosphate were identified to be suitable for use in an ophthalmic particle dispersion system based on their physical and chemical properties. The pH of the levofloxacin chitosan microsphere suspension was 5.87±0.04, the average particle diameter was 2,452±342 nm, the polydispersity index was 0.168±0.028 and the ζ potential was 28.62±1.7 mV. The chitosan temperature-sensitive gel carrying microspheres loaded with drug prevented drug burst release at the initial stage and facilitated the slow release of the drug later on. Furthermore, this delivery system markedly prolonged the contact duration of levofloxacin with the eye. The chitosan temperature-sensitive hydrogel was safe and provided a good bioavailability of the drug. The results revealed that the chitosan temperature-sensitive gel had a cytotoxicity of grade 0, and no erythematous response was observed during the entire course of the skin irritation test. The present study provided a basis for the future development of the chitosan-based temperature-sensitive hydrogel in ophthalmic drug delivery.

Introduction

Eye drops account for ~90% of ophthalmic drug delivery systems available (1). Traditional eye drops are usually lost from the ocular surface by tear washing or nasolacrimal drainage immediately after administration, with poor ocular bioavailability and numerous adverse reactions (2). In addition, due to the inconvenience of night administration, the pharmacological peak-valley phenomenon is prominent, greatly affecting the treatment effect of ocular drugs. With the development of novel technologies, novel ophthalmic drug delivery systems have been designed to effectively elevate ocular bioavailability by prolonging the retention time of drugs in the eye or improving the penetration of drugs into the cornea and conjunctiva (3,4).

Chitosan is a natural cationic polysaccharide that is prepared from chitin after partial or complete deacetylation. Due to its excellent biological properties, chitosan and its derivatives have been widely used in ophthalmic drug delivery systems (5). As a novel form of administration, in situ gel undergoes phase transition to a non-chemically crosslinked semi-solid gel preparation at the medication site immediately after administration in solution or semi-solid format. In situ gel prepared from chitosan or its derivatives forms a transparent gel that has a strong affinity to the ocular surface under ocular
physiological pH conditions. Certain chitosan derivatives are temperature-sensitive and may be prepared to form a temperature-sensitive gel in situ (6,7). The temperature-sensitive gel formation is due to changes in the physical state of polymers induced by changes in hydrogen bonds or hydrophobic interactions (8-11). Cao et al (12) synthesized a novel type of isopropyl acrylamide chitosan polymer and used it to prepare a timolol maleate temperature-sensitive in situ gel, which provides a higher bioavailability than its respective solution.

At present, certain challenges remain regarding the development of in situ gels for pharmaceutical application. First, higher concentrations of polymers and non-aqueous solvent in the gel may cause eye irritation and safety issues. Furthermore, there may be a drug burst between the time-points of dripping the formulation into the eye and the formation of the gel, leading to a significant peak-valley phenomenon (13,14). In the present study, a temperature-sensitive gel was prepared using chitosan, carboxymethyl chitosan and glycerophosphate, and levofloxacin was encapsulated into chitosan microspheres, which were embedded in the chitosan temperature-sensitive gel. In addition, the safety of chitosan temperature-sensitive gel carrying drug microspheres and the bioavailability they provided were evaluated.

Materials and methods

Preparation of chitosan microspheres. Chitosan acetic acid solution (2.0 g/l; Biotemed, Qingdao, China) was prepared and adjusted to pH 5.0. Subsequently, 20 mg levofloxacin (Wuhan DKY Technology, Wuhan, China) was dissolved in 100 ml chitosan acetic acid solution with agitation at 10.06-13.42 x g. Subsequently, sodium tripolyphosphate (1.0 g/l; Tianjin Bodi Chemical, Co., Ltd., Tianjin, China) was added to obtain a suspension after agitation for 15 min. Levofloxacin chitosan microspheres (2.4 g/l) were then obtained by filtration.

Determination of entrapment rate of levofloxacin chitosan microspheres. A 200 mg/l-stock solution of levofloxacin (20 mg) in 0.1 M acetic acid solution was prepared. A series of solutions with mass concentrations of levofloxacin of 4, 6, 8, 10, 12, 14, 16 and 18 mg/l were prepared and the absorbance at each concentration was measured at 293 nm (UV2 9200; Nanjing Xinhang Scientific Instrument Co., Ltd., Nanjing, China). Levofloxacin chitosan microspheres (2.0 ml) were centrifuged at 671 x g at 4°C for 30 min. Supernatants were obtained and diluted with 0.1 M HCl. The concentration of free levofloxacin (C_{free}) in the suspension was determined. In the meantime, levofloxacin chitosan microspheres (2.0; 2.4 g/l) which was the reaction solution from above were suspended in 8.0 ml 0.1 mol/l HCl solution and incubated at 60°C for 1 h for hydrolysis. After filtration using a microfiltration membrane (pore size, 0.02 µm; EMD Millipore, Billerica, MA, USA), the total concentration of levofloxacin in the filtrate (C_{total}) was determined. The entrapment rate of levofloxacin chitosan microspheres was calculated using the following equation: Entrapment rate = (1 - C_{free}/C_{total}) x 100%. Each test was repeated for 5 times.

Characterization of the physical and chemical properties of levofloxacin chitosan microspheres. A suspension of levofloxacin chitosan microspheres was used to determine the pH, ζ potential, particle diameter (Zetasizer 3000 HSA; Malvern Instruments, Malvern, UK) and particle size distribution. After diluting the suspension of levofloxacin chitosan microspheres, a scanning electron microscope (JEOL7500F; Jeol, Tokyo, Japan) was used to observe the morphology of the particles. In addition, an infrared spectrum was used to analyze pure chitosan powder, blank chitosan microspheres and levofloxacin chitosan microspheres. The scanning range was 400-4,000 cm⁻¹ and the resolution was 4 cm⁻¹.

Preparation of chitosan-carboxymethyl chitosan temperature-sensitive gel loaded with chitosan nanoparticles. Sodium glycerophosphate (0.78 g) and carboxymethyl chitosan (0.2 g) were dissolved in 2 and 3 ml double-distilled (dd)H₂O, respectively. Furthermore, chitosan (0.2 g) was dissolved in 5 ml 0.1 M acetic acid. With mixing, sodium the glycerophosphate solution and carboxymethyl chitosan solution were consecutively added to the chitosan solution to thereby obtain the chitosan-carboxymethyl chitosan temperature-sensitive gel system. Subsequently, 0.1 g levofloxacin chitosan microspheres were added to 10 ml chitosan-carboxymethyl chitosan temperature-sensitive gel, followed by thorough mixing.

Evaluation of in vitro release of levofloxacin by chitosan temperature-sensitive gel loaded with drug microspheres. In the different experimental groups, the drug delivery systems were prepared via three different methods as follows: Group 1, levofloxacin was added into chitosan-carboxymethyl chitosan solution; group 2, levofloxacin was added into chitosan-carboxymethyl chitosan-sodium glycerophosphate solution; group 3, temperature-sensitive gel containing levofloxacin nanoparticles was prepared as described above. The liquid samples were put into dialysis bags (molecular weight cut-off, 7,000 Da), which were soaked in 200 ml phosphate-buffered saline (pH 7.4) at 37°C. After standing still for 20 sec, 5 ml phosphate buffer solution was transferred to a sample tube. After the formation of a solid gel in dialysis bag at 37°C, 5 ml phosphate buffer solution was also transferred to individual sample tubes at 5, 10, 15, 20, 30, 60, 120, 180, 240, 300, 360, 420, 480, 540, 600, 660 and 720 min. Each time after withdrawal of phosphate buffer solution, another 5 ml of fresh solution was replenished. For group 1, in which no gel was formed, phosphate buffer solution was also aspirated at the abovementioned time-points. The absorbance of levofloxacin in each sample tube was measured at 293 nm (UV2 9200; Nanjing Xinhang Scientific Instrument Co., Ltd.). Each sample was tested in triplicate. Using the levofloxacin standard curve, the concentrations of levofloxacin from each group at the different time-points were calculated and levofloxacin in vitro release curves were plotted.

Eye retention test of chitosan temperature-sensitive gel. The experimental animal protocols of the present study were approved by the Ethics Committee of Qingdao Agricultural University (Qingdao, China). On ice, fluorescein sodium (mass fraction, 0.05%) was added into a levofloxacin drug loading system in groups 1-3. A total of 18 healthy New Zealand white rabbits (Qingdao Medicine Inspecting Institute, Qingdao,
Cell compatibility test. The chitosan-based temperature-sensitive hydrogel was prepared as a preparation of chitosan-carboxymethyl chitosan temperature-sensitive gel loaded with chitosan nanoparticles, and the thickness of the hydrogel was controlled at 1-2 mm, which could be regarded as a membrane. The membrane was cut into circular sheets with a diameter of 1.1 cm. The circular sheets were soaked in 25, 50 and 75% ethanol for 2-3 h each time and then with 75% ethanol for 1-2 days, followed by transfer to sterile D-Hank's buffer for soaking over 10 h with the buffer being replaced 4-5 times. The membranes were then soaked in D-Hank's buffer prior to use. The membranes were placed into 48-well cell culture plates containing 100 µl Dulbecco's modified Eagle's medium (DMEM; Tianjin Bodi Chemicals, Tianjin, China) supplemented with 10% fetal bovine serum. Each time-point had 5 repeats. Subsequently, rabbit corneal endothelial cells (15) were trypsinized and adjusted to a concentration of 5x10³/ml. Cell suspension (200 µl) was added into predefined wells. The negative control contained DMEM only. The cells were cultured at 37°C and 5% CO₂. On days 3 and 5, the cells were observed and images were captured. The cells were then trypsinized and transferred to 96-well plates, followed by incubation over 12 h. Subsequently 5 mg/ml MTT (20 µl) was added to each well, followed by further incubation for 4 h. After discarding the medium, dimethyl sulfoxide (DMSO; 150 µl) was added and the wells were incubated at 37°C for 10 min. After agitation for 40 sec, the absorbance of each well was measured at 490 nm on a UV2 9200 plate reader (Nanjing Xinhang Scientific Instrument Co., Ltd.).

Cytotoxicity test. The L929 mouse fibroblast cell line at the logarithmic growth phase was trypsinized, the suspension was adjusted to a concentration of 1x10⁴/ml, and the cells were seeded onto 96-well plates (200 µl/well). After incubation at 37°C and 5% CO₂, the medium was replaced with DMEM that had been used to soak the chitosan-based temperature-sensitive hydrogel membrane for 24 h in the experimental group. For the control group, normal medium was replaced with DMEM only. After incubation at 37°C for 10 min. After shaking for 40 sec, the absorbance of each well was measured at 490 nm (UV2 9200; Nanjing Xinhang Scientific Instrument Co., Ltd.). The relative growth rate (RGH) was calculated as follows: RGH = mean absorbance in experimental group/mean absorbance in control group x 100%. Cytotoxicity grading was as follows: 0, RGH≥100%; 1, 75%<RGH<95%; 2, 50%<RGH<74%; 3, 25%<RGH<49%; 4, 1%<RGH<24%; and 5, RGH=0%. For grade 1, the cytotoxicity was considered acceptable; for grade 2, cytotoxicity was evaluated in combination with cell morphological analysis; for grades 3-5, cytotoxicity was considered unacceptable.

Skin irritation test. Sterile and pyrogen-free chitosan-based temperature-sensitive hydrogel membrane (5 g) was soaked in 5 ml saline for 72 h. At 4-24 h prior to the experiments, the hair of three healthy rabbits was removed on both sides of the dorsal spine. Six points were selected on each side of the dorsal spine. The six points on the left side were included in the control group and those on the right side were included in the experimental group. Saline (0.1 ml) was injected subcutaneously into the six points of the control group, while chitosan-based temperature-sensitive hydrogel membrane extract liquid (0.1 ml) that was DMEM soaked with the gel for 24 h, was injected subcutaneously into the six points of the experimental group. At 12, 24 and 48 h after injection, each injection point of each rabbit was evaluated according to Table I to determine the artificial vascular stimulation fraction. On days 3, 5 and 7 after surgery, subcutaneous tissues were obtained from all animals under anesthesia with 1 mg/kg sodium pentobarbital and subjected to hematoxylin and eosin (H&E) staining. The samples were paraffin-embedded, sliced, and washed with xylene for 15 min twice, 100% ethanol for 5 min twice, 80% ethanol for 5 min once, and ddH₂O for 5 min once, followed by staining with hematoxylin at 25°C for 5 min. After washing with water for 1-3 sec, the samples were washed with 1% hydrochloric acid (1 ml) and 75% ethanol solution (99 ml) for 1-3 sec prior to washing with water for 10-30 sec. After washing with ddH₂O for 1-2 sec, the samples were stained using 0.5% eosin at 25°C for 1-3 min. Subsequently, the samples were rehydrated/dehydrated in a graded ethanol series. The samples were then washed with xylene for three times of 2 min and mounted with neutral balsam.

Table I. ISO 10993.10-2002 scoring standard.

| ER Grade | Edema response | Grade |
|----------|----------------|-------|
| None     | 0              | None  |
| Mild     | 1              | Mild  |
| Clear    | 2              | Clear |
| Moderate | 3              | Moderate |
| Severe ER or escharosis | 4 | Severe |

ED, edema; ER, erythematus response.

Statistical analysis. The results were analyzed using SPSS v17.0 software (SPSS, Inc., Chicago, IL, USA). Values are expressed as the mean ± standard deviation. Differences were compared using Student’s t-test. P<0.05 was considered to indicate a statistically significant difference.
Results

Levofloxacin chitosan microspheres have an entrapment rate of 26.5±1.31%. To determine the entrapment rate of levofloxacin chitosan microspheres, the measurement data were subjected to regression analysis, resulting in the following standard equation: A=0.08752+59.951C (r=0.9998 when C<100 mg/l) with A being the absorbance and C the mass concentration (mg/l). The measurement data were the absorbance values of the levofloxacin standard solutions at different concentrations. The linear range of the standard curve was 6-16 mg/l. Using this equation, the concentration of each diluted sample was calculated and then converted to the concentrations prior to dilution to obtain C_free and C_total. The mean entrapment rate of the levofloxacin chitosan microspheres was calculated from the measurement data of five experiments (Table II). The result suggested that the entrapment rate of levofloxacin chitosan microspheres was 26.5±1.31%.

Levofloxacin chitosan microspheres formed by chitosan and sodium tripolyphosphate have physical and chemical properties rendering them a suitable ophthalmic particle dispersion system. To characterize the levofloxacin chitosan microspheres regarding their physical and chemical properties, the pH and ζ potential, as well as the particle diameter and distribution were determined, and a scanning electron microscope was used to observe the morphology of the particles. In addition, an infrared spectrum was recorded to analyze pure chitosan powder, blank chitosan microspheres and levofloxacin chitosan microspheres. The results indicated that the pH of levofloxacin chitosan microsphere suspension was 5.87±0.04, the average particle diameter was 2,452±342 nm, the polydispersity index was 0.168±0.028 and the ζ potential was 28.62±1.7 mV. Scanning electron microscopy revealed that the shape of the levofloxacin chitosan microspheres was almost spherical (Fig. 1). The infrared spectrum demonstrated the existence of chitosan by intense absorbance peaks at 1,063 cm⁻¹ (C-O in hydroxyl or cyclic ether) and 3,363 cm⁻¹ (O-H and N-H of polysaccharides) (Fig. 2A). In addition, the formation of microspheres by chitosan and sodium tripolyphosphate was indicated by peaks at 1,399 cm⁻¹ (amino acid cross-linked by ion) and 592.9 cm⁻¹ (interaction between phosphate and chitosan amino site) (Fig. 2B). These results indicate that levofloxacin chitosan microspheres that are formed by chitosan and sodium tripolyphosphate are suitable for use as an ophthalmic particle dispersion system due to their physical and chemical properties.

Chitosan temperature-sensitive gel containing microspheres loaded with drug prevent drug burst release at the initial stage and facilitates the slow release of the drug later on. To evaluate the in vitro release of levofloxacin by the chitosan temperature-sensitive gel, three different groups were used with different sample preparations. In group 1, a great amount of

| C_free (g/l) | C_total (g/l) | Entrapment rate (%) | Mean ± RSD |
|-------------|--------------|---------------------|------------|
| 0.312       | 0.425        | 26.6                |            |
| 0.305       | 0.418        | 27.0                |            |
| 0.319       | 0.432        | 26.2                |            |
| 0.314       | 0.429        | 26.8                |            |
| 0.322       | 0.436        | 26.1                |            |

C_free, concentration of free chitosan; C_total, maximum concentration of chitosan that can be released from levofloxacin chitosan microspheres; RSD, relative standard deviation.

Figure 1. Scanning electron microscopy image of levofloxacin chitosan microspheres. After diluting the suspension of levofloxacin chitosan microspheres, a scanning electron microscope (JEOL7500F, Jeol) was used to observe the morphology of particles (magnification, x10,000).

Figure 2. Infrared spectrum of (A) chitosan and (B) levofloxacin chitosan microspheres. The scanning range was 400-4,000 cm⁻¹ and the resolution was 4 cm⁻¹.
drug was released at the initial 20 sec and the drug burst release was dramatic within the first 10 min. For group 2, marked drug release was also observed within the first 20 sec, but later on, the drug was slowly released with time. For group 3, the slow release of the drug was similar to that in group 2 after 15 min, but the burst release of the drug was effectively prohibited within the first 15 min (Fig. 3). These results suggest that chitosan temperature-sensitive gel containing microspheres loaded with drug prevents drug burst release at the initial stage and facilitates the slow release of the drug later on.

Chitosan temperature-sensitive gel prolongs the contact duration of levofloxacin with the eye. To examine the eye retention of chitosan temperature-sensitive gel, fluorescein sodium was added into the levofloxacin drug loading system in groups 1-3. The results indicated that the retention time of chitosan temperature-sensitive gel in groups 1-3 was 30±2, 480±25 and 540±36 min, respectively, which was consistent with the results of the in vitro release assay (data not shown). This result indicated that the chitosan temperature-sensitive gel markedly prolongs the contact duration of levofloxacin with the eye.

Chitosan temperature-sensitive gel is safe and provides good biocompatibility. To evaluate the safety of chitosan temperature-sensitive gel and the biocompatibility it provides, a cell compatibility test, a cytotoxicity test and an ocular skin irritation test were performed. The corneal endothelial cells grown on top of the hydrogel in the control and experimental groups had pebble-like shapes and had formed dense monolayers by day 5 (Fig. 4A). An MTT assay demonstrated that the number of corneal endothelial cells that were treated with DMEM soaked with the gel in the experimental group was insignificantly higher than that in the control group on days 3 and 5 (P>0.05), suggesting that chitosan-based hydrogel membrane had good cytocompatibility (Fig. 4B). Microscopic observation indicated that the growth of L929 cells in the control and experimental groups was not significantly different (Fig. 5A). Consistently with this, the MTT cytotoxicity assay indicated that the
The absorbance in the experimental group was insignificantly higher than that in control group (RGH≥100%), suggesting that the chitosan temperature-sensitive gel had a cytotoxicity of grade 0 (Fig. 5B). The skin irritation test indicated that the control and experimental groups had nearly no congestion phenomenon and the experimental group scored 0. After stimulation for 48, 96 or 144 h, the rabbits in the two groups did not display any behavioral abnormalities. No erythematous response was observed during the entire course of the experiment. H&E staining of the injection sites on days 3, 5 and 7 was not indicative of any inflammatory response (Fig. 6), suggesting that chitosan temperature-sensitive gel caused nearly no skin irritation. These results suggested that chitosan temperature-sensitive gel is safe and provides good bioavailability.

Discussion

Chitosan has been widely assessed for use in drug delivery systems due to its biocompatibility, safety and other unique properties. In addition, it is included in the usage standards for food additives as a thickening or coating agent (16). At present, research on chitosan and its derivatives in ophthalmic drug delivery systems still mainly focus on improving the solubility of chitosan, increasing the drug load and enhancing the stability of preparations (17). However, only few studies have focused on the absorption, transportation, distribution and metabolism of drugs after administration (13). In the present study, the optimal proportions of the major components and manufacturing process of chitosan temperature-sensitive gel were determined, and the physical and chemical properties, the slow-release effect and the safety of chitosan temperature-sensitive gel loaded with drug-containing nanoparticles were investigated.

The pH value of artificial tears is ~8 and their addition is equivalent to that of crosslinking agent. As a result, the temperature-sensitive system easily solidifies into a gel (17,18). In the present study, a temperature-sensitive in situ gel loaded with levofloxacin-containing microspheres was prepared. Chitosan microspheres easily attach to cornea and have a slow-release effect. Therefore, the retention time of drugs on the cornea is prolonged, the local bioavailability of drugs on the eye surface is enhanced and adverse effects of the drugs are reduced (19,20).

During the preparation of levofloxacin chitosan microspheres via the ionic gelation method in the present study, levofloxacin molecules become surrounded by a network structure formed by chitosan and sodium tripolyphosphate (6,21,22). The particle size of the microspheres is in a range that meets the requirements of an ophthalmic particle dispersion system. The measured particle size determined by a particle sizing meter in the present study is the size of hydrated particles, while that determined by scanning electron microscopy is the size of dried particles. As a result, the particle size determined by scanning electron microscopy was smaller. In addition, the zeta potential suggests that the system is stable, and guaranteed the quality of the prepared temperature-sensitive in situ gel.

Chitosan nanoparticles have biological adhesion properties and may attach to conjunctival cells (9,23,24). As a result, the tight junction between epithelial cells of the cornea and conjunctiva may be transiently opened to increase the permeability of the drug in the cornea and conjunctiva. The in vitro slow release and ocular retention experiments demonstrated that chitosan temperature sensitive gel loaded with levofloxacin microspheres prolonged the contact duration between the microspheres and eyes, and ensured the steady and sustained release of the drug. Chitosan temperature-sensitive hydrogel must not have any adverse effects on the organizational structure of organisms when being in contact with tissues in vivo (25,26). The cell compatibility test, the cytotoxicity test

![Figure 6. Skin irritation test. A sterile and pyrogen-free chitosan-based temperature-sensitive hydrogel membrane (5 g) was soaked in 5 ml saline for 72 h. Saline (0.1 ml) was injected subcutaneously into six points in the control group, and DMEM soaked in chitosan-based temperature-sensitive hydrogel (0.1 ml) was injected subcutaneously into the six points in experimental groups. At 12, 24 and 48 h after injection, each injection point of each rabbit was evaluated to determine the artificial vascular stimulation fraction. On days 3, 5 and 7 after surgery, subcutaneous tissues were obtained from all animals and subjected to hematoxylin and eosin staining (magnification, x10).](image-url)
and the skin irritation test in the present study have demonstrated that chitosan-based temperature-sensitive hydrogel has good biocompatibility and safety. No adverse effects are observed. However, a limitation of the present study was that only an extract of the gel was applied to the cells. To conclude, the present study provided a basis for the future development of chitosan-based temperature-sensitive hydrogel in ophthalmic drug delivery.

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