

Injectable thermosensitive hydrogel based on chitosan and quaternized chitosan and the biomedical properties

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Abstract A novel injectable thermosensitive hydrogel (CS–HTCC/ α β -GP) was successfully designed and prepared using chitosan (CS), quaternized chitosan (HTCC) and α,β -glycerophosphate (α,β -GP) without any additional chemical stimulus. The gelation point of CS–HTCC/ α β -GP can be set at a temperature close to normal body temperature or other temperature above 25°C. The transition process can be controlled by adjusting the weight ratio of CS to HTCC, or different final concentration of α,β -GP. The optimum formulation is (CS + HTCC) (2% w/v), CS/HTCC (5/1 w/w) and α,β -GP 8.33% or 9.09% (w/v), where the sol–gel transition time was 3 min at 37°C. The drug released over 3 h from the CS–HTCC/ α,β -GP thermosensitive hydrogel in artificial saliva pH 6.8. In addition, CS–HTCC/ α,β -GP thermosensitive hydrogel exhibited stronger antibacterial activity towards two periodontal pathogens (*Porphyromonas gingivalis*, *P.g* and *Prevotella intermedia*, *P.i*). CS–HTCC/ α β -GP thermosensitive hydrogel was a considerable candidate as a local drug delivery system for periodontal treatment.

1 Introduction

Hydrogels are a special class of materials that could absorb considerable amount of water while maintaining their integrity in water [1]. In past decades, stimuli-sensitive hydrogels have gained increasing attention owing to their smart responsibility to the environmental stimuli and good biocompatibility, especially, thermosensitive physically crosslinked hydrogels. In situ, it has attracted a great deal of their practical biomedical or pharmaceutical application.

Chitosan, a polysaccharide derived from naturally abundant chitin, is currently receiving a great deal of interest for medical and pharmaceutical applications in various chemical and physical gel forms [2]. Chitosan thermosensitive hydrogels possessed many favorable properties such as nontoxicity [3], biocompatibility [4] and biodegradability [5, 6], antimicrobial activity [7, 8], wound healing, muco-adhesive properties [9], and tissue regeneration properties [10]. In recent years, chitosan thermosensitive hydrogels have been utilized widely in drug delivery [11], cell encapsulation [12], tissue engineering [13]. Chitosan-based thermosensitive hydrogel have been prepared with different methods such as grafting chitosan with poly(Nisopropylacrylamide) [13], grafting chitosan with PEG [11], mixing chitosan with poly(vinyl alcohol) and sodium bicarbonate or coupling Pluronic (a block copolymers based on ethylene oxide and propylene oxide) onto chitosan using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide and *N*-hydroxy-succinimide as coupling agents [14], chitosan–PEG diblock copolymer thermosensitive gel [15]. Chenite and Ruel [16, 17] with their co-workers successfully designed and prepared chitosan- β -glycerophosphate (β -GP) thermosensitive hydrogel.

However, the low solubility is the main drawback of chitosan. It can be only dissolved in dilute inorganic and

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organic acid solutions when pH is less than 6.5 [18]. This character restricted the direct application in many fields. So, various chemical modifications have been employed to enhance the aqueous solubility of chitosan. Quaternized chitosan which introduced quaternary amino groups into chitosan chain is both facile and effective method to render it soluble in water. Moreover, quaternized chitosan has cationic activity, bioadhesive properties, permeation enhancing effects and high efficacy against bacteria and fungi even at neutral conditions [19].

Periodontal disease is a world-wide prevalent chronic infection which is caused by accumulation of bacteria in dental plaque. Local drug delivery systems against periodontal pathogens have been focused on due to the disadvantages of systemic administration as well as resistance of antibiotics [20–26].

Accordingly, this study aimed to develop an novel in situ chitosan-based hydrogel with chitosan and quaternized chitosan by blending them with α,β -glycerophosphate (α,β -GP) as a local drug delivery system for periodontal treatment. This formulation not only can increase the solubility and inhibit activity to bacterial of the formulation, but overcome the disadvantage of HTCC. The characteristics of the hydrogel on thermosensitivity, morphology, drug release and antibacterial activity of themselves were investigated. Ornidazole (ONZ) was chosen as a model drug since it is widely used for periodontal disease treatment. Antimicrobial activity of this formulation against the periodontal pathogens—*Porphyromonas gingivalis* (*P.g*) and *Prevotella intermedia* (*P.i*) were also investigated.

2 Experiments performed

2.1 Materials

Chitosan, molecular weight 1,080 kDa, deacetylation degree 75.6%, were prepared in our laboratory through the method of acidic-degradation [27]. GTMAC (glycidyltrimethylammonium chloride) was obtained from Dongying Guofeng Fine Chemical Co., Ltd. (Shandong, China). α,β -glycerophosphate (α,β -GP) was provided by Kaiyuan Pharmaceutical & Chemical Co., Ltd. (Shanxi, China, medicine grade). ONZ was kindly donated by Xi'an Bodyguard Pharmaceutical Co., LTD (Batch No.0706083) (Xi'an city, China). All other reagents were of analytic reagent.

2.2 Preparation and characterization of HTCC

N-[(2-hydroxy-3-trimethylammonium)propyl] (HTCC), a water-soluble chitosan derivative, was prepared by reacting chitosan with glycidyltrimethylammonium chloride

(GTMAC). Briefly, 6 g chitosan was mixed and dispersed in 225 ml dimethylcarbinol. The reaction was carried out by stirring at 80–90°C for 1 h. GTMAC was dissolved in deionized water (30% w/v) to form a solution. The GTMAC solution was added to chitosan suspension slowly. The molar ratio of GTMAC to amino groups of chitosan was 4 to 1. After 4 h of reaction at 80°C, precipitations were filtered with filter-papers (Xinhua-I filter-paper, Xinhua Group, Hangzhou, China). Then the product was poured into 50 ml alcohol and washed for five times. HTCC was obtained by drying at 80°C for 48 h. The infrared spectrums of HTCC were measured with KBr pellets on FT/IR-430 Fourier Transform Infrared Spectrometer (Jasco Co. Tokyo, Japan) to confirm the presence of quaternary amino groups on HTCC at room temperature.

The degree of quaternization (DQ) was determined by titrating the amount of Cl^- ions on the HTCC with a certain concentration of aq AgNO_3 solution [28].

2.3 Preparation of CS-HTCC/ α,β -GP thermosensitive hydrogel

CS-HTCC/ α,β -GP thermosensitive hydrogel was prepared through the following steps. First, 0.18 g mixture of CS and HTCC (2% w/v) was progressively added to 7 ml 0.1 M aqueous lactic acid (LA) solution at room temperature under mechanical stirring until complete dissolution, while adding 2 ml H_2O to dilute the solution. Second, α,β -GP aqueous solution (50% w/v) was prepared in deionized water. Both of the solutions were chilled in an ice bath for 15 min. Third, α,β -GP aqueous solution (50% w/v) was added drop wise to the chitosan solution under stirring. The solution obtained was stirred for 20 min. The formulation containing ONZ was prepared by pouring the sterilized drug powder to aqueous lactic acid solution before mixing with the α,β -GP solution as described above.

2.4 Characterization of thermosensitivity

The gelation times at 25, 30, 35 and 37°C were determined by test tube inverting method [29]. The CS-HTCC/ α,β -GP solution (2.0 ml) except for the ONZ, was added into a tube (10 ml) with a diameter of 1.0 cm and kept in a water bath of 25 ± 0.5 , 30 ± 0.5 , 35 ± 0.5 and $37 \pm 0.5^\circ\text{C}$, respectively. The tube was taken out every 1 min and inverted to observe the state of the sample. The gelation point was determined by flow or no-flow criterion over 30 s with the test tube inverted.

Sol-to-gel behavior of CS-HTCC/ α,β -GP solution thermosensitive hydrogel was further studied by measuring the solution viscosity of the samples. A viscometer (NDJ-8S, Shanghai Cany Precision Instrument Co., Ltd., Shanghai, China) was used to determine the viscosities of CS-HTCC/

α,β -GP solution thermosensitive hydrogel (in 50 ml lots) at predetermined time intervals as a function of time at 37°C. The third rotator was selected with rotation speed of 1.5 rpm.

2.5 Morphological studies

When the samples of hydrogels were transformed into gels, they were observed after being frozen in liquid nitrogen and lyophilized for 48 h. The samples were then coated with platinum by an ion sputter gold under vacuum and the surface of it was investigated by using a scanning electron microscope (KYKY2800B, KYKY Technology Development Ltd., Beijing, China).

2.6 Incorporation of ONZ and in vitro release study

ONZ was introduced into the formulations at 0.5, 1 and 1.5% concentrations to evaluate the release rate of the model drugs in vitro. Samples A and C (250 mg respectively) with or without ONZ were placed in dialysis membrane (New Brunswick, New Jersey, USA) with a molecular weight cut-off of 8,000–10,000. The dialysis membranes were placed in 100 ml artificial saliva buffer [30] with pH 6.8 and 4.0 respectively as a simulation of gingival crevicular fluid (GCF) in Erlenmeyer flasks (250-ml) and tied at the top to ensure retention of the sample. The volume of dissolution medium used was dependent on the stage of the dissolution test. The Erlenmeyer flasks were placed in a water bath at $37 \pm 0.5^\circ\text{C}$, with samples being taken and the dissolution medium replaced accordingly. The buffer solutions were shaken continuously at 100 rpm in a vibrating incubator. At certain periodic intervals 4 ml buffer samples were removed and replaced by an equal volume of the fresh buffer to maintain a constant volume. The samples were examined with UV-spectrophotometer (Ultrospec2100 pro, Amersham Biosciences, U.S.A) at 318 nm.

2.7 Antibacterial activity

Porphyromonas gingivalis (*P. g* ATCC33277) and *Prevotella intermedia* (*P. i* ATCC25611) were selected for antimicrobial tests. Two kinds of strict anaerobic strains were provided by Beijing Dental Institution, Affiliated Hospital of Capital Medical University (Beijing, China). The susceptibility test was evaluated by using agar dilution method which is the NCCLS-recommended method for anaerobe susceptibility testing [31, 32]. The anaerobic strains were individually inoculated into tubes containing 5 ml of sterile 0.9% saline solution. The suspension was adjusted to match the turbidity of 1.5×10^8 CFU ml⁻¹ (equivalent to 0.5 McFarland standards). There were

150 μl of bacterial suspensions spread throughout the agar plate. Seeding was done using sterile swabs that were brushed across the agar surfaces in two directions. Sterilized stainless steel tubes of $8.0 \times 1.0 \times 10$ mm (inner diameter, 6 mm) were added to the surfaces of the media and filled with 100 μl of sterilized cooling CS/ α,β -GP solution, CS-HTCC/ α,β -GP solution and controls (0.9% saline solution) respectively. Plates were incubated at 37°C under aerobic workshop for 5 days. Zones of inhibition of microbial growth around the cylinder containing the tested substances were measured and recorded after the incubation period. The inhibitory zone was considered to be the shortest distance (mm) between the outer margin of the cylinder and the initial point of the microbial growth. Six replicates were made for each microorganism. Each assessment was performed three times to ensure reproducibility of results.

Statistical data were analyzed using SPSS13.0 and differences were considered to be significant at a level of $P < 0.05$, using one-way test.

3 Results and discussion

3.1 Characterization of HTCC and hydrogel

The HTCC synthesized was a white powder and was soluble in water. The degree of quaternization (DQ) of HTCC was 74.5% (mol/mol). The presence of quaternary amino groups on chitosan chains was proven by the IR spectra data of HTCC see in Fig. 1 [33, 34].

FITR measurement showed that there are three characteristic peaks of CS (Fig. 1a) at $3,363\text{ cm}^{-1}$ of n (OH), $1,382\text{ cm}^{-1}$ of d (C–O–C) and $1,603\text{ cm}^{-1}$ of n (NH₂). And the saccharide oxygen bridge peaks of the skeletal vibrations involving the C–O stretching appeared between $1,146\text{ cm}^{-1}$ and $1,083\text{ cm}^{-1}$. Compared with CS, HTCC (Fig. 1b) shows the disappearance of the NH₂-associated band near $1,600\text{ cm}^{-1}$ (arrow) of the N–H bending in the primary amine; and appearance of a new band at $1,483\text{ cm}^{-1}$ (arrow), which is attributed to the methyl groups of the ammonium. It proved the existence of quaternary amino groups on CS chains. Characteristic peaks of alcohol and second alcohol between $1,160$ and $1,030\text{ cm}^{-1}$ did not change in HTCC confirming the lack of the introduction of an alkyl group at C-3 and C-6 of the CS[33].

The mixture of HTCC and CS with α,β -GP was initially an aqueous solution and it gradually turned into a semi-transparent hydrogel as the temperature increased. The hydrogel did not revert into liquid status when the temperature decreased. The substructure of the CS-HTCC/ α,β -GP thermosensitive hydrogel (Table 1, sample C) is shown in Fig. 2a. Compared with the substructure of CS- α,β -GP

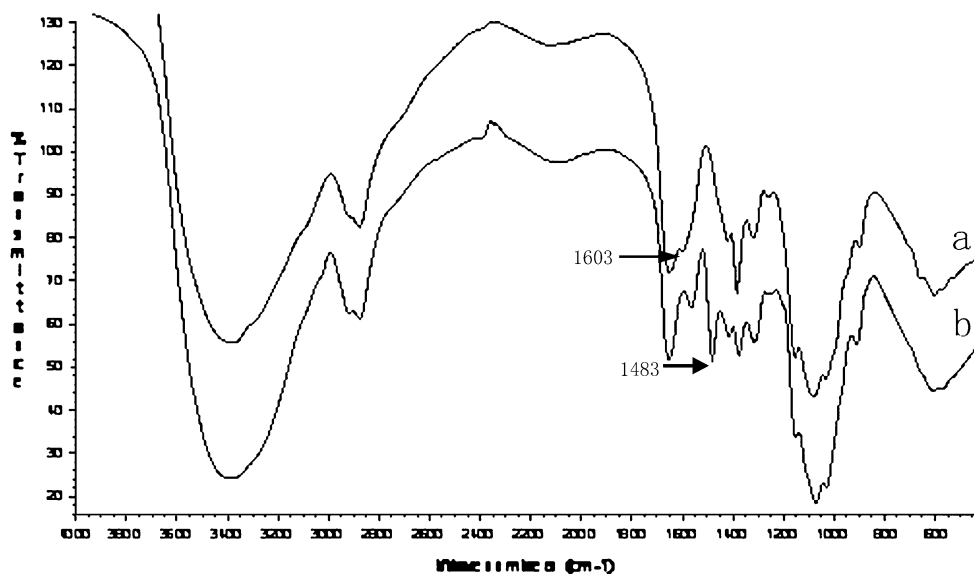


Fig. 1 IR spectra data of CS (a) and HTCC (b)

thermosensitive hydrogel (Table 1, sample A) as shown in Fig. 2b, a crosslinked network formed more porous structure in the CS-HTCC/ α,β -GP hydrogel. The surface structure of the CS-HTCC/ α,β -GP became more loose and some holes appeared in it which made it favorable for water and small molecules to move freely in the network.

3.2 Thermosensitivity of formed hydrogel

The prepared CS-HTCC/ α,β -GP system remained liquid for a desired period of time below 25°C (Fig. 3a) and turned into gel state (Fig. 3b) when the temperature was above 25°C. The weight ratio of CS to HTCC can influence the thermosensitivity of the CS-HTCC/ α,β -GP system as shown in Table 1. The sol-gel transition time would take 12 min in a CS/ α,β -GP thermosensitive system (sample A) in the present investigation at 37°C. But it did not exhibit thermosensitivity when the proportion of HTCC to the mixture of CS and HTCC was above 16.77% (w/w). The sol-to-gel transition time is only 3 min when the weight ratio of chitosan to HTCC was 83.33% to 16.67% (sample C) which possessed the shortest sol-gel transition time at 37°C. Compared with a CS/ α,β -GP thermosensitive

system, the sol-to-gel transition time of a CS-HTCC/ α,β -GP thermosensitive is significantly shorter.

This kind of phenomena could be further verified through viscosity determination as shown in Fig. 3c. The viscosity of the CS-HTCC/ α,β -GP (sample C) thermosensitive hydrogel increased significantly after 3 min at 37°C, indicating that the liquid solution has turned into a gel quickly. However, the viscosity of the CS/ α,β -GP thermosensitive hydrogel (sample A) increased slowly under the same conditions. In the first 12 min, the viscosity of CS/ α,β -GP hydrogel (sample A) was increased slightly, and then the viscosity was increased quickly with the time prolong. Compared with CS/ α,β -GP hydrogel, the viscosity change of CS-HTCC/ α,β -GP hydrogel is faster. The final viscosity of the formed CS/ α,β -GP hydrogel is higher than that of CS-HTCC/ α,β -GP hydrogel. The possible reason is mainly due to that HTCC is the chitosan water soluble derivation which possesses the low viscosity. The presence of quaternary amino groups on HTCC chains decreased crystallinity and improved the water-solubility of chitosan which both contributed to reduce the viscosity [35]. Therefore the CS-HTCC/ α,β -GP hydrogel shows a different response to the external temperature changes.

The sol-gel transition time showed a relationship with gelation temperature (Table 1). According to Table 2, the gelation time was 20 min at 25°C and the gel formed with less intensity. When the temperature was increased above 40°C, the transition process became too fast to be controlled easily. The CS-HTCC/ α,β -GP system exhibits good characteristics of response to the external temperature changes between 35 and 40°C. Sol-gel transition time was 3–5 min at temperatures in the range of the normal body

Table 1 The relationship between different gelation temperature and gelation time

CS + HTCC (w/v%)	CS (%)	HTCC (%)	α,β -GP (v/v%)	Gelation time (min)			
				25°C	30°C	35°C	37°C
2	83.33	16.67	9.09	10	8	3	3
2	83.33	16.67	8.33	20	11	10	3

Fig. 2 SEM photographs of CS-HTCC/ α,β -GP thermosensitive hydrogel (sample C) (a) and CS/ α,β -GP thermosensitive hydrogel (sample A) (b)

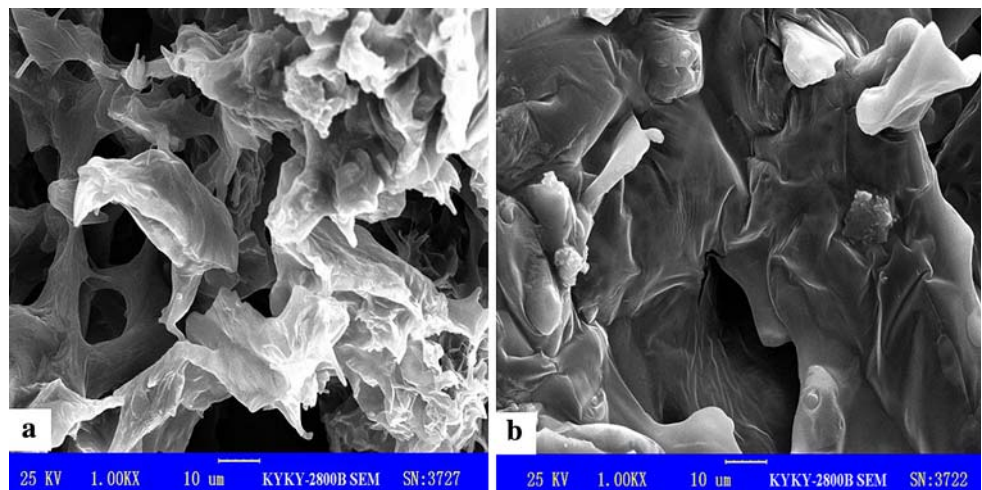
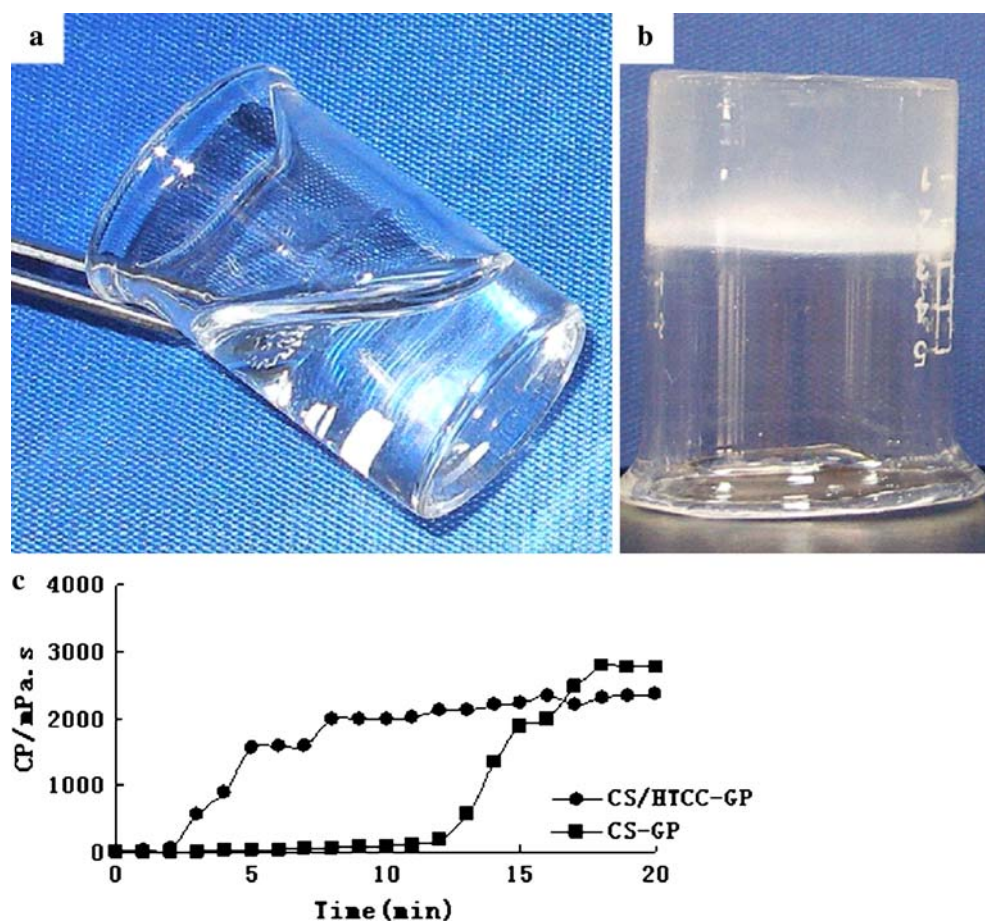


Fig. 3 a CS-HTCC/ α,β -GP solution below 25°C. b Formed CS-HTCC/ α,β -GP hydrogel at 37°C. c Viscosity of CS-HTCC/ α,β -GP thermosensitive hydrogel (sample C) and CS/ α,β -GP thermosensitive hydrogel (sample A) at 37°C



temperature scale (35–37°C) and the gel formed exhibited good strength.

The final concentration of α,β -GP was found to have an influence on the thermosensitive hydrogel (sample C) as listed in Table 3. The sensitive concentration of α,β -GP is 4.84–9.09% (w/v). When the concentration of α,β -GP is 4.84% in the CS-HTCC/ α,β -GP system (sample C), the gelation time is 10 min at 37°C. The sol-to-gel transition

takes only 3 min at 37°C when the α,β -GP concentration is increased to 8.33 and 9.09%. The results indicated that the gelation time can be controlled through the different concentration of α,β -GP. α,β -GP is a weak base which would neutralize the acid solution [34]. With the increase of the α,β -GP amount, electrostatic binding between the ammonium group of the CS, HTCC and the phosphate group of the glycerophosphate was reinforced. With the chitosan

Table 2 The relationship between different weight ratio of CS to HTCC and gelation time

Sample	CS + HTCC (w/v%)	CS (%)	HTCC (%)	α, β -GP (v/v%)	Gelation time (min) at 37°C
A	2	100	0	8.33	12
B	2	88.89	11.11	8.33	12
C	2	83.33	16.67	8.33	3
D	2	66.67	33.33	8.33	No
E	2	50	50	8.33	No
F	2	33.33	66.67	8.33	No
G	2	16.67	83.33	8.33	No
H	2	11.11	88.89	8.33	No
I	2	0	100	8.33	No

Table 3 The relationship between different concentration of α, β -GP and gelation time

CS + HTCC (w/v%)	CS (%)	HTCC (%)	α, β -GP (v/v%)	Gelation time (min) at 37°C
2	83.33	16.67	4.84	10
2	83.33	16.67	5.56	8
2	83.33	16.67	8.33	3
2	83.33	16.67	9.09	3

solution becoming closer to neutralization with α, β -GP, hydrogen bonding between the chitosan chains reduced electrostatic repulsion. In addition, ammonium groups on HTCC were unprotonated while quaternary amino groups were protonated at this pH value. Quaternary amino groups showed stronger cationic properties than amino groups. The static repulsive forces between the quaternized amino groups would be weakened. With the increasing of the α, β -GP amount, the HTCC chains became more flexible and approach each other more easily, which helped the formation of hydrogen bonds between HTCC chains. More quaternary amino groups were combined with α, β -GP by ionic interaction. As a result, electrostatic repulsive force between quaternary amino groups was weakened, and the growth rate of polymer chains increased. Therefore, the gelation time was markedly decreased.

3.3 Drug release behavior in vitro

ONZ as a model drug was trapped during the preparation of hydrogel and it could be slowly released in vitro. The release profile was changed according to the different PH value of release medium, the concentration of ONZ as well as α, β -GP.

The compared release profile of ONZ from the CS-HTCC/ α, β -GP (sample C) and the CS/ α, β -GP hydrogel (sample A) in artificial saliva buffer PH 6.8 is shown in

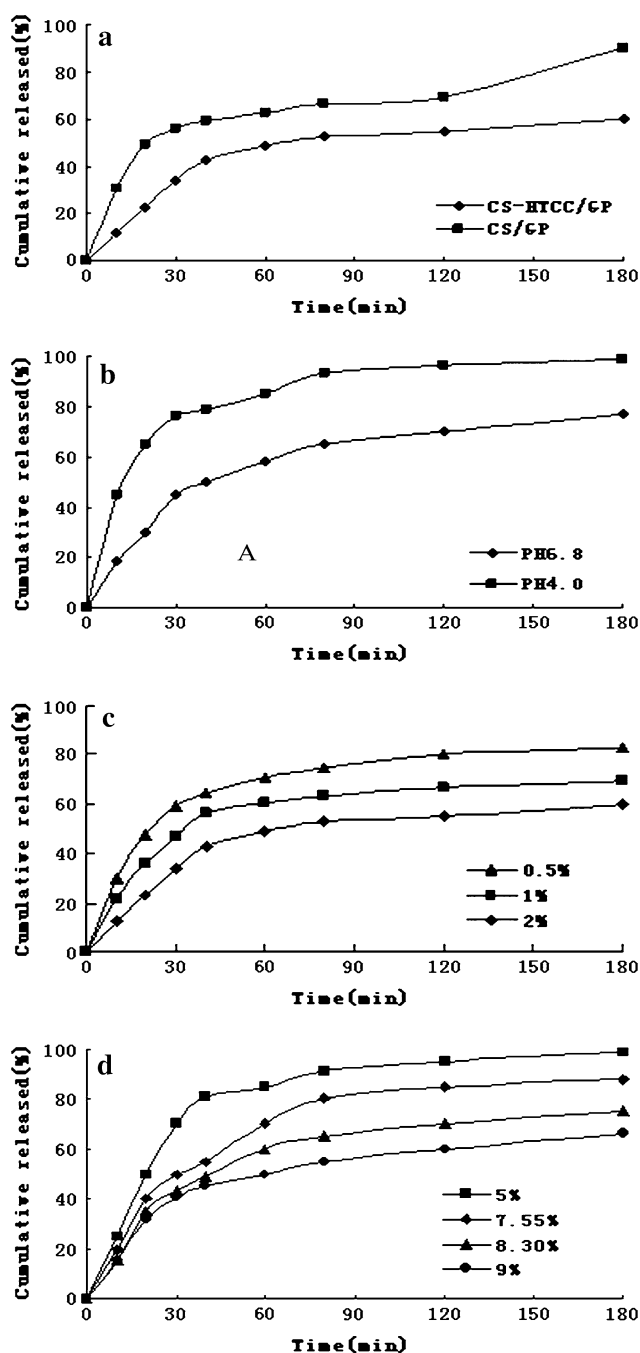


Fig. 4 a Ornidazole release profiles from CS-HTCC/ α, β -GP thermosensitive hydrogel (sample C) and CS/ α, β -GP thermosensitive hydrogel (sample A) in artificial saliva pH = 6.8 ($n = 3$). b 1% Ornidazole release profiles from CS-HTCC/ α, β -GP thermosensitive hydrogel (sample C) from artificial saliva with different PH ($n = 3$). c Ornidazole release profiles from CS-HTCC/ α, β -GP thermosensitive hydrogel (sample C) with different concentration of ornidazole ($n = 3$). d Ornidazole release profiles from CS-HTCC/ α, β -GP thermosensitive hydrogel (sample C) prepared with different concentration of α, β -GP ($n = 3$)

Fig. 4a. According to Fig. 4a, in the first 30 min, there was 34 and 55.8% ONZ released from the CS-HTCC/ α, β -GP system and the CS/ α, β -GP system, respectively. Also, 55

and 69% of drug was released from the CS–HTCC/ α,β -GP system and the CS/ α,β -GP system respectively in the first 2 h respectively. The cumulative release rate of ONZ is much slower in the CS–HTCC/ α,β -GP system than in the CS/ α,β -GP system. The difference of ONZ release profiles observed may well be due to that hydrophobic interaction in the CS-HTCC/GP system is much heavier than that of in the CS/ α,β -GP system which effectively retarded the drug release from the hydrogel.

Difference pH value was found to have a relationship with the cumulative release profiles of ONZ (1% w/v) from sample C (Fig. 4b). An artificial saliva buffer solution with pH 6.8 simulated the normal condition while pH 4.0 represents the acid environment of a pathologic status of oral cavity surroundings. Almost 80% ONZ was released in the first 40 min at pH 4 artificial saliva buffer solution, while 100% ONZ was released at pH 4 in about 2–3 h. However, at near neutral condition ONZ was released relatively slowly during the first 40 min. About 30% loaded drug was released during the first 20 min, and 77% was released in the following 3 h. The CS–HTCC/ α,β -GP gel formation effectively retarded the release of ONZ.

The effect of trapped ONZ concentration in the hydrogel on the release profile was investigated at 37°C as shown in Fig. 4c. The cumulative release profile of three different concentrations of ONZ is similar, in which the formulation with a high concentration of ONZ released faster than that of a low concentration. Because ONZ has hydroxy groups, it can form hydrogen bonds with HTCC and CS, which strengthened the crosslinked network and slowed drug release. Therefore, the higher the amount of ONZ added, the slower the observed drug release rate became.

Figure 4d shows the ONZ release profiles from hydrogels prepared with different concentrations of GP. The release rate of ONZ was decreased with the increasing of the final concentration of α,β -GP. In the process of gelling, GP played an essential role influencing the hydrogel formation by ionic interaction with CS and HTCC. The reaction strengthened the crosslinked network and retarded drug release. Therefore, with a higher amount of GP added, more compact hydrogel structure was obtained and much slower drug release profile was observed.

3.4 Antimicrobial activity

Agar diffusion assay was used for antibacterial assay. The results showed that CS–HTCC/ α,β -GP (sample C) and the CS/ α,β -GP (sample A) thermosensitive hydrogel exhibited significant antimicrobial activity against *P.g* and *P.i* as compared with the negative control ($P < 0.001$). CS–HTCC/ α,β -GP (sample C) had the biggest inhibitory zone to *P.g* and *P.i*. The antibacterial activity of CS–HTCC/ α,β -GP (sample C) hydrogel is a slight stronger than that of CS/ α,β -

GP (sample A) to two representative periodontal pathogens. In addition, the effects of CS–HTCC/ α,β -GP and CS/ α,β -GP against *P.g* was higher than *P.i*. However, these differences were not statistically significant ($P > 0.05$).

4 Conclusion

The CS–HTCC/ α,β -GP thermosensitive hydrogel is a natural polymer-based physically crosslinked hydrogel without any additional chemical stimulus for its formation, and its gelation point can be set at a temperature close to normal body temperature or other temperature above 25°C. The transition process can be controlled by adjusting the weight ratio of CS to HTCC, or different final concentration of α,β -GP. The optimum formulation is (CS + HTCC) (2% w/v), CS/HTCC (5/1 w/w) and α,β -GP 8.33% or 9.09% (w/v), where the sol–gel transition time was 3 min at 37°C. The drug released over 3 h from the CS–HTCC/ α,β -GP thermosensitive hydrogel in artificial saliva pH 6.8 more effectively retarded the release of ONZ than the CS/ α,β -GP thermosensitive hydrogel under the same conditions. In addition, CS–HTCC/ α,β -GP thermosensitive hydrogel exhibited stronger antibacterial activity towards two periodontal pathogens than CS/ α,β -GP thermosensitive hydrogel.

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