Preparation and characterization of catechol-functionalized chitosan thermosensitive hydrogels

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Abstract. Catechol functionalization of chitosan was modified by EDC coupling reaction and then catechol-functionalized chitosan thermosensitive hydrogels were prepared by catechol-functionalization of chitosan with β-glycerophosphate disodium as a thermosensitizer. The hydrogels were then characterized by FTIR, 1H-NMR, SEM and rheology analysis. The results demonstrated that the thermosensitive hydrogels were prepared at 37°C within several minutes by mixing 2% catechol-functionalized chitosan with 30% β-glycerophosphate disodium at a ratio of 8:2, and these thermosensitive hydrogels with porous network structure were expected to be developed as biomedical materials with wide applications.

1. Introduction

Hydrogels, novel functional polymeric materials with three-dimensional network structure, are hydrophilic but not soluble in water. Owing to the excellent water adsorption, water retention, and biocompatibility, they have been widely studied and applied [1]. Thermosensitive hydrogels are one of numerous environment-sensitive hydrogels that have been mostly studied, mainly characterized in ability to respond to temperature change and give rise to sol-gel transition. Compared with conventional hydrogels, thermosensitive hydrogels have significant advantages when being applied in tissue engineering, drug delivery, and 3D cell culture.

As tissue engineering materials for wound healing, surgical tissue adhesion, hemostasis in surgical procedure, and local drug delivery, tissue adheres attract wide attention [2,3]. It’s found in many studies that, adhesion behaviors of mussels are mainly affected by 3,4-dihydroxy-L-phenylalanine (DOPA), while a major adhesive component of DOPA is ortho-dihydroxyphenyl functional group that is able to form strong covalent bonds and noncovalent bonds with various organic/inorganic/metallic substrates, which plays an important role in hydrogel-like adhesives [4-6]. Ryu et al synthesized temperature-sensitive and adhesive catechol-functionalized Chitosan/Pluronic-SH hydrogel (CHI-C/Plu-SH) after chitosan backbone conjugated with multiple catechol groups was crosslinked with terminally thiolated Pluronic F-127 triblock copolymer [7]. Both in vitro and in vivo assessments indicate that CHI-C/Plu-SH hydrogel has excellent mechanical properties and stability.

Chitosan (CS) is a natural polycationic polysaccharide prepared from deacetylated chitin, and has good bioactivities such as promoting trauma healing, inhibiting scar formation, coagulation, and antimicrobial property. Due to its good biocompatibility, non-immunogenicity and non-irritability, it was approved by FDA as GRAS (generally regarded as safe) substance in 2001 [8]. As chitosan is not readily

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soluble in water but soluble in weak acids, its application is partially limited; moreover, adhesion capabilities of chitosan hydrogels are limited, too, so we conceived of grafting catechol functional groups onto chitosan backbone in order to increase adhesiveness while modifying its water solubility, so that it can work better.

In this paper, we mainly developed thermosensitive tissue adhesive hydrosols based on catechol-functionalized chitosan (CS-C) and β-glycerophosphate disodium(β-GP), which are expected to be applied as bio-adhesive materials.

2. Experimental

2.1. Materials
Chitosan, DD≥90%, MW=100 kDa; 3,4-dihydroxyhydrocinnamic acid (HCA), 1-ethyl-(3-dimethylaminopropyl)-carbodiimide hydrochloride (EDC) and N-hydroxysuccinimide (NHS) were purchased from Shanghai Aladdin Biochemical Technology Co., Ltd; β-glycerophosphate disodium(β-GP), 98%, was purchased from SIGMA.

2.2. Synthesis and characterization of CS-C
The procedure in literatures [6,7] was slightly modified. Briefly, 5 g chitosan was dissolved in 250 mL of 1% acetic acid overnight to result in 2% CS solution for later use. 0.1M HCA was dissolved in 50 mL of deionized water to result in HCA solution for later use. 0.2M EDC and NHS were dissolved in 200 mL of ethanol/water mixture (1:1 by volume). Secondly, HCA solution was added into CS solution and stirred, then EDC/NHS was added dropwise into CS/HCA solution for reaction for 10 hours while pH was kept at 4.5–5.5. After the reaction was completed, the reaction product was dialyzed with pH 5 hydrochloric acid solutions for three days, and then dialyzed with purified water for 4 hours; thereafter the product was freeze-dried to result in CS-C. CS-C was structurally characterized using Spectrum 100 FTIR spectrometer, and AVANCE III NMR spectrometer.

2.3. Preparation of CS-C/β-GP thermosensitive hydrogels
A calculated amount of CS-C was weighed and dissolved in distilled water to result in CS-C solution at a certain concentration; a calculated amount of β-GP was dissolved in distilled water to result in β-GP solution at a certain concentration. At a given ratio by volume, β-GP solution was added dropwise into CS-C solution; thereafter, the mixture was further stirred for 2 min and then put into a thermostat water bath at 37°C to result in CS-C/β-GP-based thermosensitive hydrogel.

2.4. Characterization of CS-C/β-GP thermosensitive hydrogel

2.4.1. Gel time. Gel time was determined by inversion method. 1 mL of the mixture was added into a 3 mL screw neck vial, then the vial was placed into the thermostat water bath at 37°C; 15 min later, gel state was observed every 1 min, and the gel time is the time at which the mixture stops flowing in 30 sec when the vial is inverted.

2.4.2. IR characterization. The freeze-dried CS-C/β-GP hydrogel sample was tabletted with potassium bromide powder, and its IR absorption characteristics were analyzed by Spectrum 100 FTIR spectrometer within a wavenumber range of 4000–400 cm⁻¹ with a resolution of 4 cm⁻¹ over 16 scans.

2.4.3. Microstructure. A small part of the freeze-dried sample was placed on the stage for gold spray treatment and then placed in S-4800 SEM, observed and photographed at an acceleration voltage of 10 kV.

2.4.4. Rheology analysis. Rheology analysis was performed on HAAKE Rheometer (MARSIII, Haake, Germany) equipped with cone/plate combination (C35/1: φ=35mm, angle=1°) as a measuring system. 1 mL of a hydrogel sample was put into the rheometer, and at a constant frequency of 1 Hz, (1)
rheological properties of the sample were determined at a heating rate of 1°C/min within 20°C–60°C, changes in storage modulus G’(Pa) and loss modulus G”(Pa) were recorded as functions of temperature so as to assess gel temperature of the sample; (2) sample rheology was determined at a physiological temperature fixed at 37°C for a longer time, and changes in storage modulus G’(Pa) and loss modulus G”(Pa) were recorded as functions of time so as to assess gel time of the sample. To prevent moisture evaporation, one layer of oil was added around the sample in temperature and time scans.

3. Results and discussion

3.1. Characterization of CS-C

The prepared CS-C was characterized in a number of ways. As can be seen from IR spectrum of CS in figure 1(A), absorption peak at 3433 cm⁻¹ is assigned to stretching vibration of O-H and N-H associated via a hydrogen bond, absorption peaks at 1650 cm⁻¹ and 1599 cm⁻¹ are assigned to stretching vibration of amide group in chitosan, absorption peaks at 1082 cm⁻¹ and 1033 cm⁻¹ are assigned to stretching vibration of C-O typical of chitosan. Compared with IR absorption of CS, IR spectrum of CS-C varied significantly as a new absorption peak occurred at 1533 cm⁻¹, which is attributed to stretching vibration of aromatic C=C, demonstrating that catechol group has been successfully grafted onto macromolecular chain of chitosan.

CS and CS-C were further structurally analyzed by ¹H-NMR (CD₃COOD/D₂O, 500 MHz, 20°C) (figure 1B). In CS spectrum, signal peaks are resolved very well in agreement with what reported in literature [9,10]. In the spectrum of CS-C, the multiplet at chemical shifts of 3.5–4.1 ppm represents absorption peaks of H₃–H₆ on saccharide ring of CS, the absorption peak at a chemical shift of 4.92 ppm is assigned to H₁ on saccharide ring of CS (partially covered by strong solvent peak), the absorption peak at a chemical shift of 3.16 ppm is assigned to H₂, absorption peaks at chemical shifts of 2.64 ppm and 2.81 ppm are assigned to two methylene groups (H₈,H₉) linked to a benzene ring, absorption peaks at chemical shifts of 6.71–6.81 ppm are assigned to hydrogens on aromatic ring (H₁₀, H₁₁, H₁₂), and the ratio of their peak area to methylene peak area was 3:2. Based on relative peak area of catechol functional groups (H₁₀, H₁₁, H₁₂) grafted onto chitosan backbone against peak area of H₂ in ¹H-NMR, grafting ratio was calculated to be 17.3%.

![Figure 1](image.png)

**Figure 1.** FTIR (A) and ¹H-NMR (B) spectrum of CS-C.

3.2. Preparation of CS-C/β-GP-based thermosensitive hydrogels

Based on preliminary work, we selected 2% or 2.5% CS-C, 25% or 30% β-GP, and 7:3 or 8:2 as the ratio of CS-C to β-GP by volume; compositions of thermosensitive gels in various groups are shown in table 1. Sol-gel transition of CS-C/β-GP thermosensitive hydrogels is shown in figure 2.
Table 1. Compositions of CS-C/β-GP thermosensitive hydrogels.

| Group | CS-C: β-GP (V/V) | CS-C (% W/V) | β-GP (% W/V) | Gel Time (min) |
|-------|------------------|--------------|--------------|----------------|
| A     | 8:2              | 2            | 25           | 18             |
| B     | 2                | 30           |              | 16             |
| C     | 2.5              | 25           | 17           |                |
| D     | 2.5              | 30           | 20           |                |
| E     | 7:3              | 2            | 25           | 28             |
| F     | 2                | 30           | 24           |                |
| G     | 2.5              | 25           | 30           |                |
| H     | 2.5              | 30           | 27           |                |

Figure 2. Sol and gel of CS-C/β-GP thermosensitive hydrogel.

3.3. IR characterization of CS-C/β-GP thermosensitive hydrogels
Hydrogel samples from groups A and B were selected for IR characterization. As shown in figure 3(A), IR spectra of CS-C, β-GP, and CS-C/β-GP hydrogel samples (A,B) do not differ much, significant blue shift occurred in absorption peaks of CS-C/β-GP hydrogel samples near 3433 cm\(^{-1}\) and these absorption peaks became broader, meanwhile symmetric stretching vibration peaks of phosphoric acid at 977 cm\(^{-1}\) became weaker, which might be caused by stacking of hydroxyl groups in the modified CS and hydroxyl groups in β-GP, or due to hydrogen bond formation after interactions between amino groups on CS-C molecular chain and hydroxyl groups on β-GP.

Figure 3. FTIR(A) and SEM(B) of CS-C/β-GP hydrogels.

3.4. SEM examination of CS-C/β-GP thermosensitive hydrogel
The thermosensitive hydrogel sample from Group A was selected and its microscopic morphology was studied using SEM. As shown in figure 3(B), the lyophilized hydrogel prepared from CS-C and β-GP exhibited porous network microstructure that is favorable for water and small molecular drugs to pass.
through freely, and can be used in wound healing, drug release, etc.

3.5. Rheological study of CS-C/β-GP thermosensitive hydrogels

Hydrogel samples from groups A and B were preferentially selected to evaluate rheological properties, and storage modulus $G'(\text{Pa})$ and loss modulus $G''(\text{Pa})$ were plotted against temperature and time respectively, as shown in figure 4. In general, as temperature rises or time extends, curves of storage modulus $G'(\text{Pa})$ at lower viscosity intersect with curves of loss modulus $G''(\text{Pa})$ at higher viscosity, and the intersection points indicate sol-gel transition [11]. Each intersection point between the curve of storage modulus $G'(\text{Pa})$ and the curve of loss modulus $G''(\text{Pa})$ corresponds to gel temperature or gel time at corresponding temperature, at which the sample transits from sol state to gel state [12]. In the case of group A, gel temperature of the hydrogel was 39°C (figure 4A1); while in the case of group B, when the amount of β-GP was increased to 30%, its gel temperature was 37.5°C (figure 4B1) close to body temperature. With increasing temperature and time, storage modulus $G'(\text{Pa})$ increased more quickly while loss modulus $G''(\text{Pa})$ increased more slowly, indicating presence of more networked gel structures in the system; when $G'(\text{Pa})=G''(\text{Pa})$ or at the critical point of sol-gel transition, large-sized physical gel-like network came into being, that is, a hydrogel was formed; in addition, it can be seen after the gel point that $G'(\text{Pa})>G''(\text{Pa})$, in other words, compared with the increase in loss modulus, the increase in storage modulus was higher, indicating that highly elastic gel network was formed during continuous gelation. In the case of group A, gel time was 13 min (figure 4A2); while in the case of group B, gel time was 12 min (figure 4B2). Gel times measured by rheology test were shorter than those measured by test tube inversion, as the latter ones were 18 min and 16 min respectively (table 1); such differences should be caused by difference in test methods, and the test tube inversion method would be subjectively affected by observers.

![Figure 4](image-url)

Figure 4. Rheology curves of CS-C/β-GP thermosensitive hydrogels.

4. Conclusion

CS-C/β-GP thermosensitive hydrogels were successfully prepared from catechol-functionalized
chitosan by ionic gel reaction. The structural studies indicated that these thermosensitive hydrogels formed porous network structures via intermolecular hydrogen bonds, which were beneficial for adsorption of substantial fluid, and the hydrogels are expected to be developed as biomedical materials.

Acknowledgments
We gratefully acknowledge the financial support by Guangdong Provincial Natural Science Foundation of China (2016A030308009), Project of Science and Technology Plan of Guangdong Province (2015A020216019, 2017A010103023), Project of Enhancing School with Innovation of Guangdong Ocean University (2017KTSCX090) and Postgraduate Education Innovation Project of Guangdong Ocean University (201927).

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