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Synthesis and characterization of *in situ* photogelable polysaccharide derivative for drug delivery

Rong Hu, Yu-Yun Chen, Li-Ming Zhang*

Key Laboratory for Designed Synthesis and Application of Polymer Materials, School of Chemistry and Chemical Engineering, and Key Laboratory for Polymeric Composite and Functional Materials of Ministry of Education, Sun Yat-Sen (Zhongshan) University, No. 135, Xingangxi Road, Guangzhou 510275, China

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ABSTRACT

A novel polysaccharide derivative with photoreactivity was prepared by the conjugation of carboxymethylated chitosan with *N*-hydroxyl succinimide-activated nitrocinnamate in the presence of *N*,*N*-dicyclohexylcarbodiimide, and characterized by IR, ^{1}H NMR, UV–vis and rheological analyses. It was found that such a modified polysaccharide could exhibit an unique photogelation ability in the absence of potentially toxic photoinitiator or catalyst and be suitable particularly for the *in situ* preparation of photocrosslinked hydrogel biomaterials. By changing the photoirradiation time and incorporated nitrocinnamate content, its photogelation property could be modulated. For the resultant hydrogels incorporated with various nitrocinnamate contents, their properties such as swelling, viscoelasticity, *in vitro* biodegradation and drug release were investigated. In addition, the photogelation mechanism of this polysaccharide derivative was also discussed.

1. Introduction

Naturally occurring polysaccharides are hydrophilic macromolecules suitable for the development of hydrogel biomaterials (Liu and Chan-Park, 2009; Kopeček, 2007; Hennink and van Nostrum, 2002; Zhang et al., 2005a,b; Liang et al., 2007; Zhao et al., 2006). They possess a number of favorable characteristics such as high hydrophilicity, good biocompatibility, lack of toxicity, and availability of reactive sites for chemical modification. Hydrogels derived from the crosslinking of polysaccharides and their derivatives have been investigated in a number of biotechnical and biomedical applications, ranging from controlled drug delivery systems and enzyme immobilization matrices to size-selective biomembranes and wound healing dressings (Song et al., 2009; Hoare and Kohane, 2008; Leach et al., 2003).

Among various methods used for the polysaccharide gelation, photoinduced crosslinking provides an effective and benign method of *in situ* hydrogelation. This is because that facile photogelation exhibits considerable advantages when compared to the conventional methods of physical or chemical hydrogel formation, including mild reaction conditions, minimum byproduct formation, and easily controlled processing. Therefore, there has been an increasing interest in utilizing photogelation as a means of biomaterial preparation in the field of medicinal and biomedical science. In this context, a common strategy for photoinduced hydrogel formation involves a polymerizable polysaccharide derivative having pendant acrylate groups along the polysaccharide backbone chain, which is usually obtained by the reaction of the polysaccharide with maleic anhydride or glycidyl methacrylate (Li and Zhang, 2007; Kim et al., 1999; Li et al., 2004; Smeds et al., 2001). In this method, the photosensitive polysaccharide derivatives are polymerized in the presence of a photoinitiator (such as benzophenone and xanthene dyes), and a catalyst or an accelerator, upon exposure to long-wavelength ultraviolet radiation (UV) or visible light.

Chitosan is a copolymer of D-glucosamine and *N*-acetylglucosamine derived from chitin (Mi et al., 2002). It was reported that chitosan is a potentially useful pharmaceutical material owing to its good biocompatibility and low toxicity (Nakatsuka and Andrady, 1992; Thacharodi and Rao, 1993). For drug delivery applications, chitosan needs to be crosslinked due to its hydrophilic property. Various crosslinking agents such as formaldehyde and glutaraldehyde have been used to prepare chitosan hydrogels (Illum, 1998; Risbud et al., 2000). In this work, we prepare and characterize a novel water-soluble chitosan derivative with nitrocinnamate as pendant groups. Different from the photocrosslinkable polysaccharide derivatives reported previously in the literature, this modified polysaccharide has a unique photogelation ability in the absence of potentially toxic photoinitiator or catalyst, which makes it to be suitable particularly for photoinduced formation of hydrogel biomaterials. It is known (Lewis et al., 1988) that cinnamate can undergo trans–cis

* Corresponding author: Tel.: +86 20 84112354; fax: +86 20 84112354.
E-mail address: ceszhlm@mail.sysu.edu.cn (L.-M. Zhang).

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isomerization and [2+2] cycloaddition upon UV irradiation at wavelengths longer than 290 nm. Such a photocrosslinking property has been broadly utilized in the field of photolithography and the semiconductor industry (Reiser, 1989). To our knowledge, however, the application of nitrocinnamate photoreactivity in the formation of polysaccharide-based hydrogels as well as the resultant hydrogel properties have rarely been explored.

2. Materials and methods

2.1. Materials

Chitosan (75–85% deacetylated; brookfield vicosity, 20–200 cp at 1% concentration in 1% acetic acid), lysozyme and *N*,*N*-dicyclohexylcarbodiimide (DCC) were purchased from Sigma. 4-Nitrocinnamate acid (NC) was supplied by Aladdin-reagent Inc. Doxorubicin hydrochloride was obtained from BioBasic Inc., USA. *N*-Hydroxyl succinimide (NHS) was obtained from Sinopharm Chemical Reagent Co., Ltd. All other chemicals were of analytical grade and were obtained from Aldrich.

2.2. Synthesis of water-soluble chitosan derivative (CMCS-NC) containing nitrocinnamate moiety and its characterization

A three-step reaction strategy shown in Scheme 1 was used to prepare water-soluble chitosan derivative (CMCS-NC) containing nitrocinnamate moiety. For this purpose, water-soluble carboxymethylated chitosan (CMCS) was firstly synthesized by the reaction of chitosan (10 g) and chloroacetic acid (25 g) in isopropyl alcohol/H_2O (volume ratio, 2:1) mixed solvent (150 mL) according to the procedure reported previously (Liang and Zhang, 2007), and its carboxymethyl substitution degree was determined to be 0.72 by potentiometric titration method (Riccardo et al., 1982). To obtain NHS-activated nitrocinnamate (NHS-NC), 4-nitrocinnamate acid (1.93 g) was mixed with NHS (1.20 g) and DCC (2.10 g) in *N*,*N*′-dimethylformamide (DMF, 35 mL) at 40 °C. The mixture was reacted for 24 h at room temperature. The precipitated dicyclohexyl urea was removed by filtration. The filtrate was precipitated by water, and the precipitate was recrystallized in DMF and ethanol to give yellowish NHS-NC powder (yield, 75.2%). The following ^{1}H NMR data (DMSO-d_6, ppm), which were obtained by a Varian Mercury-Plus (300 MHz) spectrometer, confirmed the formation of NHS-NC: 2.85 (s, 4H), 7.18 (d, 1H), 7.23 (d, 1H), 8.12 (d, 2H) and 8.26 (d, 2H).

For the conjugation of CMCS with NHS-NC, CMCS (0.4 g) was dissolved in aqueous sodium bicarbonate solution (50 mL, pH 8.3), and the resultant solution was diluted with DMF (40 mL) under stirring. After that, a DMF solution (10 mL) of NHS-NC (0.240 or 0.150 g) was added dropwise. The reaction was carried out for 72 h at room temperature in the dark. Upon reaction completion, the reaction mixture was poured into THF (150 mL). The resultant precipitate was separated from the solution by filtration. Reprecipitation was carried out several times in water–THF system until there was no free NHS-NC in the filtrate, which was detected by UV analysis. After the last precipitate was dried at 40 °C under vacuum, CMCS-NC was obtained. By changing the amount of NHS-NC, two CMCS-NC samples with various NC contents were prepared, namely CMCS-NC-1 (yield, 65.5%) for the sample obtained when 0.240 g NHS-NC was used, and CMCS-NC-2 (yield, 71.2%) for the sample obtained when 0.150 g NHS-NC was used. The degree of substitution (*DS*) of the introduced nitrocinnamate groups was expressed as the number of incorporated nitrocinnamate groups per 100 anhydroglucose units of CMCS, and was determined by the UV–vis spectra (TU-1901, Beijing Purkinje Co., China). As a result, the *DS* value was found to be 11.2 for CMCS-NC-1, and 1.9 for CMCS-NC-2, respectively. FTIR (Nexus 670, Nicolet Co., USA) and ^{1}H NMR analyses were used to confirm the conjugation reaction.

2.3. Photogelation of aqueous CMCS-NC solution and its characterization

Photogelation for aqueous solution (5.0%) of CMCS-NC sample (CMCS-NC-1 or CMC-NC-2, W_{solid}) was performed on a Teflon dish by photoirradiation, using a 200 W mercury UV lamp (365 nm) with an intensity of 0.9 mW/cm^2 (Philips UV lamp, Shanghai Yayuan Lighting Appliance Co., China) for a predetermined time (20, 30, 40, 50 or 60 min). The gelled sample obtained was immersed in distilled water at room temperature for 24 h to remove unreacted CMCS-NC and then weighed (W_{wet}) after the careful removal of excess water. The vacuum-dried hydrogel was weighed (W_{dry}). The gelation efficiency (*GE*) was calculated using the following equation:

$$GE(\%) = \frac{W_{dry}}{W_{solid}} \times 100 \quad (1)$$

The swelling degree (*SD*) was calculated as follows:

$$SD = \frac{W_{wet} - W_{dry}}{W_{dry}} \quad (2)$$

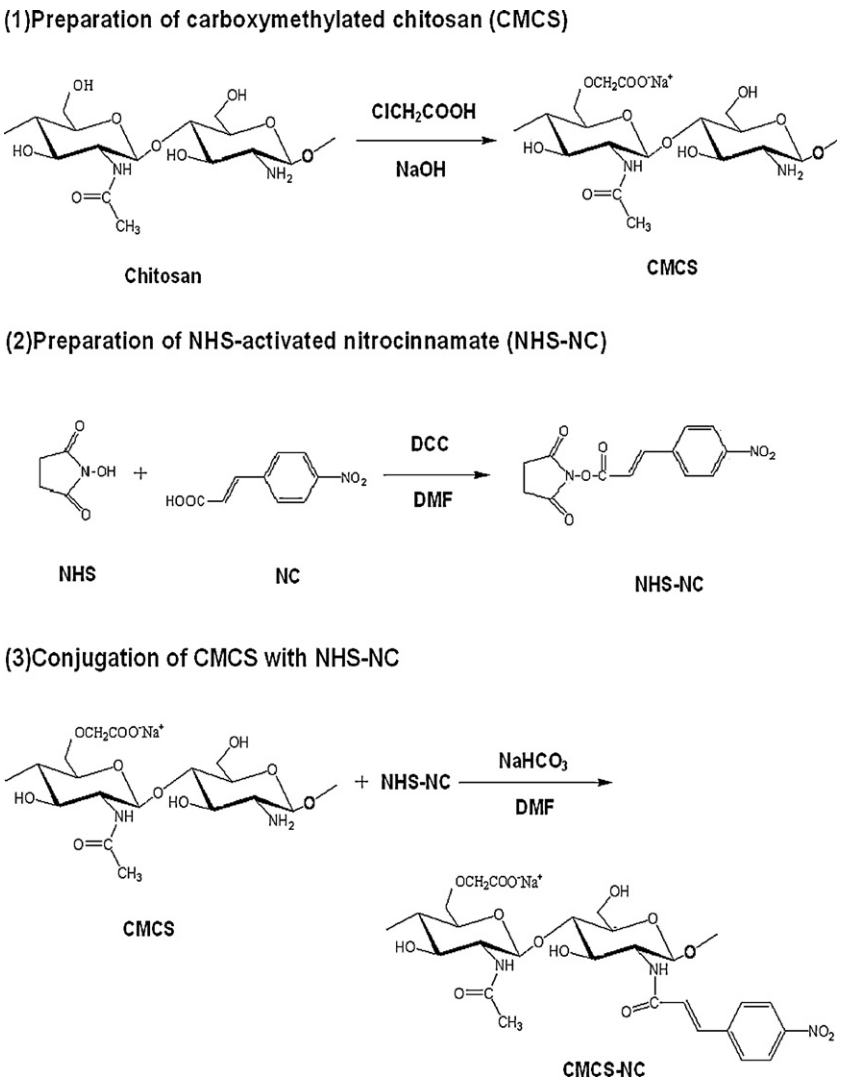
For aqueous CMCS-NC solution before and after photoirradiation, their rheological characterization was taken on an ARES rheometer (TA Co., USA). The storage and loss moduli were measured as a function of frequency under oscillatory shear at a strain of 0.5%, which was within the linear viscoelastic region. The UV/vis spectra of aqueous CMCS-NC solution during photoirradiation were investigated as a function of irradiation time (0–20 min). For the resultant hydrogels, their morphologies were observed by using a Hitachi S 4800 scanning electron microscope (Japan) with an accelerating voltage of 5 kV. The hydrogel samples were allowed to lyophilize and then coated with a thin layer of gold.

2.4. In vitro degradation of CMCS-NC hydrogels

In vitro degradation of CMCS-NC hydrogels was investigated according to the method reported by Amsden et al. (2007). For this purpose, the dried CMCS-NC hydrogels were incubated in a mixture of 4 mg/mL lysozyme from chicken egg white and 0.1% (w/v) sodium azide with a gentle shaking at 37 °C. The lysozyme mixture solution was changed every 10 days. Every 10 days, the hydrogel samples were taken out from the lysozyme solution, rinsed with deionized water, dried for 48 h and weighed to obtain the final weight after degradation. The extent of *in vitro* degradation was expressed as the percentage of the weight loss of the dried hydrogel after lysozyme treatment.

2.5. Drug loading by CMCS-NC hydrogel and in vitro release

For the *in situ* loading of doxorubicin (DOX) as the model drug, a known amount of doxorubicin was added to aqueous CMCS-NC solution according to the DOX/CMCS-NC mass ratio of 20 mg/g, and was mixed manually until homogeneous. After that, UV irradiation (365 nm) was conducted for 45 min to induce the gelation of mixed CMCS-NC/DOX solution. The formed hydrogel was soaked in 10 mL PBS solution pre-heated to 37 °C and incubated in water bath at 37 °C under mild shaking motion (50 rpm). PBS solutions were periodically renewed with fresh buffer. The amount of DOX released into the buffer was determined using UV–vis spectroscopy at a wave length of 481 nm and the total amount released was calculated from the established standard curve. All release studies were carried out in triplicate.



Scheme 1. A three-step reaction strategy for the preparation of water-soluble chitosan derivative (CMCS-NC) containing nitrocinnamate moiety.

3. Results and discussion

The water-soluble chitosan derivative (CMCS-NC) with photoreactivity was prepared in this study by the conjugation of carboxymethylated chitosan (CMCS) with NHS-activated nitrocinnamate (NHS-NC) in the presence of DCC, as illustrated in Scheme 1. To confirm the conjugation reaction, FTIR and ^{1}H NMR analyses were carried out for purified CMCS and it's conjugate (CMCS-NC-1). From the FTIR spectra shown in Fig. 1, it was found that the spectrum of the conjugate showed not only the characteristic stretching vibrations of CMCS at 3427 cm^{-1} (O–H stretch overlapped with N–H stretch), 2924 cm^{-1} (C–H stretch), 1602 and 1415 cm^{-1} (C=O stretch), 1325 cm^{-1} (C–N stretch) and 1070 cm^{-1} (C–O stretch) (Wang et al., 2007), but also the additional shoulder peaks at 1500 and 1400 cm^{-1}, which could be assigned to the characteristic absorptions of benzene ring in the incorporated NC groups. Moreover, the absorption peak of the conjugate at 1662 cm^{-1} become stronger, which could be attributed to the carbonyl stretch of secondary amides (amide I band). From the ^{1}H NMR spectra shown in Fig. 2, it was found that the spectrum of the conjugate showed not only the characteristic proton peaks of CMCS at 1.95 ppm (*CH*$_3$, acetyl group of of *N*-acetamidoglucose units), 2.60 ppm (*CH*, carbon 2 of *N*-unsubstituted glucosamine units), 3.1–4.0 ppm (*CH*, carbon 3, 4, 5 and 6 of glucosamine units) and 4.35 ppm (–*CH*$_2$COOH, car-

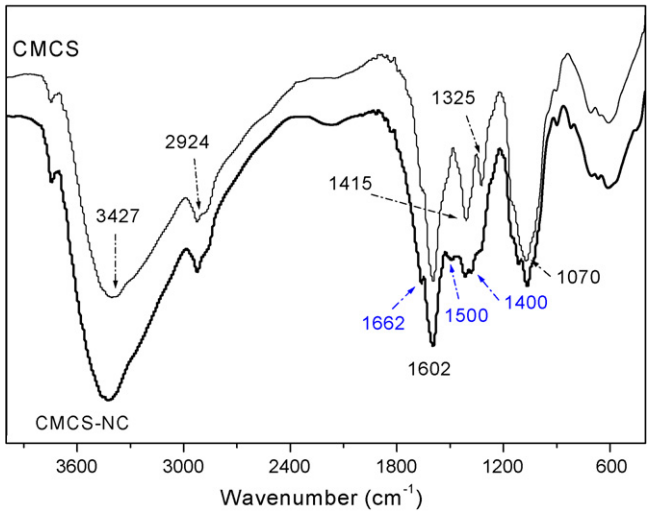


Fig. 1. IR spectra of CMCS and its conjugate (CMCS-NC-1) sample (KBr pellets).

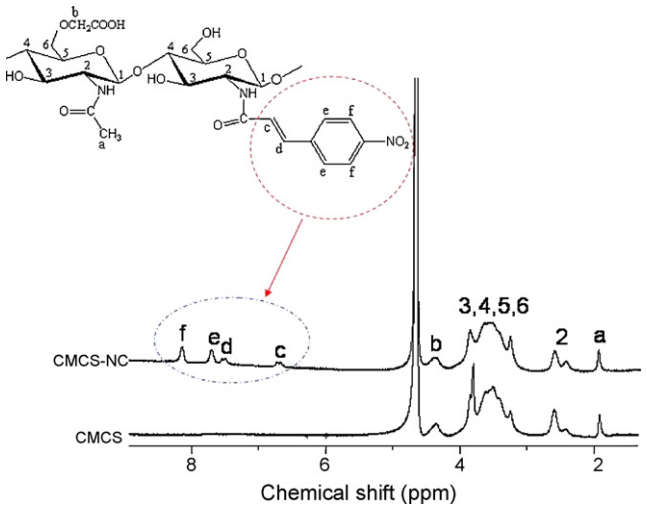


Fig. 2. ^{1}H NMR of CMCS and its conjugate (CMCS-NC-1) (D_2O, 25 °C).

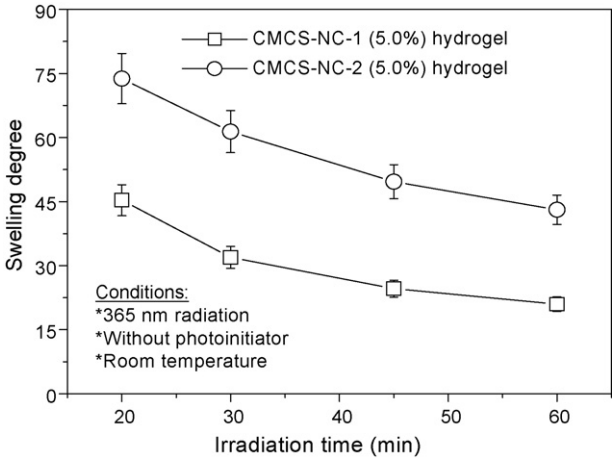


Fig. 4. The swelling degree as a function of irradiation time for the hydrogels formed from 5.0% aqueous solutions of CMCS-NC samples upon exposure to 365 nm radiation in the absence of photoinitiator.

boxymethyl group) (Wang et al., 2007), but also the additional proton peaks of incorporated NC groups at 6.72 ppm (CH=C*H*–CO), 7.53 ppm (Ar–*CH*=CH), 7.71 and 8.14 ppm (*H*–Ar). These results confirmed the formation of an amide linkage between the primary amino groups of CMCS and the carboxyl groups of NHS-NC.

Fig. 3 shows the gelation efficiency (*GE*) as a function of irradiation time for aqueous solutions of CMCS-NC samples upon exposure to 365 nm radiation in the absence of photoinitiator. With the increase of irradiation time, the *GE* was found to have an obvious improvement. When the irradiation time increased from 20 to 60 min, for example, the *GE* of aqueous CMCS-NC-1 solution increased from 29.6 ± 1.4% to 78.5 ± 3.9%, and the *GE* of aqueous CMCS-NC-2 solution increased from 9.1 ± 0.5% to 35.0 ± 1.7%. This implies that an increase of the irradiation time will be favorable for the photogelation. In comparison with 5.0% aqueous solution of CMCS-NC-2 incorporated with a low nitrocinnamate (NC) content (*DS* = 1.9), 5.0% aqueous solution of CMCS-NC-1 incorporated with a high NC content (*DS* = 11.2) has a higher *GE* value, indicating the contribution of the incorporated NC groups to the UV-induced gelation. Fig. 4 gives the swelling degree (*SD*) as a function of irradiation time for the hydrogels formed from 5.0% aqueous solutions of CMCS-NC samples upon exposure to 365 nm radiation in the absence of photoinitiator. On prolonging the irradiation time, the *SD* was found to have an obvious decrease irrespective of incorporated NC content. In contrast, CMCS-NC-1 hydrogel with a high NC content has a lower *SD* than CMCS-NC-2 hydrogel with a low NC content. It is clear that the irradiation time and incorporated NC content affect the network structure of the resultant hydrogel. A longer irradiation time or higher NC content would result in the formation of a denser hydrogel network with a lower swelling degree.

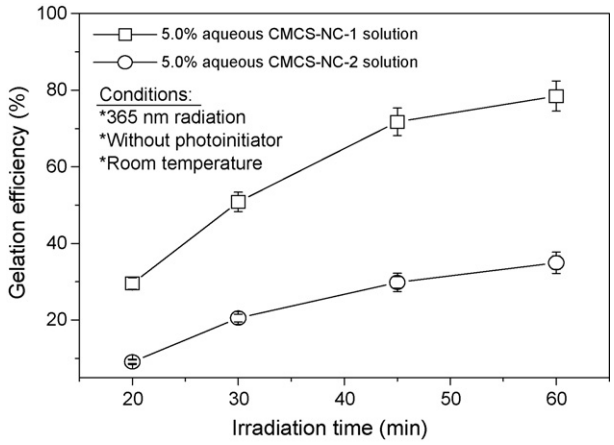


Fig. 3. The gelation efficiency as a function of irradiation time for 5.0% aqueous solutions of CMCS-NC samples upon exposure to 365 nm radiation in the absence of photoinitiator.

This gelation phenomenon of aqueous CMCS-NC solution upon exposure to 365 nm irradiation in the absence of photoinitiator may be caused by the intermolecular cycloaddition formation (crosslinking) of the incorporated NC groups under the photoirradiation and subsequent network structure formation, as shown in Fig. 5. To confirm this hypothesis, UV–vis absorbance of aqueous CMCS-NC solution during UV irradiation was determined, as indicated in Fig. 6. Before UV light irradiation (irradiation time = 0), aqueous CMCS-NC solution exhibits an absorption maximum at 314 nm due to the incorporated NC groups. Upon exposure to the near-visible–UV light (365 nm), the absorbance peak at 314 nm underwent a hypsochromic shift and a decrease in its intensity. Within 20 s of irradiation, this maximum absorbance peak decreased its intensity by approximately 16% and blue-shifted about 5 nm. Although at a slower rate, this trend continued with irradiation time. A similar response has been reported for other cinnamate derivatives when incorporated into polymeric materials, and it was found to be the result of cis–trans isomerization and photodimerization reactions (Lee et al., 2003; Yuan et al., 2005). In this case, the NC moieties were covalently bonded to adjacent NC moieties by forming the cyclobutane ring via the photocycloaddition reaction, which could act as crosslinking points and result in the formation of hydrogel network structure. Obviously, a greater number of crosslinking points, which may be induced by a higher NC content or a longer irradiation time, would lead to a higher *GE* and make the photocrosslinked hydrogel to have a lower *SD* value.

Further work was dealt with the rheological characterization of aqueous CMCS-NC system before and after photoirradiation. Fig. 7 gives the angular frequency dependence of storage modulus (G') and loss modulus (G'') for 5.0% aqueous CMCS-NC-1 solution before and after UV irradiation for 30 min in the absence of photoinitiator and corresponding optical photos for their flow states. As seen, the system before the irradiation was in sol state, and showed the viscoelastic behavior with dominating viscous property. In particular, the G' and G'' values were too small to be measured accurately in the low frequency region. In contrast, the system after the irradiation was in gel state, and the G' value was observed to be considerably greater than the G'' value over the entire range of frequency.

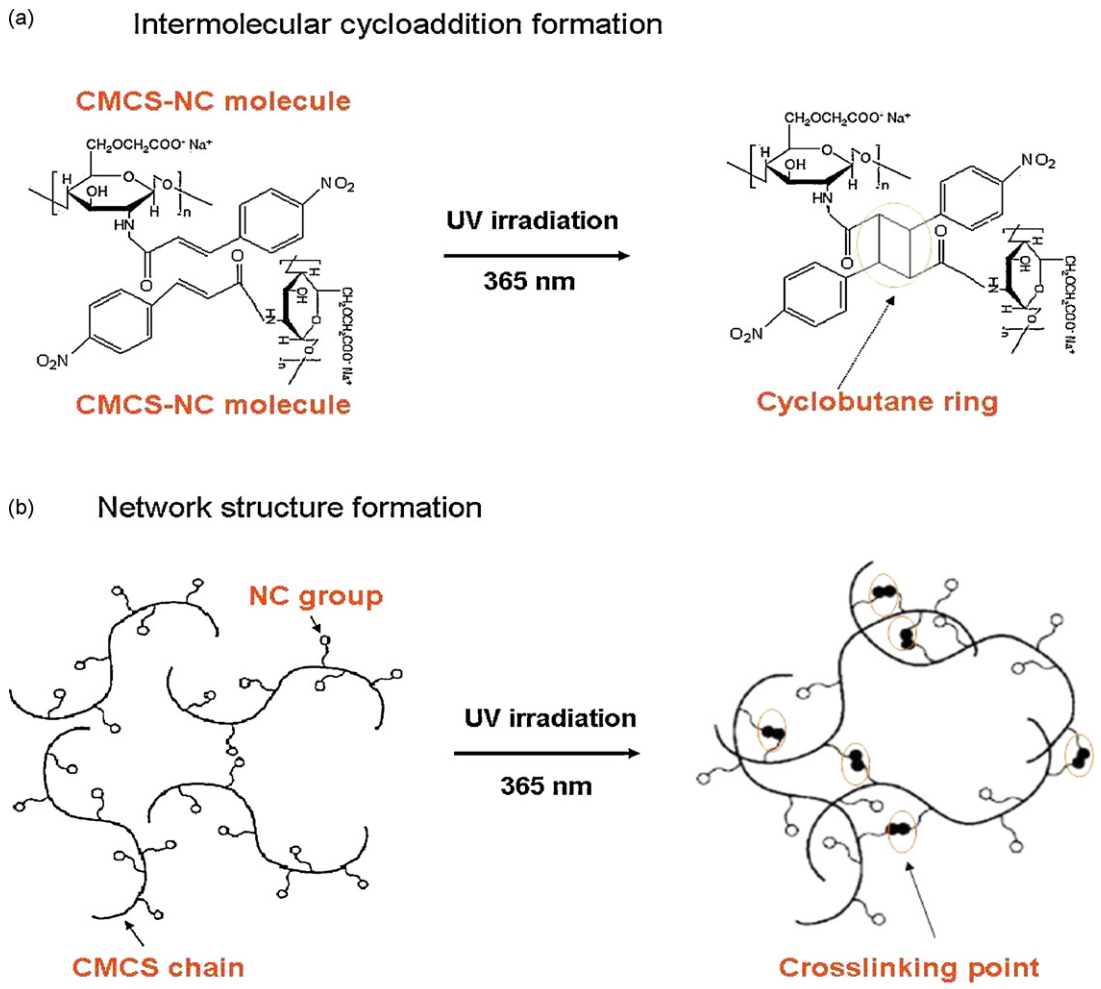


Fig. 5. A possible mechanism for the photogelation of aqueous CMCS-NC solution.

Moreover, the G' value is fairly constant throughout the entire frequency region, although a slight increase is observed with the increase of frequency. These phenomena indicate that the system after the irradiation displays a predominantly solid like behavior, which can be attributed to the UV-induced crosslinking. In addition, the incorporated NC content has a great influence on the mechanical strength of the resultant CMCS-NC hydrogel. We investigated the storage modulus as a function of frequency for two CMCS-NC hydrogels incorporated with various NC contents (Fig. 8), and found that CMCS-NC-1 hydrogel with a high NC content had a higher G' value in the entire frequency region when compared to CMCS-NC-2 hydrogel with a low NC content. This may be due to the formation of a denser network structure in CMCS-NC-1 hydrogel. We carried out the SEM observation for the structural morphology of two CMCS-NC hydrogels and confirmed such a deduction (Fig. 9).

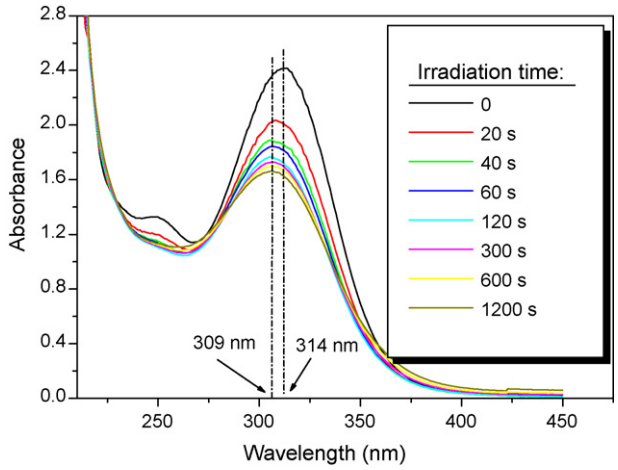


Fig. 6. UV–vis absorbance of aqueous CMCS-NC solution with a concentration of 0.24 mg/mL during UV irradiation at $\lambda = 365$ nm (irradiation time, 0–1200 s).

For the resultant CMCS-NC-1 and CMCS-NC-2 hydrogels, their *in vitro* degradation was evaluated in 4 mg/mL lysozyme solution of pH 7.4 at 37 °C, as shown in Fig. 10. In general, natural polysaccharides including chitosan and chitin are degraded by enzymatic hydrolysis (Amano and Ito, 1978; Pangburn et al., 1982). In this study, we found that two chitosan-based hydrogels had also a biodegradation property. As seen, both of two hydrogels lost weight during the degradation period. In contrast, CMCS-NC-2 hydrogel incorporated with a low nitrocinnamate content had a faster degradation rate due to its looser network structure (Fig. 9). In this case, a lower crosslink density of CMCS-NC-2 hydrogel allows for more ready access of the enzyme to the chitosan backbone. Moreover, the penetration of the enzyme into the hydrogel bulk would increase as the crosslink density decreased. Both of these effects would increase the rate of biodegradation.

To explore their drug delivery application, two CMCS-NC hydrogels loaded with doxorubicin hydrochloride (DOX, an anticancer drug) during their formation were investigated for the *in vitro* drug

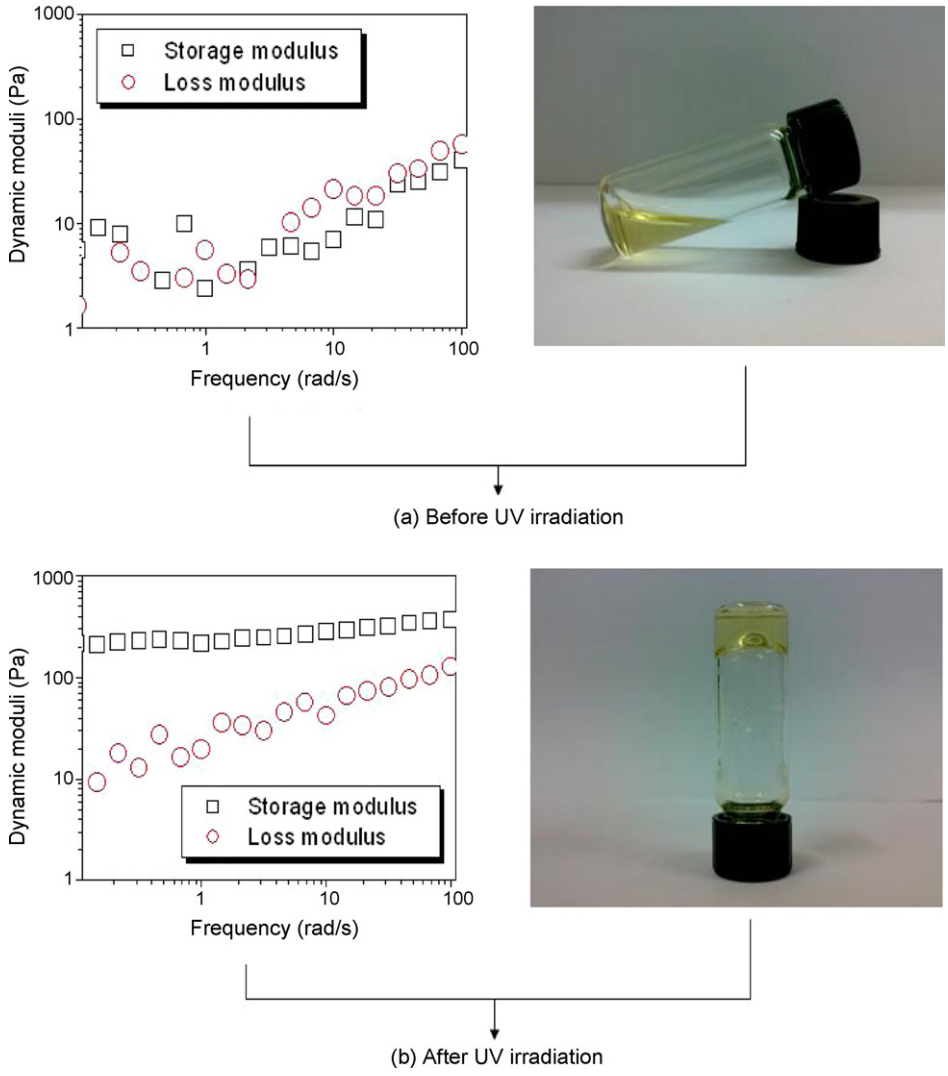


Fig. 7. Dynamic storage and loss moduli of 5.0% aqueous CMCS-NC-1 solution before and after UV irradiation (365 nm, 30 min, room temperature) in the absence of photoinitiator and corresponding optical photos for their sol and gel states.

release behavior. Fig. 11 gives the cumulative DOX release against time for each hydrogel sample in a pH 7.4 phosphate-buffered saline at 37 °C. As seen, there is a sustained release behavior in these cases. For example, CMCS-NC-1 hydrogel released only about 30.5% of the loaded DOX in the first 5 h and another 17.1% in 20 h, while CMCS-NC-2 hydrogel released only about 35.4% of the loaded DOX in the first 5 h and another 25.2% in 20 h. In contrast, the DOX-loaded CMCS-NC-1 hydrogel has a slower release rate due to the effect of its higher crosslink density on the drug diffusion. The relatively low amount of DOX released from two CMCS-NC hydrogels was probably related to the stronger interactions between the hydrogel matrix and DOX. To understand the release mechanism of the entrapped DOX molecules from the CMCS-NC hydrogels, we fitted the release curves using the following semi-empirical equation (Korsmeyer et al., 1983):

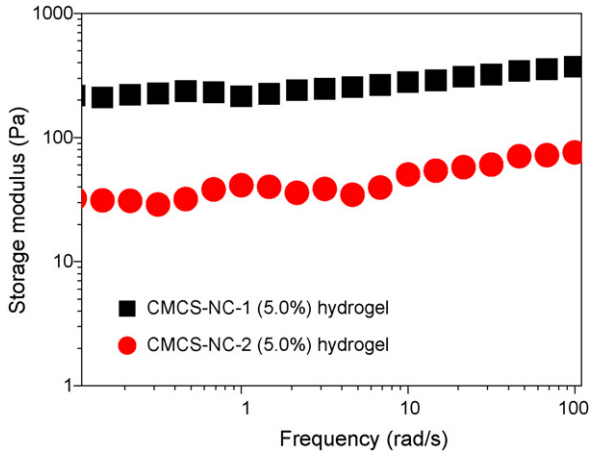


Fig. 8. Storage modulus as a function of frequency for two CMCS-NC hydrogels incorporated with various NC contents.

$$\frac{M_t}{M_\infty} = kt^n \tag{3}$$

where k is the kinetic constant and n is an exponent characterizing the diffusional mechanism, M_t and M_∞ are the cumulative amount of the drug released at t and equilibium, respectively. Only in two cases of $n = 0.5$ (pure diffusion controlled drug release) and $n = 1$ (swelling-controlled drug release or Case II transport), Eq. (3) becomes physically realistic. Other values for n indicate anomalous transport kinetics (Ritger and Peppas, 1987). In this study, the n value was obtained to be 0.45 ± 0.02 with the determination coefficient (R) of 0.986 for the DOX-loaded CMCS-NC-1 hydrogel, and 0.53 ± 0.03 with the determination coefficient (R) of 0.988

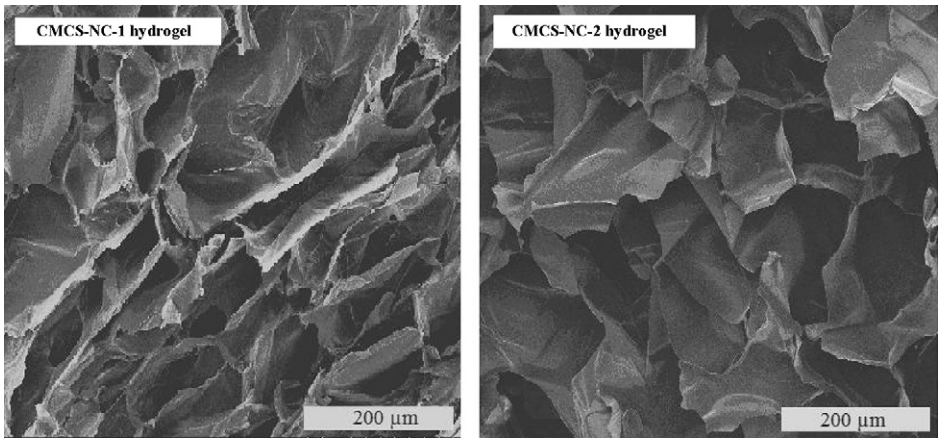


Fig. 9. SEM photos of two CMCS-NC hydrogels incorporated with various NC contents.

for the DOX-loaded CMCS-NC-1 hydrogel, respectively. It seems that the diffusion behavior of the entrapped DOX from the photocrosslinked CMCS-NC hydrogels belongs to anomalous transport kinetics.

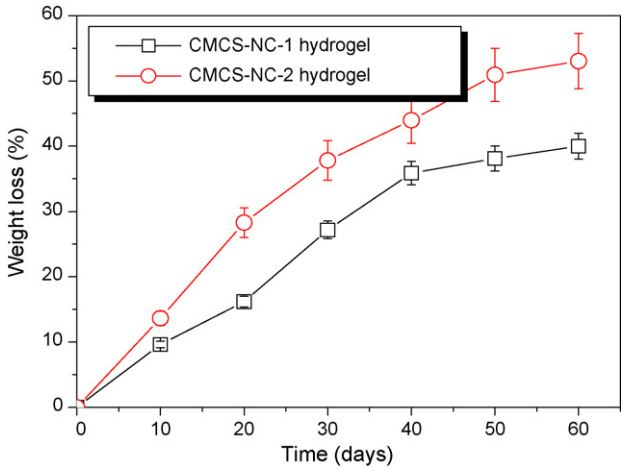


Fig. 10. *In vitro* biodegradation of two CMCS-NC hydrogels incorporated with various NC contents in 4 mg/mL lysozyme solution of pH 7.4 at 37 °C.

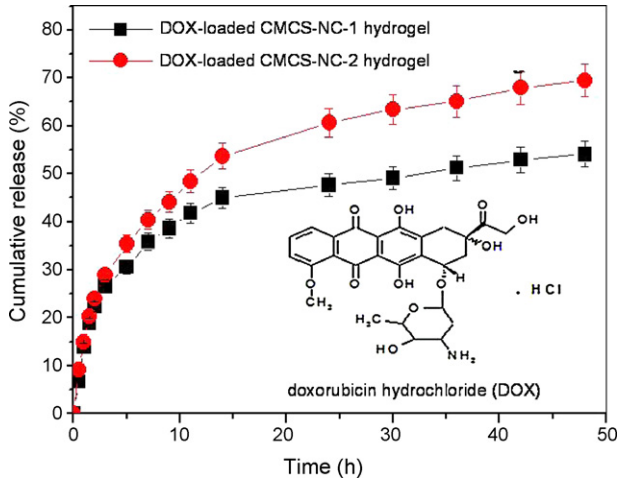


Fig. 11. *In vitro* release profiles of doxorubicin hydrochloride (DOX) from the photocrosslinked CMCS-NC hydrogels incorporated with various NC contents in a pH 7.4 phosphate-buffered saline at 37 °C.

4. Conclusion

In this study, chitosan was hydrophilically modified by carboxymethylation to increase its water solubility, and then used for the conjugation with 4-nitrocinnamate acid. This preparation process results in a novel polysaccharide derivative capable of UV photogelation in the absence of photoinitiator or catalyst. The unique gelation ability of this modified polysaccharide could be attributed to the intermolecular cycloaddition formation of the incorporated nitrocinnamate groups and subsequent network structure formation. It was found that the photoirradiation time and incorporated nitrocinnamate content affected greatly the gelation efficiency of the modified polysaccharide and the swelling degree of the resultant hydrogel in water. Moreover, a relatively high nitrocinnamate content could make the photocrosslinked hydrogel to have a denser network structure, a greater mechanical strength, a slower biodegradation and drug release rate.

Acknowledgments

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