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Preparation and characterization of double crosslinked hydrogel films from carboxymethylchitosan and carboxymethylcellulose

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ABSTRACT

A novel crosslinked hydrogel film was prepared from carboxymethylchitosan (CMCS) and carboxymethylcellulose (CMC) by ionical and covalent crosslinking with $CaSO_4$ and genipin, respectively. The swelling ratio of the crosslinked CMCS/CMC hydrogel films was investigated at different pH solutions (1–9), and the results indicated that the crosslinked hydrogels had the swelling–deswelling properties with two primary peaks of swelling ratio at pH 3 and 7. The surface morphologies of the crosslinked hydrogels at different pH values provided evidences of the swelling–deswelling properties. The mechanical properties of the hydrogel films were also examined. The ionical and covalent crosslinking were found to have the primary impact on the toughness and max load, respectively, of the crosslinked hydrogels. The cells comparatively cultured on the crosslinked hydrogels and the negative and positive controls suggested the biocompatibility of the crosslinked CMCS/CMC films. This kind of hydrogel films have potential application in drug delivery vehicles and skin tissue engineering.

1. Introduction

Hydrogels are polymers in three-dimensional network arrangement with the capability to absorb and retain large amount of water (Bakass, Mokhlisse, & Lallemant, 2002; Jao, Chen, Lin, & Yang, 2009; Peppas, Bures, Leobandung, & Ichikawa, 2000). The physically or chemically crosslinked polymer chains are usually crucial to keep the spatial structure of hydrogels (Hennink & van Nostrum, 2002; Sangeetha & Maitra, 2005). In recent decades, hydrogels based on natural polymers such as cellulose, chitosan, starch, alginate, dextrin, etc, and their derivatives have attracted much attention and shown great potential application in many fields including agriculture, medicine, pharmacy and biotechnology (Agnihotri, Mallikarjuna, & Aminabhavi, 2004; Gombotz & Wee, 1998; Nguyen & West, 2002)

Carboxymethylchitosan (CMCS) and carboxymethylcellulose (CMC), two natural polyelectrolytes derived from cellulose and chitosan by introducing carboxyl methyl group (–CH_2COOH), respectively, have attracted considerable interests in a wide range of biomedical applications (Chen, Du, Wu, & Xiao, 2002; Chen et al., 2004; Lee & Chiang, 2004). Due to the unique biodegradability, biocompatibility, good film-forming property and pH sensitivity, they have been found potential applications as wound dressings, and artificial bone and skin (Chen et al., 2004; Muzzarelli, 1988; Thanou, NihotM, Jansen, Verhoef, & Junginger, 2001). Unfortunately, the application of these bio-based hydrogels was significantly hindered by the limited mechanical strength (Hoare & Kohane, 2008).

In recent years, considerable efforts have been focused on the development of novel methods to obtain hydrogels with extremely high mechanical strength. The interpenetrating polymer network (IPN) technology, which can be achieved by double crosslinking route, is one of the possible strategies to enhance the mechanical properties (Gong, Katsuyama, Kurokawa, & Osada, 2003; Zhang, Zha, Zhou, Ma, & Liang, 2005a, 2005b). Highly stretchable and tough hydrogel was prepared through combining chemical and physical crosslinking methods (Sun et al., 2012). This hydrogel could be stretched beyond 20 times their initial length and had fracture energies of 9000 J/m^2, which was almost comparable with 10,000 J/m^2 for natural rubbers (Lake, 1995) and higher than 1000 J/m^2 for cartilage (Simha, Carlson, & Lewis, 2004), suggesting a great potential for future application. Considering the possible application as biomaterials, the biotoxicity is a very important factor for hydrogel. Therefore, it is significantly necessary to use green chemical process to prepare hydrogel materials.

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Unfortunately, it is difficult for the traditional direct crosslinking or graft-polymerization to ensure the bio-nontoxicity of the final product.

Genipin, derived from an iridoid glycoside called geniposide present in fruit of Gardenia jasminoides (Akao, Kobashi, & Aburada, 1994), is an excellent natural crosslinker for proteins, collagen, gelatin, and chitosan (Muzzarelli, 2009). It has a low acute toxicity, much less (5000–10,000 times) toxic than glutaraldehyde and other commonly used synthetic crosslinking reagents. Therefore, genipin can be widely used in medical fields especially for biological tissue repair, and regulating agent for drug delivery (Sung, Chang, Chiu, Chen, & Liang, 1999; Tsai, Huang, Sung, & Liang, 2000). In the present study, a novel CMCS/CMC hydrogel film was prepared by combining two types of crosslinking method, ionically by $CaSO_4$ and covalently by genipin. The properties of the prepared hydrogel films were characterized for the possible application.

2. Materials and methods

2.1. Materials

CMC (M_w, 70–100 kDa; DS, 0.9; viscosity, 2500–4500 mPa s) and $CaSO_4$ were purchased from Aladdin Reagent Inc. (Shanghai, China). CMCS (M_w, 100 kDa; DS, 0.9; degree of deacetylation (DD), ≥90%) was obtained from Hefei Bomei Biotechnology Co., Ltd. (Anhui, China). Genipin was bought from Fuzhou Linchuan Zhixin Biotechnology Co., Ltd. (Fujian, China). All chemicals used in this study were of analytical grade and used as received. Phosphate buffer solution (PBS) with different pH was prepared using NaH_2PO_4 and Na_2HPO_4.

2.2. Preparation of CMCS/CMC hydrogel film

A series of CMCS/CMC hydrogel films were prepared with genipin and $CaSO_4$ as dual crosslinkers under the conditions listed in Table 1. Accurately weighed CMCS (0.3000 g) was firstly added into 20 mL purified water under magnetic stirring with the rotation speed of 500 rpm at room temperature to obtain a clear solution. Then CMC (0.3 g) was slowly added portionwise into CMCS solution, and the mixture was further stirred for 3 h to ensure the homogeneity of the system. Genipin, previously dissolved in 10 mL purified water, was added dropwise with agitation into the obtained slightly yellow homogeneous solution. The required amount of $CaSO_4$, based on the total polyelectrolytes CMCS and CMC, was also added portionwise. The mixture was irradiated for 10 min with ultrasound at sonic power of 100 W on a 5 L Ultrasonic Cleaning Bath (Newpower, Guangzhou, China) to ensure the homogeneity of CMCS/CMC system. The crosslinking reaction was incubated for 12 h at room temperature. The resulted blue hydrogel solution was irradiated with ultrasound at 100 W for 15 min to degas and obtain a uniform sample, and then casted into a polytetrafluoroethylene (PTFE) mold (diameter 90 mm, depth 8 mm). After drying in blast oven for 6–8 h at 50 °C, the CMCS/CMC crosslinked hydrogel film was obtained. The blank sample was prepared from CMCS and CMC under the same condition without the addition of Genipin and $CaSO_4$. All of the films were kept away from moisture and heat before use.

2.3. Attenuated Total Reflectance (ATR)-FTIR spectra

The film samples were cut into pieces (10 mm × 10 mm) and dried in infrared drying oven. The ATR-FTIR spectra of the hydrogel films were recorded on an FT-IR spectrophotometer (Nicolet 750, Florida, USA) using ATR technique by averaging the signals of 32 scans at a resolution of 4 cm^{-1} in the range from 4000 to 400 cm^{-1}.

2.4. Swelling study

The CMCS/CMC crosslinked films were cut into small pieces (30 mm × 15 mm) and immersed in different pH (1–9) solutions (HCl solution and PBS solution) with ionic strength 0.1 M promoted with NaCl at room temperature. The swelling equilibrium of the crosslinked films could be reached within 6 h. The equilibrate films were removed from the solutions after 6 h, and the surface water on the swollen films was carefully blotted with filter paper without applying pressure. The swollen hydrogel films were weighted and the swelling ratio (g/g) was measured by the following equation (Kumar et al., 2010).

$$\text{Swelling ratio (g/g)} = \frac{(W_s - W_d)}{W_d}$$

where W_s and W_d are the weights of the swollen and the dried hydrogels, respectively.

2.5. Scanning Electron Microscope (SEM)

To evaluate the surface characteristics, the CMCS/CMC films were swollen, freeze-dried and sputter-coated with a thin gold layer before measurements. The surface morphology was examined by a SEM (LEO 1530VP, LEO, Germany) with an accelerating voltage of 10 kV.

2.6. Mechanical properties and statistical analyses

Mechanical properties including toughness, max load, elastic modulus, and fracture energy were determined using an Instron Universal Testing Machine 5565 fitted with a 100 N load cell. The film samples were cut into rectangular shape with a length of 70 mm and a width of 15 mm, and clipped by two clamps on the testing machine for the determination of mechanical properties. The initial distance between the clamps was 30 mm, and the clamps were separated with the rate of 5 mm/min. The elastic modulus was calculated from the slope of the initial linear portion of the stress–strain curves within the range of elastic limit of stretching. The toughness was estimated from the area under the stress–strain curves until rupture. The fracture energy (Γ) was calculated by the following equation (Sun et al., 2012).

$$\Gamma = \frac{U(L_c)}{(a_0 \times b_0)}$$

where $U(L_c)$ is the area beneath the stress–strain curves giving the work done until rupture at the critical distance L_c between the clamps, and a_0 and b_0 are width and thickness of samples clipped by clamps, respectively.

The data were expressed as the mean standard deviation (SD) of the means. Analysis of variance was performed by LSD-test using SPSS 19.0 for the comparison of max load and toughness between the crosslinked samples and the blank sample or between the crosslinked samples. The data were considered to be significant differences at $p < 0.05$.

2.7. Biocompatibility

Direct contact tests were performed to examine the biocompatibility of the crosslinked films according to ASTM F-813-83 (ASTM, 1983) and ISO 10993-5 (ISO/EN, 1992). The hydrogel film samples (C1, C2, C3 and C4) were sterilized by keeping them in a 70% ethanol/sterile water solution for 48 h. The sterilized film samples were placed in a 24-well plate, immersed in culture medium Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD, USA) with 10% fetal calf serum (FCS)(Invitrogen, Karlsruhe, Germany) and kept at 37 °C for 24 h. After the required time, the

Table 1
Mechanical properties of the double crosslinked CMCS/CMC film samples.

Entry	Genipin concent. (mM)	$CaSO_4$ concent.[a] (%)	Toughness (MJ/m^3)	Max load (N)	Elastic modulus (GPa)	Fracture Energy ($\times 10^6$ J/m^2)
Blank	0	0	3.5 ± 0.1	25.7 ± 2	0.93 ± 0.10	0.3 ± 0.05
A1	0.25	0	4.1 ± 0.3	36.6 ± 3	1.07 ± 0.10	0.9 ± 0.08
A2	0.25	2.5	13.5 ± 1.0	41.3 ± 2	0.87 ± 0.08	0.7 ± 0.07
A3	0.25	5	15.1 ± 1.0	36.9 ± 2	1.52 ± 0.15	1.7 ± 0.06
A4	0.25	10	6.9 ± 0.7	45.7 ± 3	0.86 ± 0.10	0.5 ± 0.08
B1	0.5	0	10.7 ± 0.9	40.9 ± 4	0.41 ± 0.05	0.4 ± 0.06
B2	0.5	2.5	12.3 ± 1.0	40.1 ± 2	4.26 ± 0.50	4.0 ± 0.08
B3	0.5	5	14.8 ± 1.5	49.7 ± 4	3.21 ± 0.50	3.0 ± 0.09
B4	0.5	10	5.7 ± 0.5	47.9 ± 3	1.09 ± 0.10	0.8 ± 0.10
C1	0.75	0	5.8 ± 0.5	43.5 ± 4	0.62 ± 0.05	0.6 ± 0.10
C2	0.75	2.5	6.9 ± 0.7	45.9 ± 3	0.61 ± 0.05	0.6 ± 0.09
C3	0.75	5	10.7 ± 1.0	57.1 ± 3	0.39 ± 0.08	0.3 ± 0.09
C4	0.75	10	8.4 ± 0.7	55.9 ± 2	0.65 ± 0.10	0.5 ± 0.08
D1	1.0	0	10.3 ± 1.0	53.3 ± 2	0.56 ± 0.05	0.5 ± 0.10
D2	1.0	2.5	12.3 ± 2.0	58.7 ± 2	0.51 ± 0.07	0.4 ± 0.02
D3	1.0	5	14.8 ± 2.0	59.5 ± 2	0.51 ± 0.08	0.4 ± 0.05
D4	1.0	10	8.8 ± 1.0	61.6 ± 3	0.81 ± 0.10	0.7 ± 0.05

[a] Weight ratio of $CaSO_4$ to the total polyelectrolytes CMCS and CMC.

culture medium was removed and a suspension of NIH 3T3 (Mouse embryonic fibroblast cell line) (Sigma-93061524, Sigma–Aldrich, USA) cells, a fibroblast cell line, in culture medium with 10% of FCS (5×10^5 cells/mL) was added to the films. Cells inoculated on glass coverslips which have been put in the wells were as positive control, and the cells directly inoculated to the wells were as negative control. The plate was then incubated at 37 °C for 48 h. After this period, the cells were examined by light microscopy (EVOS, AMG, USA).

3. Results and discussion

3.1. ATR-FTIR analysis

In the present study, ATR-FTIR spectroscopy was used to characterize the chemical structure of the crosslinked hydrogel films to confirm the crosslinking of CMC/CMCS with genipin. Fig. 1 shows the ATR-FTIR spectra of the blank sample and the CMCS/CMC films crosslinked with different genipin concentration. The overlapped bands at 1583 cm^{-1} are associated with the asymmetric stretching of carboxylic anions COO^- and the stretching of amino group. That at 1410 cm^{-1} is originated from symmetric stretching vibration of carboxylic anions. The absorbance at 1055 cm^{-1} relates to C–O–C pyranose ring vibration in CMCS and CMC (Yang, Yan, Chen, Lee, & Zheng, 2007). In comparison with the blank sample, the crosslinked CMCS/CMC samples possessed two new small peaks at 1731 and 1660 cm^{-1}. The former is originated from the stretching of carbonyl in ester bond formed after genipin crosslinking, and the latter arises from the absorption of heterocyclic amine. The presence of these two small absorbances confirmed the attachment of groups containing heterocyclic amine and ester bond onto CMCS/CMC hydrogels, indicating the occurrence of genipin crosslinking with active amino group on CMCS. According to the literature (Mi, 2005; Mi, Sung, & Shyu, 2000), the presumed crosslinking mechanism between genipin and CMCS is illustrated in Scheme 1. As a nucleophilic reagent, genipin initially reacted with amino groups on CMCS via a nucleophilic attack to form the heterocyclic amino compound, the intermediate **1**. After the elimination of hydroxyl group, the intermediate **2** was formed. The two intermediate heterocyclic amines of genipin derivatives further covalently conjugated to dimmers **3** and **4**, via the reaction of intermediate **1** with **2** and both intermediate **2**, respectively. After crosslinking with genipin, the amino groups on CMCS were partly converted into heterocyclic amines. Genipin was reported to produce blue pigments upon spontaneous reaction with methylamine, and more importantly, only primary amines could react with genipin rather than secondary or tertiary amines (Touyama et al., 1994). As shown in the insert picture in Scheme 1, the colorless transparent solution of CMCS/CMC and genipin became light or even dark blue with the increase of reaction time, which was probably due to the reaction of genipin with CMCS.

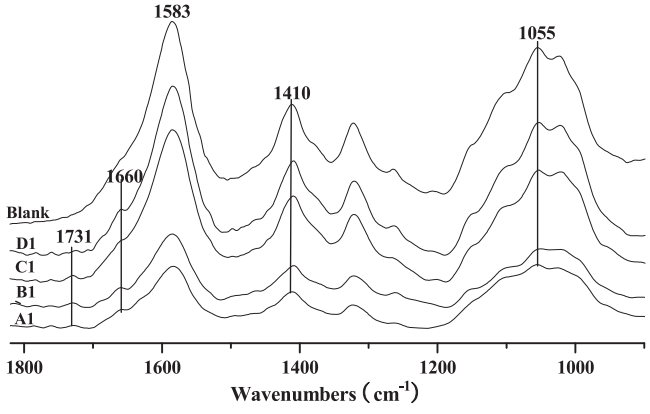
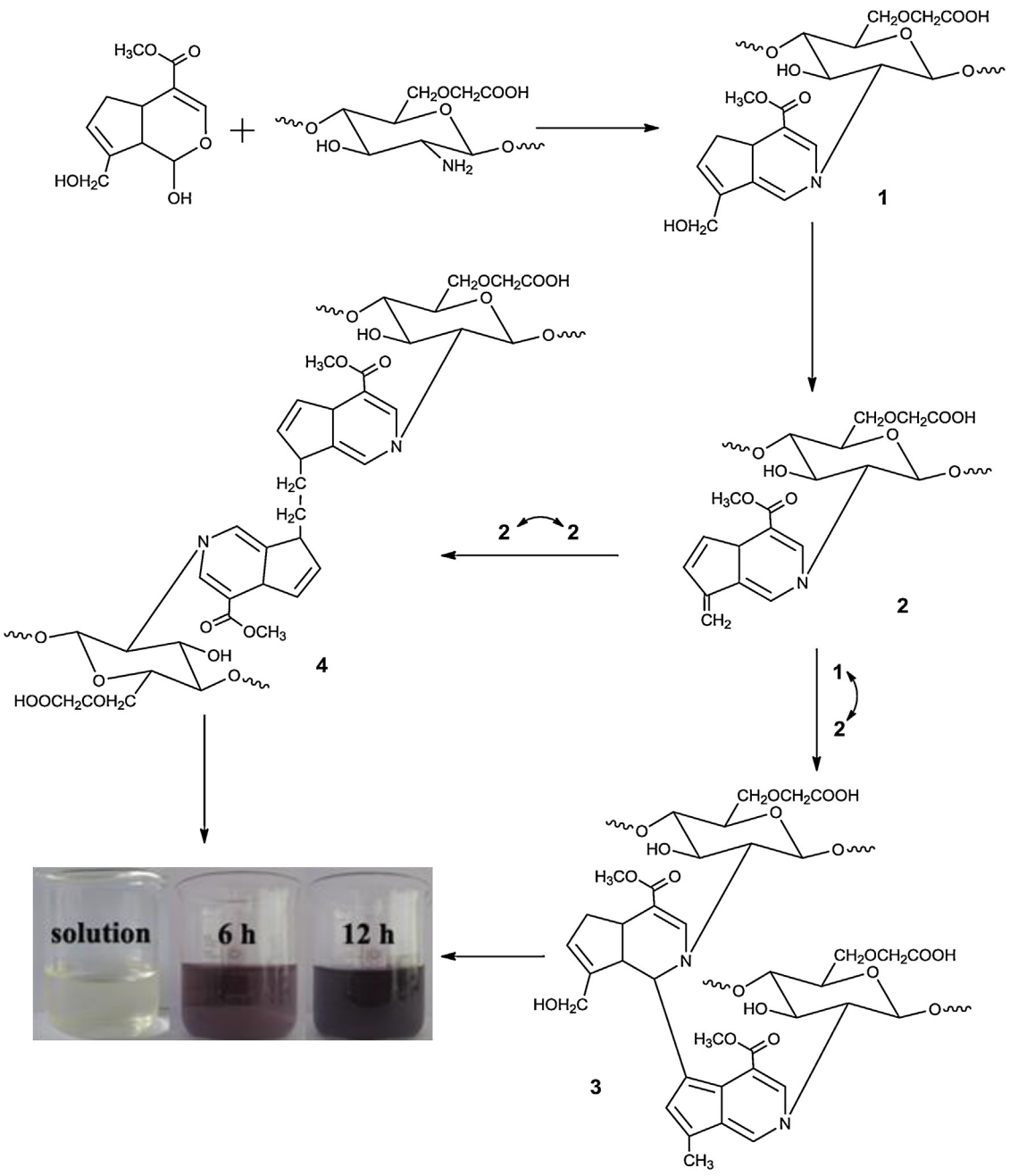


Fig. 1. ATR-FTIR spectra of the blank sample and the crosslinked CMCS/CMC samples A1, B1, C1 and D1.

3.2. Swelling study

Swelling ratio of the double crosslinked hydrogels was investigated in different solutions with ionic strength 0.1 M at pH in the range of 1–9. The effect of pH on the swelling ratio is depicted in Fig. 2. The main potential interacting species affecting the swelling ratio of the double crosslinked hydrogels in acidic or basic media include carboxylate anions (COO^-, pK_a 4.6) and amino group (NH_2, pK_b 6.5) (Pourjavadi, Mahdavinia, & Zohuriaan-Mehr, 2003). Therefore, the swelling behaviors of the CMCS/CMC hydrogel films depended on the different changes of COO^- and NH_2.

At pH 1, COO^- and NH_2 on CMCS and CMC are protonated to carboxylic acid (COOH) and the ammonium cations (NH_3^+), respectively, resulting in the NH_3^+–NH_3^+ electrostatic repulsion as the primary interaction affecting on the swelling ratio. However, the charge of NH_3^+ is shielded by the Cl^- screening effect and an efficient repulsion is prevented, which leads to a remark decrease in swelling ratio, as shown in Fig. 2. Increasing pH to 3, the NH_3^+–NH_3^+ electrostatic repulsion is intensive and the hydrogel network is efficiently swollen, resulting in the high swelling ratio compared with



Scheme 1. Crosslinking mechanism of CMCS with genipin.

that at pH 1. However, due to the pK_a of carboxylic groups 4.6 and the pK_b of amino groups 6.5, further improving pH to 5, carboxylic acid comes into action. NH_3^+ begins to deprotonate to form NH_2, and COOH starts to deprotonate to COO^-. The involving species are NH_3^+, NH_2, COO^-, and COOH. The decreased NH_3^+–NH_3^+ electrostatic repulsion, and the increased NH_2–COOH interchain hydrogen bonding as well as the NH_3^+–COO^- interchain attraction lead to a decrease in swelling ratio. All of NH_3^+ and COOH deprotonate at pH 7 to NH_2 and COO^-, respectively, and the COO^-–COO^- electrostatic repulsion become the primary interaction affecting on the swelling ratio, resulting in an increase in swelling ratio. However, further improving pH to 9 led to a decrease in swelling ratio due to the weakened COO^-–COO^- electrostatic repulsion by Na^+ screening effect. The results indicated that the prepared hydrogels possess the swelling–deswelling properties in the wide pH range. In addition, the increase of genipin concentration from 0.25 to 0.75 mM resulted in the enhanced swelling ratio due to the increased crosslinking density. However, further improving genipin concentration from 0.75 to 1.0 mM led to a decrease in swelling ratio, which was probably due to the limited swelling of the hydrogels with the over-crosslinked dense network structure.

3.3. SEM

Fig. 3 shows the surface morphologies of sample C1 freeze-dried following swollen at pH 1, 3 and 7, respectively. The pictures confirmed the macroporous structure of the prepared hydrogels. At pH 1, a complex networks consisting of many polygonal cross-sections with pore size of about 100 μm were observed. The polygonal macropores were the regions of water permeation and interaction sites, and the cross-sections were attributed to the polymer matrices. The regular shape in picture **a** implied the compact structure of

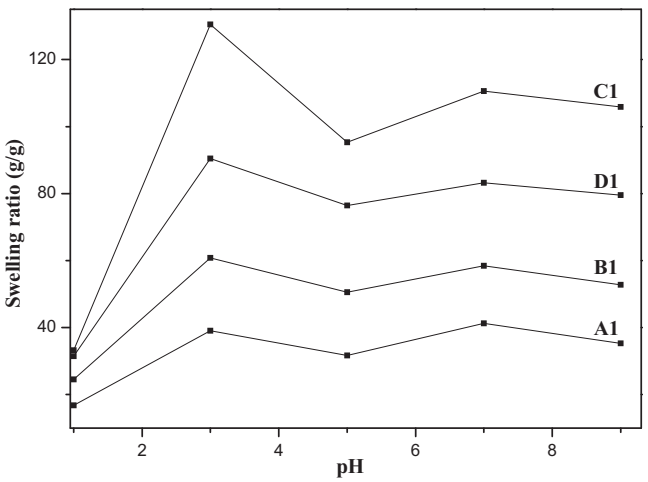


Fig. 2. The effect of pH on the swelling ratio of the crosslinked CMCS/CMC hydrogel films (A1, B1, C1 and D1) in different pH solutions (ionic strength 0.1 M).

the hydrogel network. The inner structure of the macropores in the magnified picture **a**′ was highly dense, corresponding to the relatively low swelling ratio of the hydrogel at pH 1. An open and highly porous structure of three-dimensional network was clearly shown. The pore size in the interconnected porous network structure increased to about 400 μm in picture **b**, indicating the well swollen hydrogel at pH 3. The inner structure of the macropores in the magnified picture **b**′ showed the interconnected highly porous network, providing more evidence of highly swollen hydrogel, which was in agreement with the results from swelling studies. In comparison with that at pH 1 and pH 3, the highly porous structure at pH 7 disappeared, and only a few macropore was present in picture **c**, consistent with the decreased swelling ratio. To further explore the structure of the hydrogel, the picture **c** was magnified and a great deal of dense and compact micropores with the pore size below 10 μm were found in picture **c**′, which were probably responsible for the water uptake-capacity of the hydrogel at pH 7. Based on the above analysis, the morphology of CMCS/CMC hydrogel was consistent with the results from swelling study at different pH values.

In comparison with crosslinked CMCS/CMC sample C1, the blank sample prepared without any crosslinking agents exhibited relatively smooth structure, as shown in Fig. 3, indicating the less crosslinking network structure. Clearly, genipin played an important role in the formation of the crosslinking network of CMCS/CMC hydrogel films.

3.4. Mechanical properties

To investigate the effects of the double ionical and covalent crosslinking with genipin and $CaSO_4$ on the mechanical properties of the prepared CMCS/CMC films, the max load, toughness, elastic modulus, and the fracture energy of the blank sample and the crosslinked films were determined and the results are summarized in Table 1. Clearly, compared with the blank sample without addition of the crosslinker genipin and $CaSO_4$, the crosslinked CMCS/CMC films possessed relatively high mechanical properties.

Fig. 4 displays the detailed effects of $CaSO_4$ and genipin concentration on the max load of the crosslinked films. In comparison with the blank sample, the film ionically and covalently crosslinked with $CaSO_4$ and genipin showed the significantly ($p < 0.05$) higher max load. Without the addition of $CaSO_4$, the increase of genipin concentration from 0 to 0.25, 0.5, 0.75, and 1.0 mM resulted in a significant ($p < 0.05$, as shown in Table 2) increase in the max load from 25.7 to 36.6, 40.9, 43.5, and 53.3 N, respectively. This increment may be due

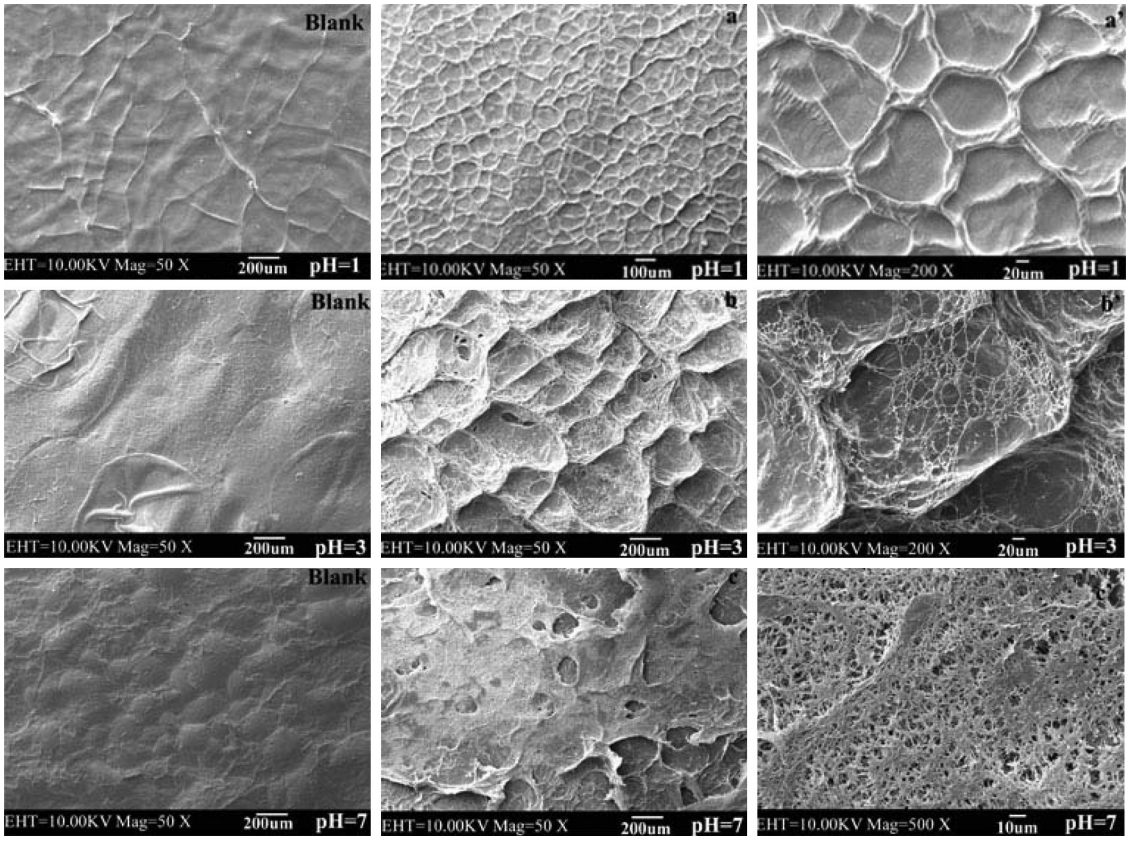


Fig. 3. SEM pictures of freeze-dried CMCS/CMC blank sample and sample C1 swollen at pH 1 (a), 3 (b), and 7 (c).

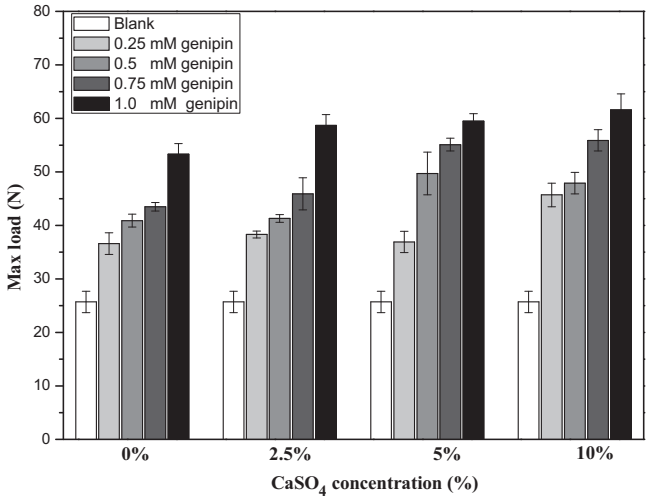


Fig. 4. Max load of the double crosslinked CMCS/CMC films prepared at different genipin (0, 0.25, 0.5, 0.75 and 1.0 mM) and $CaSO_4$ concentration (0%, 2.5%, 5% and 10%).

Table 2
The significant difference (p) of max load obtained at different genipin concentration between 0.25–0.5, 0.5–0.75, and 0.75–1.0 mM.

Genipin concent.	$CaSO_4$ concent.			
	0%	2.5%	5%	10%
0.25–0.5 mM	0.004	0.047	0.003	0.039
0.5–0.75 mM	0.042	0.015	0.023	0.014
0.75–1.0 mM	0.002	0.003	0.043	0.018

to the more order and stable network structure of the crosslinked hydrogels at higher crosslinking density. The similar observations were also obtained at $CaSO_4$ concentration of 2.5%, 5%, and 10%. The maximum max load of the crosslinked hydrogels reached 61.6 N at $CaSO_4$ concentration 10% and genipin concentration 1.0 mM, which was about 2.4 times that of the blank sample.

Similarly, ionical and covalent crosslinking with $CaSO_4$ and genipin could significantly ($p < 0.05$) improve the toughness of the crosslinked films compared to that of the blank sample. Fig. 5 shows the detailed effects of $CaSO_4$ and genipin concentration on the toughness of the films. Keeping the genipin concentration at 0.25 mM, increasing the $CaSO_4$ concentration from 0 to 2.5% and 5% resulted in a significant ($p < 0.05$, as listed in Table 3) increase

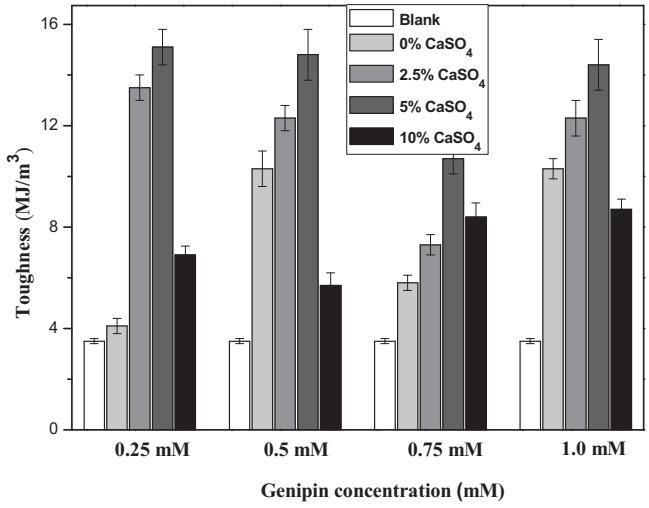


Fig. 5. Toughness of the double crosslinked CMCS/CMC films at different $CaSO_4$ (0%, 2.5%, 5% and 10%) and genipin concentration (0.25, 0.5, 0.75 and 1.0 mM).

Table 3
The significant difference (p) of toughness obtained at different $CaSO_4$ concentration between 0–2.5%, 2.5–5%, and 5–10%.

$CaSO_4$ concent.	Genipin concent.			
	0.25 mM	0.5 mM	0.75 mM	1.0 mM
0–2.5%	<0.001	0.045	0.024	0.047
2.5–5%	0.041	0.016	<0.001	0.023
5–10%	<0.001	<0.001	0.003	<0.001

in the toughness from 4.1 to 13.5 and 15.1 MJ/m^3, higher than that of the blank sample (3.5 MJ/m^3). This increase indicated the positive effects of ionical and covalent crosslinking. However, further improving $CaSO_4$ concentration to 10% resulted in a significant ($p < 0.05$) decrease in $CaSO_4$ concentration to 6.9 MJ/m^3, suggesting the detrimental effect of excessive crosslinker. The role of $CaSO_4$ was relied on the Ca^{2+} interaction with the anionic ions COO^- on CMCS and CMC to form the ionical crosslinking between the polymer chains. At low $CaSO_4$ concentration, the anionic sites on the polymers could accommodate Ca^{2+} to increase the interactions between the polymer chains, resulting in an increase in the film toughness. However, at high $CaSO_4$ concentration, the excess Ca^{2+} may compete for anionic sites disrupt some of the cross-linkages due to repulsive forces (Tang, Lelievre, Tung, & Zeng, 1994; Tang, Tung, & Zeng, 1995, 1996; Yang, Paulson, & Nickerson, 2010), leading to the decreased film toughness. The similar observations were also obtained at the genipin concentration of 0.5, 0.75, and 1.0 mM. To further confirm the effect of $CaSO_4$ concentration on the film toughness, Fig. 6 depicts the stress–strain curves of the crosslinked CMCS/CMC samples prepared and different $CaSO_4$ concentration at genipin concentration 0.75 mM. As shown in Fig. 6, the toughness of the double crosslinked hydrogel samples C1–C4 exhibited the following order: C3 > C4 > C2 > C1, which indicated the important effect of ionical crosslinking with $CaSO_4$ on the toughness of crosslinked hydrogels, corresponding to the above-analyzed high relation between $CaSO_4$ concentration and film toughness. Elastic modulus and fracture energy of the crosslinked hydrogels were also estimated, and the results indicated that the crosslinked hydrogel films possessed the elastic modulus in the range of 0.39–4.26 GPa and fracture energy in the range of 0.3–4.0 $\times 10^6$ J/m^2. However, there was no clear relation found between these two properties and crosslinker concentration. Anyway, the double crosslinked hydrogel films from CMCS and CMC showed good mechanical properties, suggesting the potential as biomaterials like skin replacement (Costa-Júnior, Barbosa-Stancioli, Mansur, Vasconcelos, & Mansur, 2009).

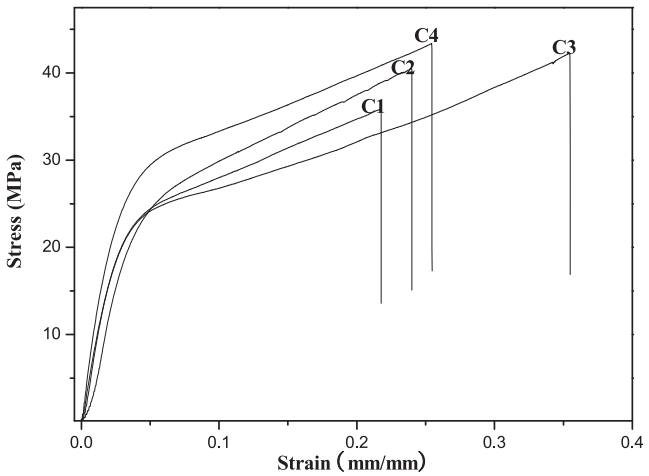


Fig. 6. Stress–strain curves of the crosslinked CMCS/CMC samples C1, C2, C3 and C4 prepared with genipin concentration 0.75 mM.

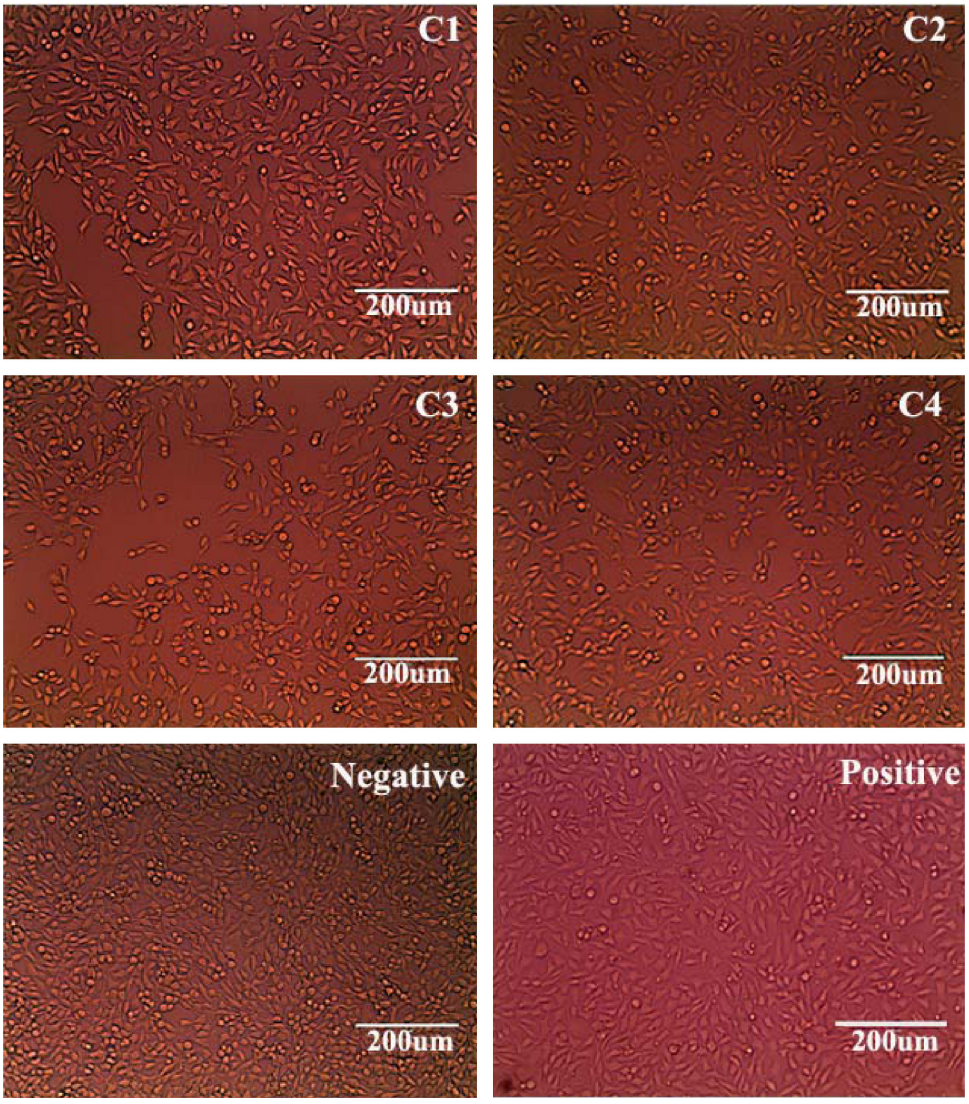


Fig. 7. Micrographs of 3T3 cells cultured for 48 h on the surface of CMCS/CMC films (C1, C2, C3 and C4) and the controls.

3.5. Biocompatibility

Besides the necessary functions for the specific application, biocompatibility is essential for ideal biomaterial (Lopes & Felisberti, 2003). To evaluate the potential application as biomaterials, the biocompatibility of the prepared hydrogels was investigated with the in vitro cytotoxicity. Photographs of the 3T3 cells cultured for 48 h on the double crosslinked CMCS/CMC hydrogel samples C1 to C4 and the controls are shown in Fig. 7. The cells with the perfect morphological characteristics were observed on the negative and positive control. Comparatively, the cells cultured on the prepared hydrogel films remained relatively flat and well-spread without apparent impairment, indicating the absence of low molecular components harmful for cells released from the prepared hydrogels. It could be stated that cells grew on the crosslinked hydrogels as well as on negative and positive control and the hydrogels were bio-nontoxic. These results suggested that the double crosslinked CMCS/CMC hydrogel samples did not contain any toxic substrate influencing cellular growth and had promising application as biomaterials.

4. Conclusion

The novel double crosslinked hydrogel films were prepared from CMCS and CMC with genipin and $CaSO_4$ as ionical and covalent crosslinker, respectively. The results showed that the hydrogels possessed swelling–deswelling properties in the wide pH range with two dominant peaks of swelling ratio at pH 3 and 7. SEM images confirmed the swelling–deswelling properties at different pH values based on the surface morphologies of the hydrogels. The detailed effects of crosslinker concentration on the mechanical properties of the hydrogel films were also discussed, and the results indicated that the ionical and covalent crosslinking exhibited the primary impact on the toughness and max load, respectively, of the crosslinked hydrogels. The cells comparatively cultured on the crosslinked hydrogels and the negative and positive controls suggested the biocompatibility of the crosslinked hydrogel films. Considering the good properties, the double crosslinked hydrogel films have potential application as biomaterial for drug delivery vehicles and skin tissue engineering.

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