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Fabrication, properties and bioapplications of cellulose/collagen hydrolysate composite films

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ABSTRACT

In this work, a series of cellulose/collagen hydrolysate (RC/CH) films were prepared in NaOH/urea aqueous solution via a simple, low-cost and green pathway. To overcome the disadvantages (brittleness, poor water resistance) of CH as biomaterials, CH was combined with regenerated cellulose (RC) film to construct RC/CH composite materials, leading to vast improvement of the water resistance of CH. Crosslinking with genipin further improved the mechanical properties of the RC/CH films in the wet state. Their structure and properties were characterized by elemental analysis, Fourier transform infra-red (FT-IR) spectra, ultraviolet–visible (UV–vis), scanning electron microscopy (SEM), amino acid analysis, tensile testing, cell adhesion and toxicity tests. The mechanical properties and water resistance of the crosslinked RC/CH films were significantly improved, which made collagen hydrolysate as biomaterial could be used at wet state. Moreover, the RC/CH films exhibited good biocompatibility by proliferation of COS7 cells on the surface, supporting cell adhesion and growth.

1. Introduction

Recently, natural polymers have attracted much attention due to the effects of environmental pollution of synthetic polymers and the limited oil resources. Collagen, a form of structural protein in animal skin, bone and tendon, is an important biomaterial with widespread applications in fields such as surgical sutures, cosmetics, wound healing, tissue engineering (Cen, Liu, Cui, Zhang, & Cao, 2008; Lee, Singla, & Lee, 2001). Collagen hydrolysate (CH) is a polypeptide composite made by further hydrolysis of denatured collagen. CH has been used as a natural fertilizer in agriculture applications due to the relatively high nitrogen content (Alexy et al., 2003a); as a promoter for blend degradation based on water-soluble matrices (Alexy et al., 2003b); as an efficient hydrating agent in cosmetic preparations for skin treatment (Langmaier, Mládek, Kolomazník, & Sukop, 2001, 2002; Morimura et al., 2002); as a food additive as its edibility; and as a therapeutic agent of potential utility in the treatment of bone and joint diseases (Moskowitz, 2000). It also can be utilized for manufacturing biodegradable (potentially even edible) packaging materials (Langmaier, Mládek, Mokrejš, & Kolomazník, 2008a; Langmaier, Mokrejs, Kolomaznik, & Mládek, 2008b). It has desirable characteristics for various applications as essential biological nutrient, such as its innocuity to human health and water retention capability. Compared with gelatin, CH has smaller molecular weight and better absorbency and biodegradability.

As the most abundant plant resource on the earth, it is well known that cellulose has many excellent features such as its renewability, non-toxicity, biocompatibility, biodegradability, and derived properties (Bodin, Concaro, Brittberg, & Gatenholm, 2007; Gavillon & Budtova, 2007; Kim, Kuga, Wada, Okano, & Kondo, 2000; Kim, Nishiyama, & Kuga, 2002; Read & Bacic, 2002). Cellulose will be one of the important industrial raw materials because of its potential to substitute for some petrochemicals (Klemm, Heublein, Fink, & Bohn, 2005; Mascal & Nikitin, 2008; Nogi & Yano, 2008). However, cellulose is insoluble in most solvent because of the strong intra- and inter-molecular hydrogen bonding, resulting in its limited utilization (Fengel & Wegener, 1989). Our laboratory has exploited a low-cost green solvent and environmental-friendly method to dissolve cellulose. The native cellulose could be dissolved in NaOH/urea aqueous solution. High-quality cellulose fiber, films, hydrogels and microspheres have been fabricated (Cai et al., 2007; Chang, Zhang, Zhou, & Kennedy, 2010; He, Chang, Peng, & Zhang, 2011; Liu et al., 2009; Luo & Zhang, 2010). Moreover, some biopolymer composites such as cellulose/casein films, cellulose/corn protein films, as well as cellulose/alginate hydrogels have been constructed in NaOH/urea aqueous solution, showing good mechanical properties (Chang, Duan, & Zhang, 2009; Yang et al., 2009; Yang, Zhang, Han, & Zhou, 2001).

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The development of biomaterial-based membranes is one of the most exciting fields in many potential applications such as bioseparation, tissue engineering and food processing (Liang, Zhang, Li, & Xu, 2007). There were some previous studies involving cellulose/gelatin or collagen composites. While, most of the cellulose used in these works were bacterial cellulose (Cai & Kim, 2010; Lin, Hsu, Chen, & Chen, 2009; Wiegand, Elsner, Hipler, & Klemm, 2006) or cellulose derivatives (Jeschke, Sandmann, Schubert, & Klein, 2005; Lii, Tomasik, Zaleska, Liaw, & Lai, 2002; Rathna, Rao, & Chatteriji, 1996; Rokhade et al., 2006), which would cause potential cost and yield issues in applications. Based on native cellulose, cellulose/casein (Yang et al., 2001) and cellulose/corn protein films (Yang et al., 2009) were prepared in our previous works, which also caused protein loss or degradation problems due to the directly adding protein to alkaline solution. In view of the above problems, in this work, we tried to combine the advantages of the above two biopolymers from native cellulose and facile protein by a simple way avoiding protein loss to a great extent. Combining cellulose with CH is expected to introduce amino acid groups into cellulose film scaffold for biomedical applications. CH can provide nourishment for cells and has been widely used as a biomaterial in the biomedical field. In addition, cellulose can provide good stability, optical and mechanical properties in the wet state. In this article, a series of regenerated cellulose/collagen hydrolysate (RC/CH) films were derived from NaOH/urea aqueous solution via a simple and green pathway. Their structure and properties were characterized by element analysis, Fourier transform infra-red (FT-IR) spectra, ultraviolet–visible (UV–vis), ^{13}C solid NMR, scanning electron microscopy (SEM), X-ray diffraction (XRD), amino acid analysis, and tensile testing. Their biocompatibilities were evaluated by cell adhesion and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) test.

2. Experimental part

2.1. Materials

Cellulose (cotton linter pulps) with α-cellulose content of more than 95% was provided by Hubei Chemical Fiber Co. Ltd. (Xiangyang, China). Its viscosity-average molecular weight (M_η) was determined by using an Ubbelohde viscometer in a LiOH/urea aqueous solution at 25 ± 0.05 °C and calculated by the equation $[\eta] = 3.72 \times 10^{-2}\ M_w^{0.77}$ to be 1.1×10^5 g/mol (Cai, Liu, & Zhang, 2006). Collagen hydrolysate was purchased from Jinjian Gelatin Co. Ltd. (Cangzhou, China). Its weight-average molecular weight (M_w) was 20 kDa estimated by sodium dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) analysis. All other reagents were of analytical grade and obtained from commercial sources in China.

2.2. Preparation of cellulose/collagen hydrolysate (RC/CH) films

Cellulose solution was prepared according to a previously described method (Cai & Zhang, 2005). The solvent was prepared as a mixed solution of NaOH/urea/H_2O (7:12:81, by weight) and then precooled to −12.5 °C in a refrigerator. Cellulose was added immediately with vigorous stirring for 5 min at ambient temperature to obtain a transparent cellulose dope with a concentration of 4 wt%. The resulting blend solution was degassed at 5 °C by centrifugation at a speed of 7200 r/min, and then cast on a glass plate to provide a gel sheet, which was immediately coagulated with a 5 wt% H_2SO_4 aqueous solution for 5 min to obtain transparent films with the thickness of about 0.8 mm. The films obtained were washed with deionized water to remove the NaOH and urea for 3–5 days. Collagen hydrolysate aqueous solutions of different concentrations (2%, 5%, 8%, 12%, and 15% by weight) were prepared by dissolving collagen hydrolysate in distilled water at ambient temperature. The washed films were immersed individually in collagen hydrolysate solutions with different concentrations at ambient temperature for 24 h to get cellulose/collagen hydrolysate (RC/CH) films. The RC/CH films and the regenerated cellulose film were code as RC/CH2, RC/CH5, RC/CH8, RC/CH12, RC/CH15 and RC (the numbers meant the concentrations of CH solutions that RC films were immersed), respectively. The RC/CH films then were immersed in 0.2 g/100 mL genipin aqueous solution for 2 h to get the crosslinked RC/CH films, which were coded as cro-RC/CH2, cro-RC/CH5, cro-RC/CH8, cro-RC/CH12, and cro-RC/CH15 respectively. All the above films were fixed on a plastic plate, and dried on air at room temperature to obtain the dried membranes for the characterization of structure and properties.

2.3. Characterizations

The dried samples were ground into powder and then vacuum dried for 24 h before the following measurements. The N contents (W_N) in the RC and RC/CH films were measured by an elemental analyzer (Heraeus Co., Germany). The protein weight (W_{pro}) of the films could be calculated by Eq. (1):

$$W_{pro} = W_N \times 6.25 \quad (1)$$

FT-IR test were carried out on a FT-IR spectrometer (NICOLET 5700, USA) in the wavelength range from 4000 to 500 cm^{-1}. Wide-angle X-ray diffraction (WXRD) measurements were carried out on a X-ray diffraction diffractometer (D8-Advance, Bruker). The patterns with CuKα radiation (λ = 0.15406 nm) at 40 kV and 30 mA were recorded in a region of 2θ from 5° to 45°. Samples were ground into powders and dried in a vacuum oven at 60 °C for 24 h. The crystallinity χ_c (%) of the RC, RC/CH and cro-RC/CH films were estimated by Rabek's method, using Eq. (2):

$$\chi_c = \left[\frac{S_c}{(S_c + S_a)}\right] \times 100\% \quad (2)$$

where S_c and S_a are the areas of crystal and amorphous diffraction peaks of samples, respectively. Solid-state ^{13}C NMR spectra of the samples were recorded on a Bruker AVANCE-300 operating at 75 MHz using the combined technique of proton dipolar decoupling, magic angle spinning (MAS) and cross-polarization (CP). The spinning rate was set at 75.47 Hz for all samples. The contact time was 5 ms, the recycle delay 5 s and the spectrum accumulated 5000 times. FESEM (SIRION TMP, FEI) was used at an accelerating voltage of 10–12 kV. The wet films were frozen in liquid nitrogen, immediately snapped and then vacuum dried for SEM observation. The surface and the cross section of the samples were sputtered with gold, then observed and photographed. The percentage of weight loss (W_{loss}, %) of the films in distilled water were measured. Before and after immersed in distilled water at room temperature for 24 h, the film samples were vacuum dried at 60 °C for 24 h. The initial weight (W_0) and the final weight (W_1) were measured. The W_{loss} value of the samples can be calculated by Eq. (3):

$$W_{Loss} = \frac{[(W_0 - W_1)]}{W_0} \times 100\% \quad (3)$$

Amino acid analysis was carried out by a hydrolyzing process according to GB/T 5009-124-2003 in the Hubei Academy of Agriculture Science. The RC/CH8 blend film was immersed in distilled water at room temperature for 10 days before analysis, coded as WC/CH8. The amino acid contents of the collagen hydrolysate (W_{CH}), RC/CH8 (W_a) and WRC/CH8 (W_b) were measured. The total amino acid contents were sum of all amino acid contents in the

samples. The residual amounts of the amino acid in the WRC/CH (W_{Rb}) can be calculated by Eq. (4):

$$W_{Rb} = \left(\frac{W_b}{W_a}\right) \times 100\% \quad (4)$$

2.4. Optical transmittance properties and mechanical test

The optical transmittance of the films was measured using an ultraviolet–visible (UV–vis) spectroscope (China) in the wavelength range from 200 to 800 nm. The tensile strength (σ_b) and elongation at break (ε_b) of the films in the dry state were measured on a universal testing machine (CMT6503, Shenzhen SANS Test Machine Co. Ltd., Shenzhen, China) according to ISO572-2,1993 (E) at a speed of 5 mm min^{-1}. The σ_b and ε_b values were the averages of five measurements, respectively. Before the test, all the samples were stored in glass desiccators equipped with saturated NaCl solutions to maintain a humidity of 75%. The test was carried on the same environmental temperature and humidity. The mechanical properties in the wet state were measured in the same way as above after samples immersed in water at room temperature for 1 h.

2.5. Cell adhesion and cytotoxicity assay

African green monkey SV40-transformed kidney fibroblast (COS7) cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10% fetal bovine serum and 1% antibiotics (penicillin-streptomycin, 10,000 U/mL) in a humidified atmosphere of 5% CO_2 at 37 °C (Deng et al., 2011; Sun, Zhang, Cheng, Cheng, & Zhuo, 2008). The films were deposited in a 24-well plate and sterilized under the ultra-violet light for 2 h. COS7 cells were then seeded at a density of 6×10^4 cells/well and cultured in 1 mL DMEM containing 10% FBS. The morphologies of the COS7 cells were directly observed and the images were obtained by optical microscope (IX 70, Olympus, Japan) after incubation at 37 °C for 24 h. The cytotoxicity of the films was measured by MTT assay. With additional 24 h incubation, MTT (5 mg/mL) solutions were added for 4 h. Then, the medium was replaced by DMSO (Huang et al., 2010; Zeng, Sun, Zhang, Cheng, & Zhuo, 2009). The absorbance was measured at 570 nm using a microplate reader (BIO-RAD 550). The relative cell viability was calculated as Equation 5:

$$\text{Cell viability}\ (\%) = \left(\frac{OD_{570\ (sample)}}{OD_{570\ (control)}}\right) \times 100 \quad (5)$$

where $OD_{570\,(control)}$ was obtained in the absence of the films and $OD_{570\,(sample)}$ was obtained in the presence of the films.

3. Results and discussion

3.1. Structure and miscibility of RC/CH composite films and the interactions between cellulose and CH

The N content and the protein content are summarized in Table 1. The W_N value of the RC films was 0, suggesting that urea in the solvent was removed completely from the RC films. With the increase of the CH concentration, the N content and the protein content in the RC/CH films increased. Thus, the protein content of the RC/CH films could be adjusted by changing the concentrations of the CH solutions. Compared to the regenerated cellulose/gelatin (C/G) films prepared by the same concentration of the protein solution, the RC/CH films had the higher W_N content and protein content (Pei, Zhang, Wang, Zhang, & Xu, 2011). Gelatin had the higher molecular weight than collagen hydrolysate, and lower content of protein. Compared with gelatin, collagen hydrolysate as the smaller molecules had the better mobility, leading to the higher

Table 1
The N content (W_N, %) and the protein content (W_{pro}, %) of the RC/CH films immersed in CH solution with different concentrations (c, wt%) for 24 h.

Samples	c	W_N	W_{pro}
CH	100	15.84	99
RC/CH2	2	2.94	18.38
RC/CH5	5	5.98	37.38
RC/CH8	8	6.67	41.69
RC/CH12	12	8.71	54.44
RC/CH15	15	9.47	59.19
RC	0	0	0

W_N content of the RC/CH films after having been immersed in the same concentration of protein solution.

As shown in Fig. 1A(a), the RC film exhibited homogeneous porous structure, and the average diameter of the pores was 300–400 nm. The surface SEM images of RC/CH and cro-RC/CH films are shown in Fig. 1A(b–g). With an increase protein contents, the pore size of the RC/CH films increased. The larger pore size could occur because the wet RC/CH films attracted more water than the wet RC film, and bigger ice crystals were formed in wet RC/CH films upon contact with liquid nitrogen. With increasing the protein contents in the RC/CH films, the pores were filled up gradually. Consequently, we can suppose that collagen hydrolysate molecules firstly combined with the pore wall of cellulose film and then filled into the pores through physical interaction between CH and cellulose. Genipin (GP) is derived from gardenia fruit, which has recently been utilized as a crosslinking agent for biomaterials (e.g., chitosan, gelatin and collagen) (Bigi, Cojazzi, Panzavolta, Roveri, & Rubini, 2002; Muzzarelli, 2009; Sundararaghavan et al., 2008). Genipin can be utilized to crosslink functional amine groups present in macromolecules with less toxic effects compared to glutaraldehyde (Chang, Chang, Lai, & Sung, 2003). The schematic illustration of collagen hydrolysate crosslinked with genipin is shown in Fig. 1B. It has been reported that the crosslink agents in the protein system can promote the formation of network structures, indicating that the cro-RC/CH film has both cellulose framework and protein crosslinked network (Rault, Frei, Herbage, Abdul-Malak, & Huc, 1996). After crosslinked by genipin, the cro-RC/CH films changed to more intensive porous structures than the RC and RC/CH films. There were many tiny pores on the surface of the RC films (Fig. 1A(h)). It was noted that the pores were filled with protein molecules gradually with increasing protein content (Fig. 1A(i–k)). When W_{pro} = 59.19%, there was a protein layer on the cellulose film. After crosslinked by genipin, the surfaces of the cro-RC/CH films formed protein crosslinked network structures (Fig. 1A(l–n)).

The IR spectra of the RC and the RC/CH films are shown in Fig. 2A. The OH stretching vibration bands around 3420 cm^{-1} in the RC film broadened and shifted to a lower wavenumber in the RC/CH films, as a result of the increasing intermolecular hydrogen bonds due to the introduction of protein into the cellulose (Yang, Zhang, & Liu, 2000). In this case, new hydrogen bonds were formed easily between cellulose and collagen hydrolysate (Kondo, Sawatari, Manley, & Gray, 1994). As shown in Fig. 2A, the protein carbonyl group (amide I bond) appeared at 1649 cm^{-1}. This band shifted to a lower wavenumber in the RC/CH films, suggesting the formation of new hydrogen bonds. In addition, the relative intensity of the band at 1544 cm^{-1} (amide II bond) in the RC/CH films increased, broadened and shifted to lower wavenumber, indicating the intermolecular hydrogen bonds between cellulose and collagen hydrolysate. Similar results were also reflected in C/G films (Pei et al., 2011).

Fig. 2B shows the X-ray diffraction patterns of the RC and RC/CH films. The regenerated cellulose films exhibited three peaks at 2θ = 12.1°, 19.8°, 21.0°, assigned to the (1 1 0), (1 −1 0) and (2 0 0)

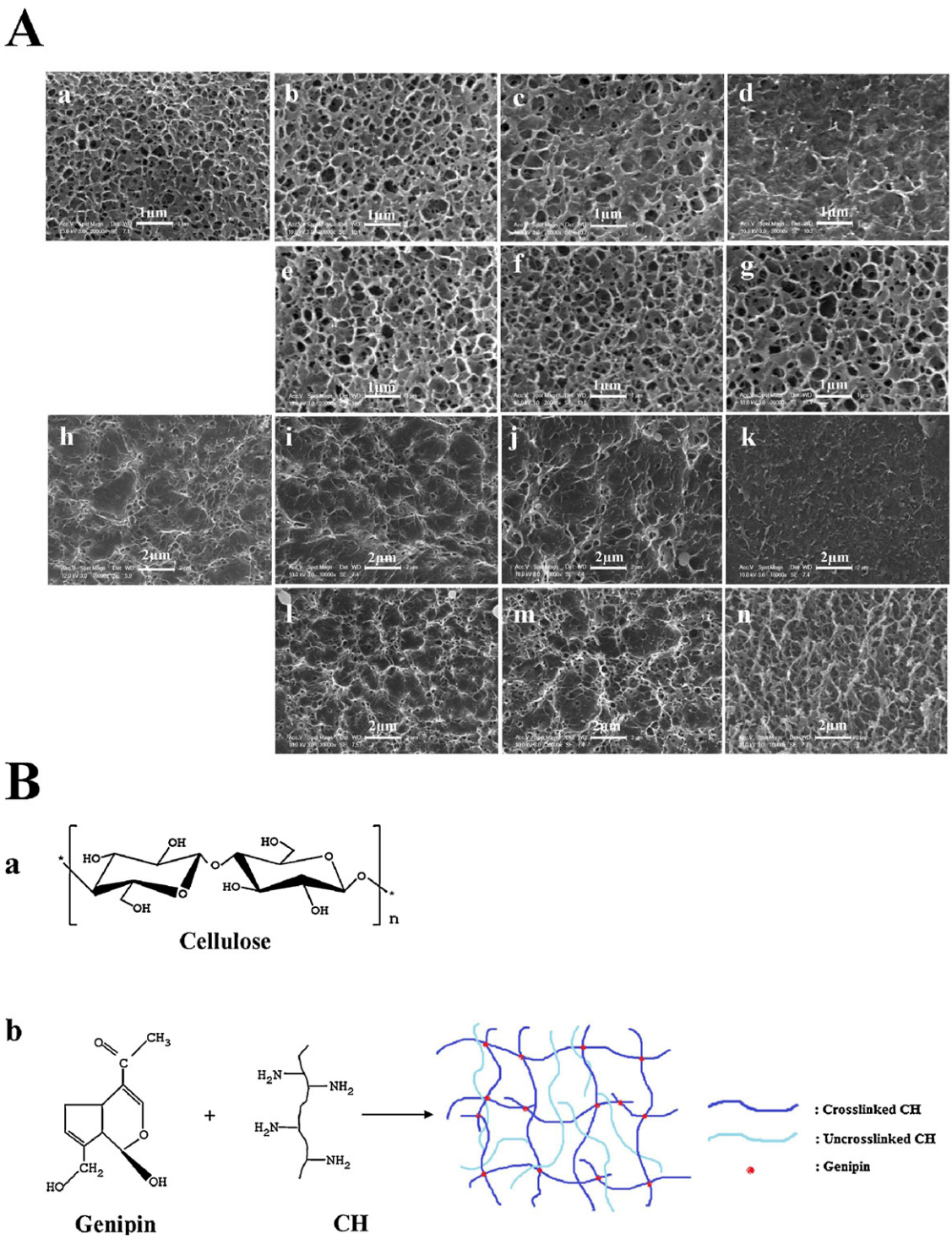

Fig. 1. (A) SEM images of the cross sections of RC (a) RC/CH2 (b), RC/CH8 (c), RC/CH15 (d), cro-RC/CH2 (e), cro-RC/CH8 (f) and cro-RC/CH15 (g) films (the bar is 1 μm); SEM images of the surface of RC (h), RC/CH2 (i), RC/CH8 (g), RC/CH15 (k), cro-RC/CH2 (l), cro-RC/CH8 (m), and cro-RC/CH15 (n) films (the bar is 2 μm); (B) the structure of cellulose (a) and schematic illustration of CH crosslinked with genipin (b).

planes of cellulose II respectively (Isogai, Usuda, Kato, Uryu, & Atalla, 1989). The diffraction angles of the RC/CH films were similar to the RC film because the crystallinity of the collagen hydrolysate was very low. The crystallinity indexes of RC, RC/CH films and CH were calculated to be 51%, 47%, 44%, 43%, 42%, 40% and 11%, and decreased with an increase of W_{pro}. However, the structure of the cellulose in the RC/CH films changed hardly. It was indicated that the interaction between cellulose and collagen hydrolysate was physical, and the results supported the conclusion from FTIR. The diffraction angles of the cro-RC/CH films hardly changed because the crystallinity of the films was primarily owe to cellulose.

The solid-sate ^{13}C NMR spectra of the CH, RC/CH and RC films are shown in Fig. 2C. The assignment of the observed signals to the various types of carbons was also demonstrated. Collagen hydrolysate is a mixture of smaller molecular polypeptides, and the amino components are similar to that of collagen. The peak assignment was carried out according to earlier reports (Huster, Schiller, & Arnold, 2002; Saitô & Yokoi, 1992). However the overall spectral

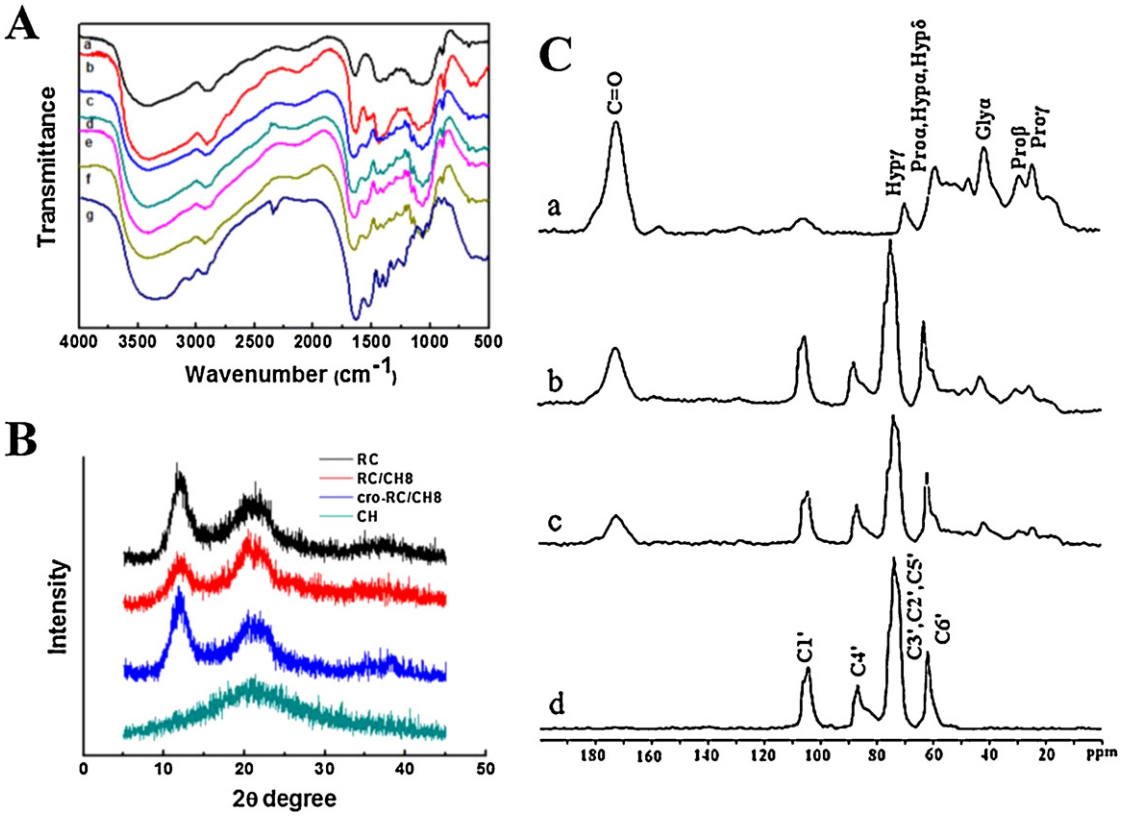

Fig. 2. (A) FT-IR spectra of RC film (a), RC/CH2 (b), RC/CH5(c), RC/CH8 (d), RC/CH12 (e), RC/CH15 (f) and CH (g); (B) X-ray diffraction patterns of the RC, RC/CH8, cro-RC/CH8 and collagen hydrolysate; (C) CP/MAS ^{13}C NMR spectrum of collagen hydrolysate (a) and RC/CH8 (b) cro-RC/CH8 (c) and RC (d).

signals were consistent with that of collagen. The strongest peak was assigned to the glycine (Gly) Cα at 43.0 ppm. Gly was the most abundant amino acid in collagen. Also the Cα resonances of proline (Pro), hydroxyproline (Hyp), alanine (Ala) could be assigned. These four residues accounted for approximately 33% Gly, 13% Pro and 11% each of Ala and Hyp (Reichert et al., 2004). There were four significant peaks at 105.8, 88.0, 73.2, (75.0, 72.5) ppm in the spectrum of the RC film, assigned to the C1, C4, C5 (C3, C2) of cellulose molecular, and the C6 peak at 63.3 ppm. The spectra of RC/CH8 and cro-RC/CH8 had signals of both RC and CH. No other new peaks appeared in the spectra, suggesting no derivatization occurred and that the interaction between RC and CH was due to hydrogen bonding interactions between the OH groups of cellulose and the amino acid groups on CH.

To study the components of collagen hydrolysate, the residual amounts of amino acid in the films that had been immersed in water for prolonged periods were measured. The results of the analysis of the amino acid compositions in the samples are listed in Table 2. Both collagen hydrolysate and RC/CH8 were rich in glycine, proline, hydroxyproline, glutamic acid and alanine, revealing that there were many intermolecular hydrogen bonds between the —OH of the cellulose and the $-NH_2$, —COOH and —CONH— groups in the collagen hydrolysate. However, the W_{Rb} value of the water-immersed RC/CH8 film that contained amino acids with —OH and basic amino acids were much higher than those of the acidic amino acids and the non-polar amino acids. This indicated that there were strong interactions caused by the hydrogen bonding between the hydroxyl groups of cellulose and the hydroxygroups of the amino acids with —OH and amino groups of the basic acids in the collagen hydrolysate in the RC/CH films. Collagen hydrolysate is rich in glycine, proline and hydroxyproline, resulting in higher W_a and W_b of these three amino acids than other amino acids. However, after the RC/CH8 film had been immersed in water for 10 days, the W_{Rb} values of tyrosine, histidine and cystine were much greater than others, indicating that the hydrogen bonds between these amino acid residues were much stronger than those of the others. There is a hydroxyphenyl group in the tyrosine residue, an imidazolyl group in the histidine, and a mercapto group in the cystine. The hydroxyphenyl and imidazolyl groups were conjugated cyclic polar groups, leading to the extraordinarily strong hydrogen bonds with

Table 2
The results of analysis results for the amino acid compositions in the samples.

Class	Amino acid	W_{CH}	W_a	W_b	W_{Rb}
Acidic	Asparagine	4.64	2.15	0.0237	1.10
	Glutamic acid	11.1	5.08	0.545	10.73
Polar	Threonine	1.42	0.65	0.073	11.23
	Serine	3.59	1.67	0.188	11.26
	Tyrosine	0.03	0.04	0.012	30.00
	Hydroxyproline	9.92	4.26	0.46	10.80
	Glycine	23.71	10.96	1.174	10.71
	Cystine	0.32	0.16	0.049	30.63
Non-polar	Alanine	9.78	10.96	0.45	4.11
	Methionine	0.56	0.27	0.01	3.70
	Phenylalanine	2.00	1.06	0.126	11.89
	Proline	11.78	5.14	0.55	10.70
	Leucine	2.82	1.33	0.153	11.50
	Isoleucine	1.38	0.65	0.074	12.33
	Valine	2.26	1.03	0.142	13.79
Basic	Lysine	4.01	1.84	0.213	11.58
	Arginine	6.42	2.92	0.364	12.47
	Histidine	0.26	0.18	0.029	16.11
W_{Total}		96	50.35	4.22	8.38

Abbreviations: W_{CH}, the amino acid contents of the collagen hydrolysate; W_a, the amino acid contents of RC/CH8; W_b, the amino acid contents of WRC/CH8; W_{Rb}, the residual amounts of the amino acid in the WRC/CH.

Table 3
The percentage of weight loss (W_{loss}, %) in the films after bathing in distilled water for 24 h.

Samples	RC	RC/CH2	RC/CH5	RC/CH8	RC/CH12	RC/CH15
RC/CH	0	2.817	3.534	4.177	4.812	5.652
cro-RC/CHH	–	0.308	0.455	0.721	1.124	2.056

the —OH of cellulose. In addition, it is well known that hydrophobic interactions between cellulose and aromatic rings of amino acids of protein is the primary driving force of binding (Liu et al., 2011). The results were in accordance with the interactions between corn protein and cellulose (Yang et al., 2009), further confirming that there were hydrogen interactions between protein and cellulose in good agreement with the results of FTIR and XRD. In view of the results of RC/CH mentioned above, the miscibility of cellulose and collagen hydrolysate in the RC/CH composite films was good.

3.2. Stability of RC/CH and cro-RC/CH films in the wet condition

Collagen hydrolysate as a kind of protein having small molecular structure can hardly keep good shape and mechanical properties in a wet state. In our findings, it was demonstrated that the RC/CH films exhibited relatively better mechanical strength in the wet state, especially for the cro-RC/CH films. It is conceivable that the RC/CH and cro-RC/CH films could be used under moist and liquid conditions. In addition, both CH and cellulose are non-toxic, biocompatible and biodegradable, which makes the application of collagen hydrolysate in biomedical fields viable. The percentage of weight loss (W_{loss}, %) values of the films after being immersed in water for 24 h are shown in Table 3. Because cellulose was insoluble in water, the weight loss should be resulted from of the change in the CH component. The low W_{loss} values indicated that most of the protein remained in the RC/CH films. Especially after being crosslinked by genipin, the cro-RC/CH films presented much lower W_{loss} values, which meant the crosslinking can lock CH molecules into the cellulose framework. Therefore, CH was mainly kept in composite films after bathing in water for hours. This provided a way for CH to be used as a biomaterial in the wet state.

Fig. 3a shows the photographs of the RC film and RC/CH films possessed a good optical transmittance, which further confirmed good compatibility between cellulose and collagen hydrolysate. The color of the cro-RC/CH films turned dark bluish, as a result of the reaction of genipin with the amino groups on the collagen hydrolysate molecules. It is known that genipin may form blue pigments upon spontaneous reaction with amino groups (Touyama et al., 1994). It has also been reported that only primary amines, rather than secondary or tertiary amines can react with genipin. The optical transmittance T_r of the films are shown in Fig. 3b and c. The values of T_r (from 450 to 800 nm) for the RC/CH films with 18.38–59.19 wt% of W_{pro} were close to each other, which were higher than 75%. The result revealed homogeneous structure in RC/CH films. Interestingly, the T_r values of the RC/CH films were higher than those of the RC films. The increase in opacity was possibly caused by the strong interaction between cellulose and collagen hydrolysate. In addition, an obvious UV resistivity appeared in the wavelength range of 200–300 nm, which suggested that the RC/CH films can absorb UV radiation and this absorbance was also observed in the optical transmittance of the crosslinked RC/CH films. The maximum absorbances of the genipin-amino reactions in the wavelength range of 595–605 nm (Lee, Lim, Bhoo, Paik, & Hahn, 2003), which were observed in the crosslinked RC/CH films. The results suggested that genipin really reacted with the amino groups in CH molecules. Optical transmittance property as one of the proofs is used to estimate miscibility between materials. The results of optical transmittance property test further proved the good miscibility of RC/CH films. In addition, good optical transmittance was required for materials when observing the cell growth and adhesion during biological experiments.

3.3. Mechanical properties

The mechanical properties of the RC and RC/CH films in the dry and wet states are shown in Fig. 4. Compared with other polysaccharide films, such as chitosan, gellan and alginate films (Dong, Wang, & Du, 2006; Lee, Shim, & Lee, 2004; Olivas & Barbosa-Canovas, 2008; Tanabe, Okitsu, Tachibana, & Yamauchi, 2002), cellulose films processed good mechanical property. The tensile stress (σ_b) of the RC/CH films in the dry state decreased with an increase of W_{pro}, and RC/CH2 exhibited the highest tensile strength (77 MPa). The lower tensile stress of the composite films was caused by the weak mechanical properties of proteins and their less cohesive structure compared with the pure cellulose. The similar result was shown in the study of chitosan/whey protein films (Ferreira, Nunes, Delgadillo, & Lopes-da-silva, 2009). The elongation at break (ε_b) of the RC/CH films increased on the whole compared with the cellulose films, suggesting that the collagen hydrolysate acts as a plasticizer. The RC/CH15 (W_{pro} = 59.19 wt%) displayed the highest ε_b value (18.2%). The formation of protein crosslink network resulted in that the σ_b values of the RC/CH films in the wet state also decreased and the ε_b increased with an increase of W_{pro}. Especially, the maximum ε_b value of the wet RC/CH15 was up to 75.3%, and the ε_b values of the wet RC/CH films increased on the whole. Collagen

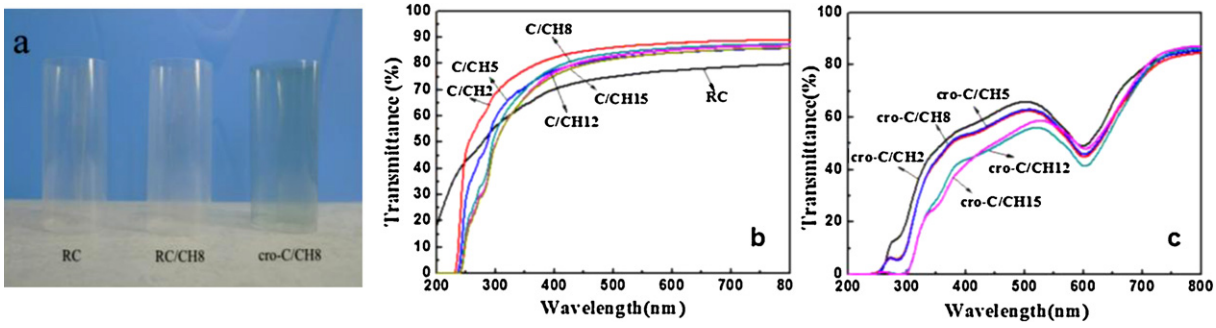

Fig. 3. The photographs of RC, RC/CH8 and cro-RC/CH8 films (a); the optical transmittance (T_r, %) curves of the RC and RC/CH films (b), cro-RC/CH films (c).

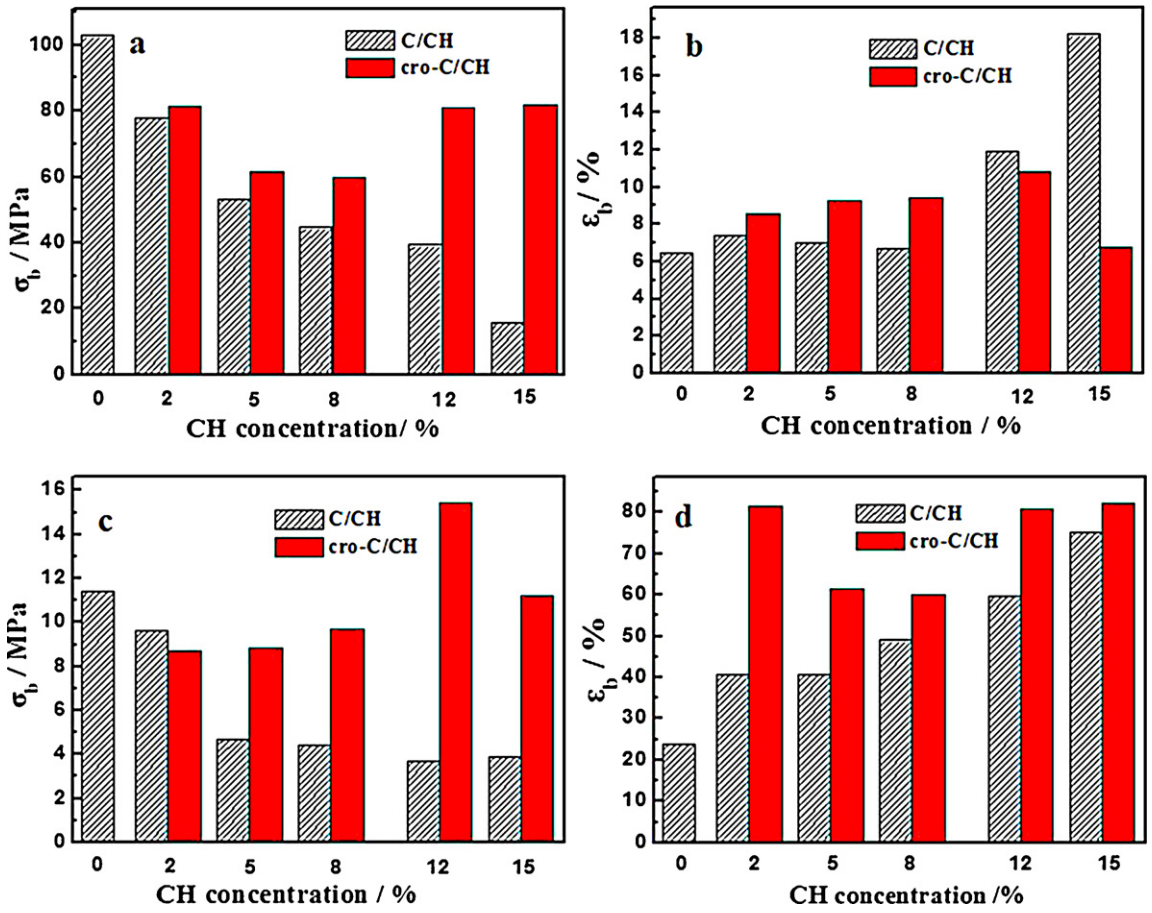

Fig. 4. The tensile stress (σ_b, MPa) and elongations at break (ε_b, %) of the RC, RC/CH and cro-RC/CH films in the dry state (a, b) and the wet state (c, d).

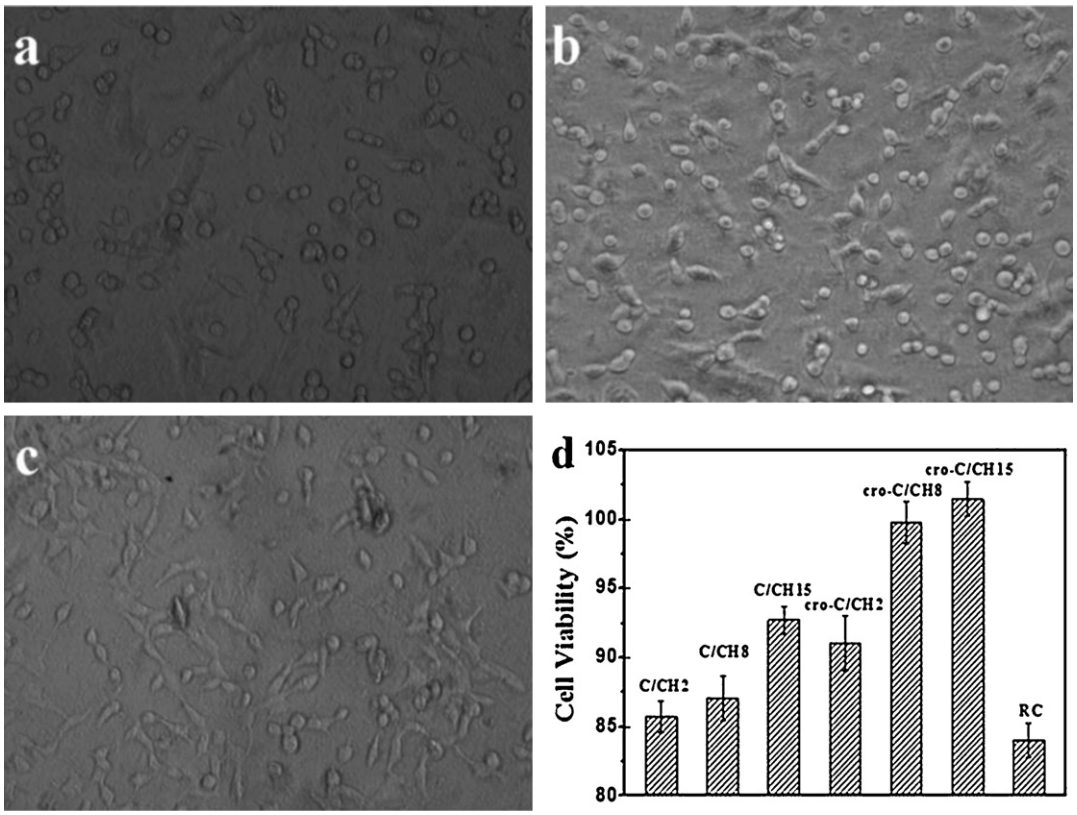

Fig. 5. Optical photographs (100×) of COS7 cells proliferation on RC (a), RC/CH15 (b) and cro-RC/CH15 (c) and the result of the cytotoxicity test (d).

hydrolysate and water molecules held by protein molecules acted as plasticizer together, leading to higher σ_b values of the RC/CH films in the wet state. Genipin as a crosslinking agent might make the collagen hydrolysate molecules firmly locked in the cellulose framework, and the cro-RC/CH films had higher σ_b values than the RC/CH films in both the dry and wet states. The ε_b values of cro-RC/CH films were a bit higher than that of RC/CH films except for cro-RC/CH12 and cro-RC/CH15 in the dry state. While the ε_b values of cro-RC/CH films were a bit lower than that of RC/CH films in the wet state. This could be explained by the decrease of the free CH molecules as plasticizers due to the crosslinking reaction. Therefore, through combining with cellulose film and crosslinking reaction, CH could be used as a material with good mechanical property at wet state.

3.4. Biocompatibility

To access the possibility of RC/CH films used as biomaterial, COS7 cells were employed to evaluate the biocompatibility of the RC/CH and cro-RC/CH films. Morphologies of COS7 cells incubated with RC, RC/CH15 and cro-RC/CH15 films for 24 h are shown in Fig. 5(a–c). In our findings, the spreading and proliferation of the seeded cells on the surface of RC/CH15 and cro-RC/CH15 films were better than on the RC films, revealing that adding of CH proteins could efficiently accelerated cell growths. MTT cytotoxicity tests were carried out to test the safety of the RC/CH films, and the results are shown in Fig. 5(d). Clearly, the cell viability values on the RC/CH films and RC film were greater than 85%, indicating RC/CH films posed nearly no toxicity to the COS7 cells and possessed greater cell viabilities than the RC films. RC/CH films exhibited greater cell viability than RC film, which was because feasible environment and nutrition furnished by collagen hydrolysate, especially in for the crosslinked RC/CH films. Collagen hydrolysate was well fixed in cellulose films by the crosslink reaction, and thus the film could supply nutrition for the cell in the liquid culture medium. The above results indicated that RC/CH films had good biocompatibility and were beneficial to the cell adhesion and growth. Therefore, the RC/CH films had potential applications in biomedical fields because of the good biocompatibility.

4. Conclusion

A series of cellulose/collagen hydrolysate (RC/CH) films were prepared successfully in NaOH/urea aqueous solution via a simple, low-cost and green pathway leading to the application of CH as biomaterial. In the composite films, cellulose and CH had good compatibility, as a result of strong hydrogen bondings between the hydroxyl groups of the cellulose and the hydroxyl groups and amino groups of the collagen hydrolysate. The RC/CH and cro-RC/CH films exhibited good transparence and the capacity for ultraviolet radiation absorption owing to the absorption of the collagen hydrolysate. The crosslinking with genipin further improved the mechanical properties of the RC/CH films, as well as enhancing the stabilities of the RC/CH films in distilled water. The results of cell culture experiments and MTT cytotoxicity tests confirmed that the RC/CH films and cro-RC/CH films could support cell adhesion and proliferation, showing good biocompatibility. Therefore, these composite films with features of safety, biocompatibility, and biodegradability would have promising applications in the biomaterials field.

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