Carbohydrate Polymers 84 (2011) 465–470

Contents lists available at ScienceDirect

Carbohydrate Polymers

journal homepage: www.elsevier.com/locate/carbpol

Preparation and properties of chitosan derivative/poly(vinyl alcohol) blend film crosslinked with glutaraldehyde

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ARTICLE INFO

Article history:
Received 20 July 2010
Received in revised form
19 November 2010
Accepted 2 December 2010
Available online 10 December 2010

Keywords:
Chitosan derivative
Poly(vinyl alcohol)
Crosslinking
Swelling
Antibacterial

ABSTRACT

Novel crosslinked blend films of N-(2-hydroxy)propyl-3-trimethylammonium chitosan chloride (HTCC) and poly(vinyl alcohol) (PVA) were prepared by mixing their aqueous solutions followed by crosslinking with glutaraldehyde (GA). The effects of the GA content and ratio of HTCC to PVA on the appearance, swelling, gel content, morphology and antibacterial activities of blend films were studied and a possible mechanism of crosslinking was proposed based on the results. It was found that the equilibrium degree of swelling (ESD) increased with the increasing content of HTCC, but decreased with the content of GA. The ESD was a maximum (212%) when the ratio of HTCC/PVA/GA was 60/40/2 (wt). Antibacterial activities against *Staphylococcus aureus* and *Escherichia coli* of the blend films were weakened slightly by crosslinking, but still showed substantial antibacterial activity. These results demonstrate that, not only the PVA, but also the HTCC reacted with GA. It was the amino groups on HTCC that was un-substituted by quaternary ammonium salt, which reacted with the aldehyde groups on GA.

1. Introduction

Chitosan, an aminopolysaccharide, is composed of β,1-4 glucosidic bonds. Due to its unique polycationic nature, chitosan and its derivatives have been proposed for various applications in biomedical, food, agricultural, biotechnological and pharmaceutical fields (Arvanitoyannis, Nakayama, & Aiba, 1998; Cardile et al., 2008; Feng & Huang, 1997; Wang, Turhan, & Gunasekaran, 2004). The antibacterial activity of chitosan and its antibacterial mechanism have been researched extensively. The six main antibacterial mechanisms have been proposed as follow: (1) interactions between the positively charged moieties on the chitosan molecules and the negatively charged ones on the microbial cell outer membranes leads to changes in the cell membrane structure and permeability inducing the leakage of proteinaceous and other intracellular constituents; (2) chitosan acts as a chelating agent that selectively binds trace metals and subsequently inhibits the production of toxins and microbial growth; (3) chitosan activates several defense processes in the host tissue, acting as a water binding agent and inhibits various enzymes; (4) low molecular weight chitosan penetrates the cytosol of the microorganisms and, through the binding of chitosan with DNA, and results in the interference with the synthesis of mRNA and proteins; (5) chitosan, on the surface of the cell, can form an impermeable polymeric layer which alters the cell permeability and prevents nutrients from entering the cell; and (6) finally, chitosan can adsorb the electronegative substances in the cell and flocculate them, it disturbs the physiological activities of the microorganism leading to their death (Muzzarelli et al., 2000; Vallapa et al., 2011).

In general, chitosan of high molecular weight can only be dissolved in acid solution. Solutions made from chitosan in dilute acid solution often need a repeated washing process to neutralize the acid, which restricts its applications thus the chemical modification of chitosan to improve its water-solubility is desirable. A series of water soluble compounds have been synthesized, hydroxyethylated, N-carboxymethylated, N-alkylated, oxidated, degradation of chitosan N, O-sulfated, oxygen inorganic acid esterified and quaternary ammonium cationizated chitosan derivatives (Chen, Wang, Liu, & Park, 2002; Ji et al., 2009; Ma et al., 2007). N-[(2-hydroxy-3-trimethyl-ammonium)-propyl] chitosan chloride (HTCC) could be prepared by reacting chitosan with glycidyl trimethylammonium chloride (GTMAC). The introduction of N-trimethylated quaternary ammonium salt group would greatly weaken the hydrogen bonds between chitosan molecule chains, and improve the water-solubility, antibacterial activity, moisture absorption and retention capabilities of chitosan. In addition, the reagents used in the prepa-

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doi:10.1016/j.carbpol.2010.12.006

ration of HTCC were of low cost, and the processing was relatively simple. This method has been widely used in the production of chitosan derivatives (Chiellini, Cinelli, Chiellini, & Imam, 2004; Jia, shen, & Xu, 2001; Kim, Choi, Chun, & Choi, 1997; Qin et al., 2004).

Poly(vinyl alcohol) (PVA) is one kind of hydrophilic semi-crystalline polymer. It has been widely used in biomedical applications because of its non-toxicity, biocompatibility, excellent chemical resistance and mechanical strength (Peppas & Wright, 1998).

Up to now, much work has been done on the preparation and study of chitosan/chitosan derivatives and PVA blend. Arvanitoyannis, Kolokuris, Nakayama, Yamamoto, and Aiba (1997) have prepared the blend of chitosan/PVA plasticized with sorbitol and sucrose for using as a food packaging material, and studied the effect of plasticizer on the structure, thermal, mechanical property and CO_2 permeability and also reviewed the preparation, physical properties, and potential as food packaging materials of this kind of blend based on natural and synthetic macromolecules (Arvanitoyannis, 1999). Some studies have focused on the applying of a series of crosslinking methods, e.g. irradiation, freeze-thawing and chemical methods. These studies include the combination of two crosslinking methods to prepare the hydrogel of PVA and chitosan, and the results showed that hydrogels made by irradiation followed by freeze-thawing show larger swelling capacity and mechanical strength, higher thermal stability, lower water evaporation rate, and are less turbid than those made by pure freeze-thawing and freeze-thawing followed by irradiation. Hydrogels made by irradiation alone cannot be used as wound dressing due to their poor mechanical strength. (Shen, Ruan, & Ga, 2009; Yang, Liu, Chen, Yu, & Zhu, 2008). The chemical crosslinking method has the advantages of improving the mechanical strength, thermal stability, keeping the intrinsic opaque appearance and swelling capacity (Bai, Xie, & Zhu, 2007). Most of studies focused on the effect of chemical crosslinking on the mechanical, thermal and swelling properties of PVA/chitosan blend, but the changes in antibacterial activity as a result of crosslinking have been seldom studied. Although some studies had demonstrated that the amino groups on chitosan might react to the aldehyde groups on GA through the FTIR spectrum, the changing of the absorption bands of N–H and characteristic peak of other groups were not so obvious, and there was no other evidence to support this mechanism (Milosavljević et al., 2009).

Our research aims at prepare the HTCC and HTCC/PVA blend films crosslinked by glutaraldehyde (GA), investigate the swelling properties, degree of crosslinking and antibacterial activities, and explore the crosslinking mechanism.

2. Experiments

2.1. Materials

Chitosan (K-03) used in this work was provided by FUNAKOSHI Co. (Japan). The viscosity-average molecular weight ($\overline{M}_v$) was 35,000 and the degree of deacetylation was 90%. PVA was purchased from Sinopharm Chemical Reagent Co., Ltd. (China) with a degree of polymerization of 1750 ± 50. GA was prepared in the lab. Analytical-grade sodium hydroxide (NaOH), acetic acid (CH_3COOH), isopropanol and GA aqueous (25 wt%) were used without further purification. Nutrient broth and agar culture medium (Hangzhou Microbial Reagent Co., Ltd., China), agar powder (Sinopharm Chemical Reagent Co., Ltd., China), meat-extract (Dainippon Pharmaceutical Co., Ltd., Japan) and polypeptone (Wako Pure Chemical Industries, Ltd., Japan) were used for the antibacterial activity test.

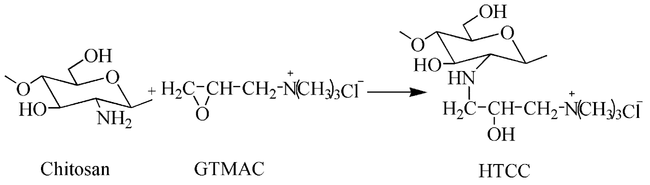

Fig. 1. Synthetic scheme of HTCC.

2.2. Sample preparation and measurement

2.2.1. Preparation of HTCC

Chitosan was dissolved in a 2 wt% acetic acid solution, and then the solution was adjusted with NaOH solution (0.1 mol/L) to pH = 9, kept for 6 h to allow sedimentation. The precipitate was then filtered out and dissolved in isopropanol, and heated to 60 °C under a nitrogen atmosphere. GTMAC was then added into the solution, and stirred for 6 h at 80 °C. After that the clear viscous solution was cooled to ambient temperature and deposited in isopropanol, then the filtered out precipitate was washed three times by isopropanol and dried at 80 °C. The synthetic scheme was represented in Fig. 1. Hydrogen atoms of $-NH_2$ on chitosan were substituted by the quaternary ammonium salt group, and the proportion of hydrogen atom being substituted was expressed by the degree of substitution (DS).

To measure the DS of HTCC, a certain quality of HTCC was dissolved in deionized water, and Cl^- in solution was titrated with silver nitrate solution using potassium chromate as the indicator. DS of the quaternary ammonium salt groups was calculated as following:

$$DS\% = \frac{VM}{VM + (W - VM \times 314)/161} \tag{1}$$

where W is the weight of HTCC in grams, V (mL) and M (mol/L) are the volume and concentration of silver nitrate solution used for titration, respectively. The numbers 314 and 161 corresponded to the molecular weight of the repeat structural unit of HTCC and CS. When the hydrogen atoms of $-NH_2$ on chitosan were fully substituted, the DS of HTCC was 200%, since there were two hydrogen atoms on each amino group which could be substituted by the quaternary ammonium salt groups. And the measured DS of HTCC was 124%, which indicates that 76% of the hydrogen atoms of $-NH_2$ on chitosan were not substituted.

2.2.2. Preparation of sample films

PVA pellets were added into deionized water (PVA/water = 1/10 in weight) and dissolved under continuous stirring at 100 °C. Then the HTCC was added into the PVA solution, and an absolutely clear mixture was obtained for a while. After that the diluted GA aqueous was added into the flask, and the mixture was stirred for 15 min at 70 °C. Then the solution was cooled down and poured into the dish, dried at ambient temperature to form the crosslinked PVA–HTCC film. Pure PVA films and HTCC films crosslinked with GA were also prepared with the same method.

2.2.3. Measurement of the swelling properties of the films

The swelling properties of PVA–HTCC blend films in buffer solutions (pH = 7.4) at 37 °C were studied. The swelling degree (SD) and equilibrium swelling degree (ESD) of films with different contents of HTCC and GA were determined gravimetrically. Dried films of appropriate size were weighed and then immersed in NaH_2PO_4–Na_2HPO_4 buffer solutions, and NaOH solutions were used to adjust pH to be 7.4. Sodium chloride was used to adjust the ionic strength of the solutions to be 0.15 mol/L. To ensure complete equilibration, the samples were allowed to swell for

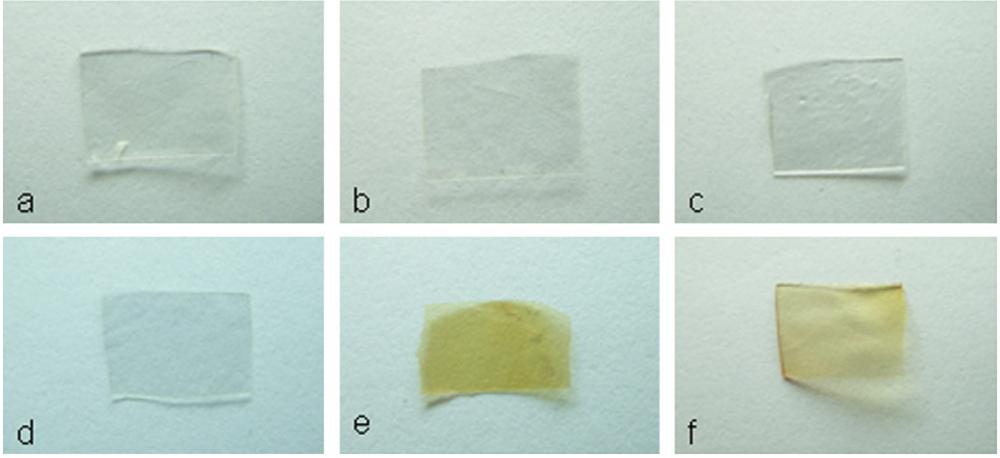

Fig. 2. Photographs of different films. (a) PVA film; (b) HTCC film; (c) HTCC–PVA blend film; (d) crosslinked PVA film; (e) crosslinked HTCC film; and (f) crosslinked HTCC–PVA blend film.

24 h. The excess surface-adhered water drops were removed by filter paper rapidly, and the swollen films were weighed (Costa-Júnior, Pereira, & Mansur, 2009; Costa-Júnior, Barbosa-Stanciolib, Mansurc, Vasconcelosc, & Mansurc, 2009; SanlI, Ay, & IsIklan, 2007). The SD and ESD were calculated as follows:

$$\mathrm{SD}\% = \frac{M_s - M_d}{M_d}100\% \tag{2}$$

$$\mathrm{ESD}\% = \frac{M_s' - M_d}{M_d}100\% \tag{3}$$

where M_s and M_d are the weights of the swollen film and dry film, respectively. M_s' is the weight of the film swollen for 24 h.

2.2.4. Measurement of gel content of films

Films with different compositions and crosslinking agent contents were dried sufficiently at 105 °C and weighed. Then the films were wrapped in a stainless steel net (150 mesh) and extracted with Soxhlet extractor at 90 °C for 12 h where water was used as solvent. Afterward, the stainless steel net encapsulated residue was taken out, then dried and weighed.

$$\text{gel content} = \frac{M_2}{M_1}100\% \tag{4}$$

where M_1 is the weight of film before extracted and M_2 is the weight of film after extracted.

2.2.5. Antibacterial activity test

The antibacterial activity test was carried out using the film adhering method. *Staphylococcus aureus* (*S. aureus*) ATCC 6538 (NBRC 13276) and *Escherichia coli* (*E. coli*) ATCC 25922 (NBRC 15034) were used as the test bacteria.

(1) The samples were cut to small pieces (50 mm × 50 mm), and pure PVA film was used as the control and cover film. All the films were rinsed with 70 wt% ethanol solution, and then fully dried and sterilized under ultraviolet light.

(2) Test bacteria were cultivated in nutrient agar at 36 ± 1 °C for 16 h. An aliquot was transferred into nutrient broth and cultivated at 36 ± 1 °C for 16 h. This cultivation broth was diluted 500 times with 1/500 nutrient broth (pH = 7.0 ± 0.2).

(3) Cultivation of test bacteria: a transferring loop of *S. aureus* or *E. coli* was taken and dispersed on the surface of slope medium evenly, then cultured at constant temperature (36 °C) for 24 h. Afterward, a transferring loop of bacteria was moved to the liquid culture medium, and shaking cultured in an oscillating incubator at 36 °C for 16 h. The bacteria content was 10^8 cfu/mL. 0.4 mL of the bacteria inocula was diluted with 20 mL liquid medium and shaking cultured for 4 h. And then diluted 10,000 times with buffer solution, the number of bacteria was $1.5\text{–}3 \times 10^{4\text{–}5}$ cfu/mL.

(4) Antibacterial activity test: the sample films were added into the conical beaker with 70 mL culture medium, then the diluted inocula (5 mL) was added and shaking cultured at 36 °C for 18 h. After that 1 mL of the above inocula was transferred by pipette to the Petri dish, 15 mL standard agar medium was added and cooled down. Next, the Petri dish was put into the constant temperature incubator and cultured for 24 h. Finally, the growing status of bacteria was observed and the number was counted. Based on the standard of QB/T 2591:^{2003} (Antimicrobial plastics-Test for antimicrobial activity and efficacy) from China, which mainly referenced the standards of ASTM G 21-1996 (Standard Practice for Determining Resistance of Synthetic Polymeric Materials to Fungi, NEQ) and JIS Z 2801-2000 (Antimicrobial products-Test for antimicrobial activity and efficacy), the antibacterial rate was calculated as the following formula:

$$\text{antibacterial rate}\,(\%) = \frac{B - C}{B}100\% \tag{5}$$

where *B*: average bacterial number per plate in the control film after 24 h incubation. *C*: average bacterial number per plate in the PVA or blend films after 24 h incubation.

I Antibacterial rate > 99% (good antibacterial efficacy).
II Antibacterial rate > 90% (normal antibacterial efficacy).
III Antibacterial rate < 90% (no antibacterial efficacy).

3. Results and discussion

3.1. Appearance of the films

Fig. 2 shows the photographs of films including the non-crosslinked and crosslinked films with GA in which GA was 2 wt% of sample. The color of pure PVA, HTCC and HTCC/PVA blend films was transparent as also the PVA film crosslinked by GA, but the HTCC and HTCC–PVA blend films crosslinked by GA are dark yellow. This was not reported in the previous studies. Some studies referenced above only reported that the aldehyde group on GA could react with the amino group on chitosan/chitosan derivatives formed the –C=N– group. In the present study, the amino groups on HTCC were not substituted totally by the quaternary ammonium salt groups. And the residual amino groups might react with GA and formed the –C=N– group, a chromophore. The adsorption band of

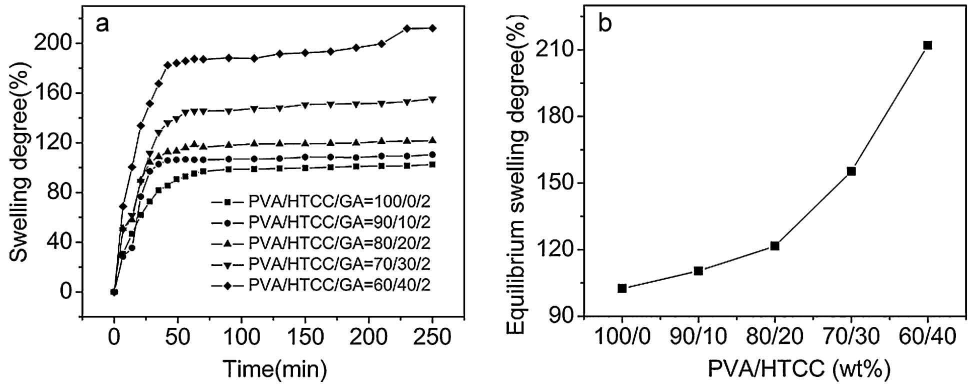

Fig. 3. The swelling kinetics (a) and EDS (b) of PVA–HTCC blend films with different compositions in buffer solutions (pH = 7.4). (GA content was the 2 wt% of the total weight of PVA and HTCC.)

–C=N– is in the extreme ultraviolet, and cannot be observed in the range of visible light. However, a cyclic group i.e. glucosamine with –OH, –OR is conjoint with –C=N–, which can act as an auxochrome. This resulted in the adsorption band of –C=N– shifting to the range of long wave, and chromogenic in the range of visible light. Therefore, the color of crosslinked blend film became darker.

3.2. Results of swelling measurement

Swelling properties in vitro test are of paramount importance for prospective evaluation of biomaterials. The swelling capacity of biomaterial can be affected by many factors, such as the crosslinking density, the hydratability of the materials, the ionic strength and pH value of the media, as well as the temperature of the environment (Yang et al., 2008). In this research the swelling capacity was studied for PVA–HTCC blend films with different compositions and dosages of crosslinking agent in NaH_2PO_4–Na_2HPO_4 buffer solutions.

Fig. 3 shows the effect of HTCC content on the swelling capacity of blend films when the dosage of GA was fixed to be 2 wt% against the total quantity of PVA and HTCC. More rapid swelling was observed for the films with higher HTCC content. When the swelling degree of the film attained 80%, 40 min was needed for the pure PVA film, but only 20 min and 10 min for the films with 10 wt% and 40 wt% HTCC, respectively. And equilibrium state of swelling was achieved within 100 min for the film with 10 wt% HTCC while more than 250 min was required for the film with 40 wt% HTCC. ESD also increased with the HTCC content. When the ratio of HTCC/PVA/GA was 60/40/2 (wt), ESD of the blend films reached 212%. A higher ESD and shorter response time were observed compared with the results obtained in the previous studies (Shen et al., 2009; Yang et al., 2008). On one hand, the high swelling rate and ESD should be attributed to the good hydratability of HTCC with large amounts of quaternary ammonium salt groups. Introduction of HTCC increased the number of positive charge, which attracted hydroxyl ion in the external environment of cross-linking networks; on the other hand, PVA was a polymer with high percentage of crystallinity, and the micro-molecular, e.g. water, CO_2, O_2 was difficult to permeate through because of the strongly hydrogen bond interaction between the molecular chains. The introduction of an amorphous polymer, e.g. chitosan (Arvanitoyannis et al., 1997, 1998) or HTCC would dilute the PVA, which induced the decrease of hydrogen bonds interaction and the percentage of crystallinity as well as improved the gas/water permeability of blend films. Therefore, both of the swelling rate and ESD increased. When swollen, the non-crosslinked molecular chains in the coiled state hindered the inhalation of liquid to a certain extent, and the stretching of HTCC molecular chains prolonged the time reached equilibrium swelling.

Fig. 4 shows the effect of GA dosage on the swelling capacity of blend films containing 10 wt% HTCC. The ESD of different films decreased with increasing dosage of GA. This could be ascribed to the fact that when the dosage of GA is increased, more hydrophilic hydroxyl and amino groups were crosslinked thus the hydratability of samples was weakened. When the GA dosage was more than 0.1 wt%, the swelling rate was higher than that for the samples crosslinked with 0.045 wt% GA. However, the swelling rate and the time reached swelling equilibrium of different films have no much difference. But as a whole, the effect of GA content on the swelling kinetics of films was not so obvious compared to the content of HTCC.

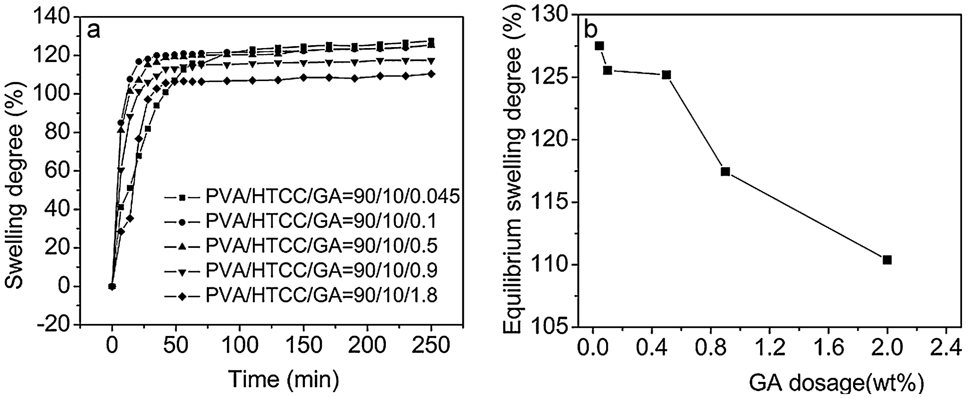

Fig. 4. The swelling kinetics (a) and EDS (b) of PVA–HTCC blend films with different GA dosages in buffer solutions (pH = 7.4). (GA content was percentage of the total weight of PVA and HTCC.)

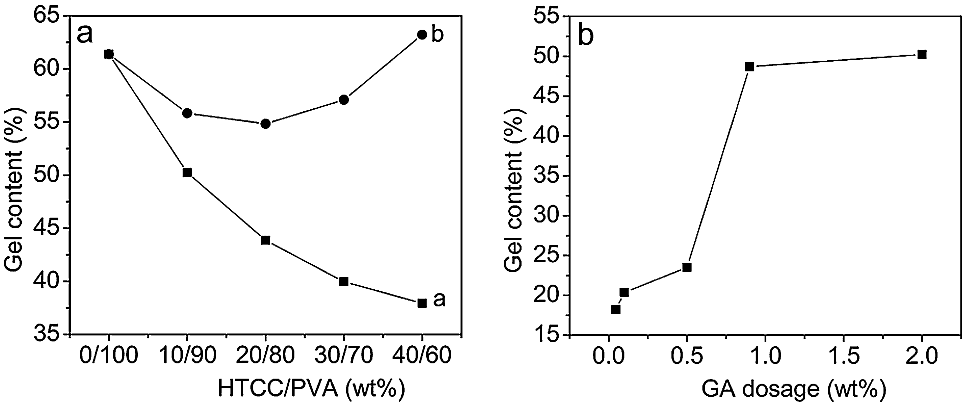

Fig. 5. Gel content of films with different HTCC–PVA compositions and 2 wt% of GA (a) and different dosages of GA while HTCC–PVA composition was fixed to be 10/90 (b).

3.3. Gel content of the cross-linked films

Gel content is one important parameter for characterizing the crosslinking degree of polymer. Fig. 5 displays the gel content of different blend films. The gel contents of films with different HTCC–PVA proportions (2 wt% GA) are showed in Fig. 5a (curve a). The gel content of the blend films decreased when the HTCC content increased. Due to most of the hydrogen atoms of $-NH_2$ on HTCC being replaced, and the reactivity of hydroxyl on HTCC was lower than that of PVA because of steric effects, and the molecular chains of HTCC hindered the crosslinking of the nearby molecular chains of PVA, thus the overall crosslinking degree of films decreased. As previously mentioned when swollen, the non-crosslinked molecular chains in the coiled state hindered the inhalation of liquid to a certain extent, and stretching process of HTCC molecular chains prolonged the time reached equilibrium swelling. These results coincided with the swelling kinetics of blend films (Fig. 3).

Considering the higher crosslinking reactivity of PVA compared with HTCC, we first assumed that only PVA was crosslinked by GA. In this case the residual gel after extracted was the crosslinked PVA networks, and then the theoretical gel content of PVA could be calculated followed formula (4) (where M_1 should be the weight of PVA in the films before extracted). Based on this assumption the calculated gel contents for the blends with different HTCC/PVA compositions were plotted as curve b in Fig. 5a. The gel content of PVA was up to 63.3% when the content of HTCC was 40 wt%, which was even higher than that of the crosslinked pure PVA (61%). The inconsistency indicates that the assumption was unreasonable, and HTCC molecules in the blends were surely crosslinked by GA at the same time. When the content of HTCC was low, the measured gel content of blend films and calculated gel content of PVA were decreased substantially by the addition of HTCC, which can be attributed to the high DS of HTCC and entanglements between molecular chains of PVA and HTCC. The gel content of PVA might be lower when the content of HTCC was higher only if PVA was crosslinked by GA. However, when the content of HTCC was up to 30 wt%, the gel content of PVA increased in contrast. This indicated that when the HTCC content was high, a portion was involved in crosslinks which resulted in an increase in gel content. But the crosslinking degree of HTCC was low and its effect on the crosslinking of PVA was not clear.

When the composition of HTCC–PVA was fixed, the gel content increased with the increase of GA dosage, especially in the range of GA dosage of 0.045–1.0 wt% (Fig. 5b). This variation tendency became unobvious with further increasing of GA dosage. When the crosslinking agent content was low, the crosslinking degree was also low, and the molecular chains were in a coiled state which did not form a fixed passage, thus reducing the liquid absorption rate and prolonged the time of reaching the swelling equilibrium. With increasing GA dosage, the crosslinking degree of film increased, and the crosslinked networks took a basic structure which could form a fixed imbibitions' passage. Therefore, as shown in Fig. 4, the swelling rate of blend films increased and samples reached the swelling equilibrium. But the difference in the swelling kinetics of the blend films was not significant for the blends while the GA dosage was over 0.1 wt%.

3.4. The antibacterial activity of blend films

Based on the QB/T 2591:^{2003} the antibacterial efficacies of the films were evaluated. Table 1 shows the results of antibacterial efficacies against *S. aureus* and *E. coli* for the samples. As can be seen, pure PVA has no antibacterial activity, and the HTCC–PVA blend film shows a strong antibacterial activity against both of *S. aureus* and *E. coli*. After crosslinked, the antibacterial efficacy of blend film was slightly weakened. This is attributed to the ammonium group with strong antibacterial activity, which had not been substituted fully by quaternary ammonium salt groups, reacted with GA (Rosa, Laranjeira, Riela, & Fávere, 2008). And this experiment provided an evidence for that the HTCC in the blend was crosslinked by GA. However, the crosslinked blend films still showed a high antibacterial activity. The reactivity of N-trimethylated quaternary ammonium salt groups in HTTCC with GA was lower than $-NH_2$ due to steric effect, and much lower than hydroxyl group in PVA. Thus much of groups with strong bacterial activity in HTCC had not reacted with aldehyde groups of GA and showed a high antibacterial efficacy. The high antibacterial efficacy and its controllability of the crosslinked PVA–HTCC

Table 1
The antibacterial activity of different films.

Samples	*S. aureus*		*E. coli*	
	Bacterial numbers (cfu/plate)	Antibacterial rate (%)	Bacterial numbers (cfu/plate)	Antibacterial rate (%)
Pure PVA	2.80×10^5	26.32	3.10×10^5	3.13
PVA/HTCC = 90/10 (wt)	2.00×10^1	99.99	4.20×10^1	99.99
PVA/HTCC/GA = 90/10/2 (wt)	1.10×10^3	99.71	3.50×10^3	98.91

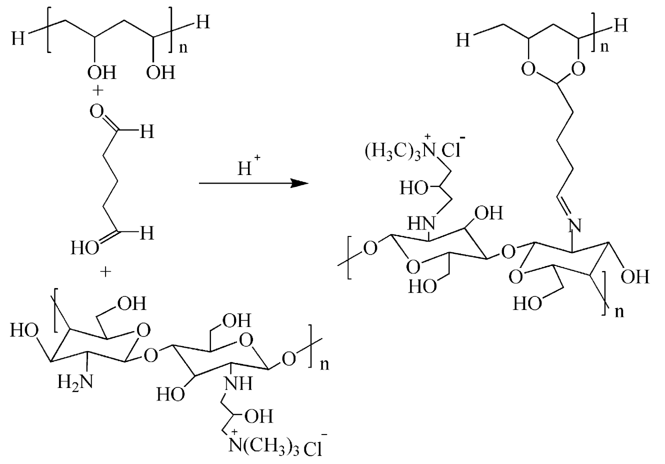

Fig. 6. Proposed crosslinking mechanism of PVA, HTCC and GA.

blend films suggested good potential applications in biomedical fields.

According to the analysis to above results (appearance, gel content, and antibacterial activity test), a possible crosslinking mechanism was proposed as Fig. 6. Both PVA and HTCC in the blends were crosslinked by GA. The reactive activity of hydroxyl group on PVA was higher than HTCC, and the ammonium group which has not been substituted fully by quaternary ammonium salt groups also reacted with GA.

4. Conclusions

In this study, a novel blend film of chitosan derivative (HTCC)/PVA was prepared by chemical-crosslinking with GA, and its swelling properties, crosslinking degree (gel content), antibacterial activities and crosslinking mechanism were studied. Results indicated that the equilibrium degrees of swelling and time reached swelling equilibrium of blend films were improved with the increase of HTCC content. The content of GA has little effect on the swelling rate and time reached swelling equilibrium. The crosslinking degree decreased with the increase of HTCC content, but contrary to the GA content. And the blend film still kept the superior antibacterial activity after crosslinking. A crosslinking mechanism HTCC and PVA with GA was proposed. The results suggested that the crosslinked PVA/HTCC blend film has good potential applications in biomedical fields, such as the drug delivery, wound-dressing.

Acknowledgements

This work was sponsored by Scientific Research Foundation for the Returned Overseas Chinese Scholars, State Education Ministry of China. The authors would like to thank Professor Teruo Nakashima in Kinki University (Japan) for his kind help and advice in measurement and evaluation of antibacterial activity of the samples.

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