Blood Compatibility and Mechanical Properties of Oxidized-Chitosan Films

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ABSTRACT: By dipping chitosan films into saturated NO_2–glacial acetic acid solution, the hydromethyl groups on the film surface could be oxidized to carboxyl groups and the blood compatibility of the films improved. Fourier transform infrared (FTIR) spectra indicated the presence of many —COOH and $—COO^-$ groups on the modified membrane surface. Scanning electron microscopy (SEM) showed that the surface of the modified membrane was rough, as compared with the chitosan film, which possessed a smooth surface. In the oxidation process, with increased dipping time of the films in saturated NO_2–glacial acetic acid solution, the tensile strength of the films decreased slowly initially, and rapidly 10 h later. The swelling ratio of the modified chitosan film increased obvious noticeably as the degree of oxidation of the film increased. All antithrombosis and hemolysis tests and blood cell morphology observation with SEM revealed that the blood compatibility of modified chitosan membranes is superior to that of chitosan films. © 2007 Wiley Periodicals, Inc. J Appl Polym Sci 106: 372–377, 2007

Key words: chitosan; 6-carboxy-chitosan; films; blood compatibility; strength

INTRODUCTION

Chitosan, a $(1\rightarrow4)$-linked 2-amino-2-deoxy-β-D-glucan, is prepared by *N*-deacetylation of chitin, which is the main structural component of crab and shrimp shells. Because of its biocompatibility and biodegradability,[1] chitosan has been developed for a variety of biomedical applications, including wound dressings and drug delivery systems.[2,3] However, chitosan, which has the common characteristics of a polycation electrolyte, was shown to adsorb erythrocytes and thrombocytes easily and could thus lead to the formation of a thrombus or cause hemolysis; it was also shown to have poor blood compatibility.[4] It is essential to improve the biocompatibility of chitosan with human tissues. Hirano et al.[5] have synthesized polysulfate chitosan derivatives and determined its antithrombin activity. These investigators have made wet spun chitosan–collagen blend fibers designed to improve blood compatibility of chitosan fibers,[6] as well. Chandy and Rao[7] found that the chitosan, cross-linked with heparin using glutaraldehyde as a crosslinking agent, has good blood compatibility. Our previous paper[8] reported that the hydromethyl group of 2-amino-2-deoxy-D-glucose unit in chitosan could be oxidized selectively to the carboxyl group with NO_2 gas to form 6-carboxy-chitosan, and that 6-carboxy-chitosan was expected to have a improvement in blood compatibility. However, our preliminary studies indicated that both polysulfate chitosan derivatives and 6-carboxy-chitosan have lower intrinsic viscosity and poor ability to form fibers or membranes.

The present work describes a method for the improvement of blood compatibility of chitosan membrane without decreasing obviously its tensile strength by selective oxidization of $—CH_2OH$ groups of chitosan molecules in the membrane surface into —COOH groups with NO_2 gas. The blood compatibilities of chitosan and modified chitosan membranes were evaluated.

EXPERIMENTAL

Materials

Chitosan was prepared by *N*-deacetylation of shrimp chitin with 50% w/v NaOH at 100°C under nitrogen. NO_2 gas was obtained from the reaction of copper with concentrated nitric acid. Other reagents were purchased from Peking Chemical Co. (Peking, China), and used without further purification.

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Preparation of chitosan and modified chitosan membranes

Chitosan films were obtained by spreading chitosan/acetic acid (0.5 mol/L) solution, degassed previously in an ultrasonic bath, on a glass plate to form a uniform thin film. After evaporation of the solvent, the membrane was removed from the plate by soaking in 0.1 mol/L phosphate buffer (pH 7.4), washed with water, and finally dried at room temperature. The modified films were prepared as follows. A chitosan film was dipped in glacial acetic acid saturated with NO_2 gas. With carefully shaking, dry NO_2 gas was slowly added to the system through a glass tube that dipped below the surface of the liquid so that the medium was saturated with NO_2 gas from beginning to end. The direct contact between the films and NO_2 gas bubbles was avoided as much as possible throughout the process. The film was carefully removed 8 h later and was washed with ethanol and water, respectively. The film was finally dried at room temperature.

FTIR spectroscopy

The infrared spectra of both chitosan and modified chitosan films were recorded (Fourier transform infrared [FTIR]) with an FTIR-8900 (Shimadzu, Tokyo, Japan) infrared spectrophotometer at the resolution of 4 cm^{-1} and 32 accumulations using the KBr pellet method.

Electron microscopy studies

The chitosan and modified chitosan films were gold-coated and visualized using a KYKY-2800 (Peking, China) scanning electron microscopy (SEM). The morphology of the blood cells was examined as follows: 4.0 mL of blood solutions (sheep blood/physiological saline solution 1 : 5) was incubated with chitosan or modified chitosan films for 4.0 h. The films and media were then removed and the blood cells were fixed in 0.25% glutaraldehyde for 24 h, followed by 1% osmium tetroxide for 1 h. Samples were then dehydrated by incubation for 5 min in phosphate-buffered saline (PBS) solutions with increasing ethanol content. Finally, they were dehydrated in hexamethyldisilazane (HMDS) and left to dry. After being gold-coated, the blood cells were visualized using KYKY-2800 SEM.

Mechanical properties and swelling ratio of the films

The breaking strength of the films was measured with a tension tester AG-2000A (Shimadzu, AUTO graph) at room temperature and a programmed elongation rate of 50 mm/min. The swelling ratio (wt %) of the films, by weighing the films before (W_1) and after

(W_2) immersion in water for 24.0 h at room temperature, was calculated from the equation:

$$wt \% = \frac{W_2 - W_1}{W_1} \times 100\%. \quad (1)$$

Determination of blood compatibility

The antithrombotic properties of chitosan and modified chitosan films were determined by spectrophotometry.[9] A film was laid on the bottom of a flask at 37°C on a constant temperature bath, and 0.25 mL of sheep blood (using EDTA as anticoagulant) was added to the center of the membrane. With careful shaking, 0.02 mL of 0.025 mol/L $CaCl_2$ solution was added and maintained for 10 min. Then 50 mL of normal physiological saline solution was carefully added. Finally the absorbance (A_S) of the solution was determined by a 722N spectrophotometer (Shanghai Tianpu Analysis Equipment, China) at 540 nm, and the blood anticoagulant index (BCI) of the sample is as follows:

$$BCI = \frac{A_S}{A_W} \times 100\%, \quad (2)$$

where A_W was the absorbance of the solution in which 0.25 mL of sheep blood was mixed with 50 mL of physiological saline solution.

The hemolylic property of chitosan and modified chitosan films was examined by spectrophotometry as well;[10] 5 mL of sheep blood was dispersed in 10 mL of physiological saline solution at 37°C, and the film was soaked in this solution for 4.0 h. After the film was removed, the suspension was centrifuged at 750g for 5 min, and the supernatant liquid containing heme was collected to determine the absorbance (A_S) at 540 nm. With the same method, the absorbance (A_N) of the supernatant liquid in which 5 mL sheep blood was dispersed in 10 mL of physiological saline solution, as well as the absorbance (A_P) in which 5 mL of sheep blood was dispersed in 10 mL of distilled water, were determined, respectively. The hemolytic ratio (HR) was as follows:
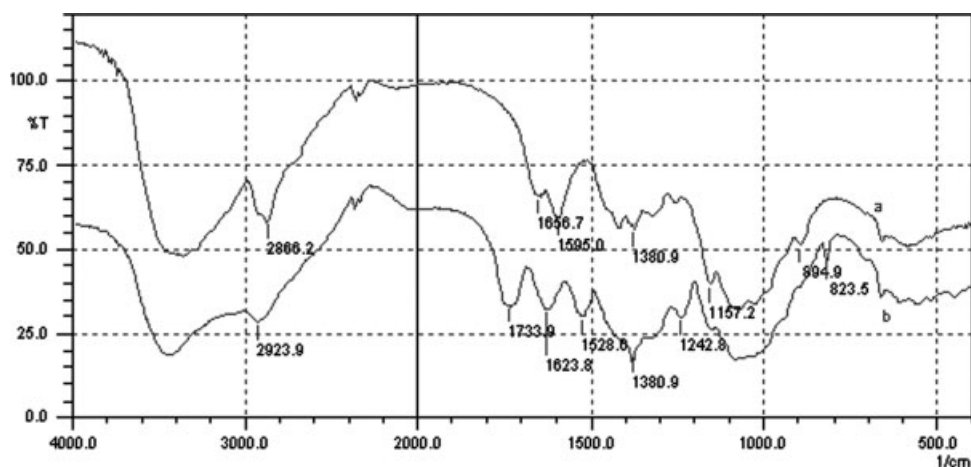
$$HR = \frac{A_S - A_N}{A_P - A_N} \times 100\%. \quad (3)$$

RESULTS

Characterization of the films

FTIR spectra

The hydromethyl group of the 2-amino-2-deoxy-D-glucose unit in chitosan could be selectively oxidized to the carboxyl group with NO_2 gas to form 6-carboxy-chitosan[8] and water. When the film was exposed

Figure 1 FTIR spectra of (a) chitosan film; (b) modified chitosan film, dipped in saturated NO_2–glacial acetic acid solution for 14 h.

to NO_2 gas some time later, the water produced during the oxidation process on the film surface could condense to tiny water drops to react with NO_2 gas to form nitric acid, and the nitric acid could induce a chitosan degradation reaction. When the oxidation procedure was executed in glacial acetic acid saturated with NO_2 gas, the water produced in the process on the film surface might be absorbed by the medium so that chitosan degradation reaction induced by nitric acid was effectively weakened.

As shown in Figure 1, all the amide $-C=O$ stretching vibration at 1656.7 cm^{-1}, the bending absorption of the $-NH_2$ group at 1595.0 cm^{-1}, and $-CH_3$ bending deformation at 1380.9 cm^{-1} appeared in the FTIR spectrum of the chitosan film [Fig. 1(a)]. In the spectrum of the modified chitosan film [Fig. 1(b)], both the new carbonyl $C=O$ stretching absorption at 1733.9 cm^{-1} and the new $C-O$ stretching absorption at 1242.8 cm^{-1} of the carboxyl group were observed. Since a part of the $-NH_2$ groups and nitric acid formed nitrate and some $-COOH$ and $-NH_2$ groups converted into $-COO^-$ and $-NH_3^+$ groups, the combination of asymmetric bending deformation of $-NH_3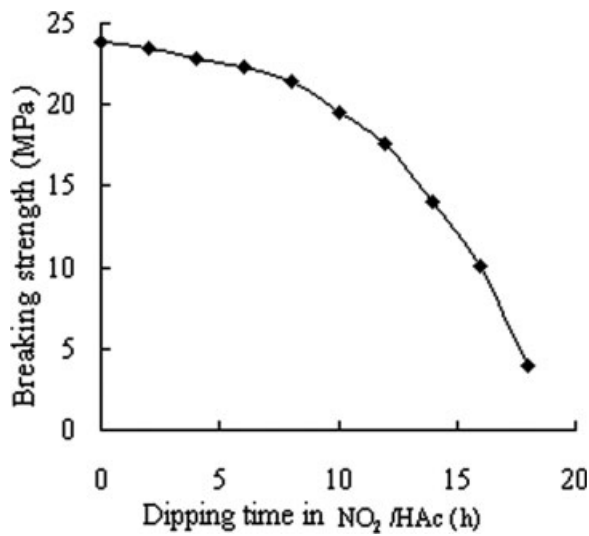^+$ groups, bending absorption of $-NH_2$ groups, and amide $-C=O$ stretching vibration gave rise to a broad absorption at 1623.8 cm^{-1} because of their similar vibration wave numbers. The symmetric bending deformation of the $-NH_3^+$ groups and the asymmetric stretching deformation of the $-COO^-$ groups overlapped at 1528.6 cm^{-1}. The absorption at 1380.9 cm^{-1} in the modified chitosan film was stronger than in the chitosan film due to the overlap of $N=O$ vibration of NO_3^- with $-CH_3$ bending deformation. The absorption at 823.5 cm^{-1} was ascribed to the $N-O$ vibration of NO_3^-. Furthermore, the characteristic absorption bands of polysaccharide combined with the $\beta(1\rightarrow4)$ glycoside bond at 894.9 cm^{-1} and 1157.2 cm^{-1} had no shift in position, but weakened in intensity. All the above indicated that the hydromethyl groups on the surface of the chitosan film were oxidized to the carboxyl groups and the surface of chitosan film was successively modified to amphoteric character, except for the slight degradation of chitosan.

SEM analysis

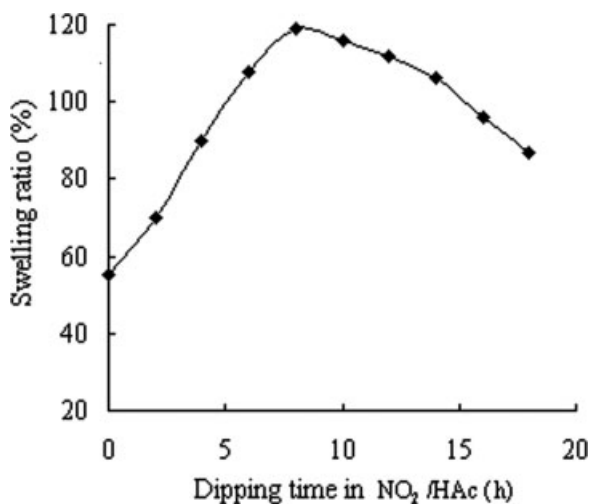Figure 2(a) shows that the surface of chitosan film is smooth. When the chitosan film contacted directly
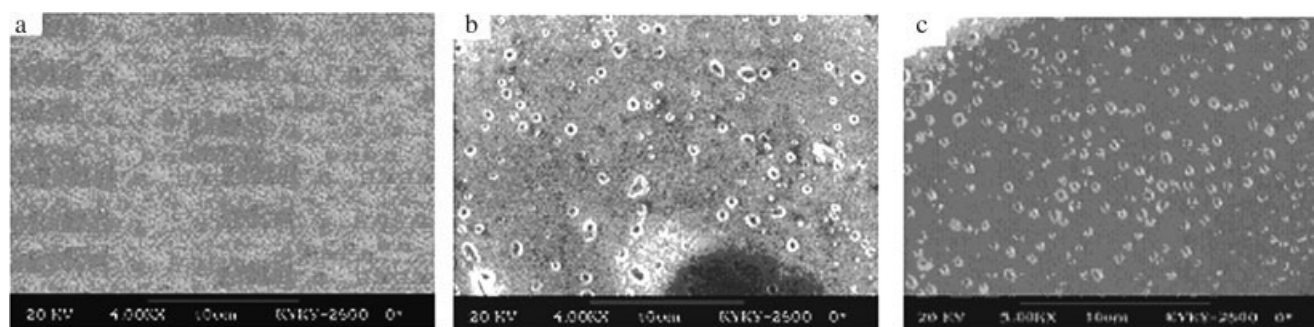
Figure 2 SEMs of (a) chitosan film; (b) modified chitosan film, exposed to NO_2 gas; (c) modified chitosan film, dipped in glacial acetic acid saturated with NO_2.
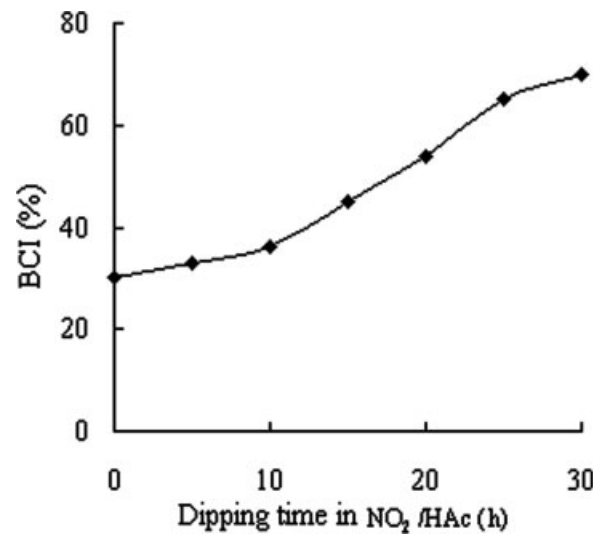
Figure 3 Influences of oxidation degree on breaking strength of the film.

Figure 5 Effects of the chitosan films with different degrees of oxidation on blood anticoagulant index (BCI).

with the NO_2 gas, not only 6-carboxy-chitosan but also the byproduct, water, were formed on the film surface. The tiny water drops formed during the oxidization process could rapidly react with NO_2 gas to give nitric acid, which induced chitosan molecules covered by the oxidized degradation of the tiny water drops, so there were a number of speckles on the modified chitosan film surface [Fig. 2(b)]. When the film was dipped into glacial acetic acid saturated with NO_2 gas, the tiny water drops produced during the oxidization process could be absorbed by glacial acetic acid, thus partly avoiding the formation of tiny nitric acid drops on the film surface, so the speckles on the film surface were smaller and more uniform [Fig. 2(c) than those on the film that was exposed to NO_2 gas directly.

Mechanical properties of films

The 6-carboxy-chitosan was an amphoteric polyelectrolyte. The electrostatic attraction between $—NH_3^+$ and $—COO^-$ groups along the same macromolecular backbone caused the conformation of 6-carboxy-chitosan molecules to fold. Unlike the chitosan molecule, which has a stretch conformation, and which forms a fiber structure easily by parallel orientation, 6-carboxy-chitosan was unable to form a fiber structure by parallel orientation. So the ability to form a fiber or membrane of 6-carboxy-chitosan was much lower than that of chitosan. By dipping chitosan film into saturated NO_2–glacial acetic acid solution, the surface molecules of the membrane could be oxidized to 6-carboxy-chitosan without obviously decreasing its
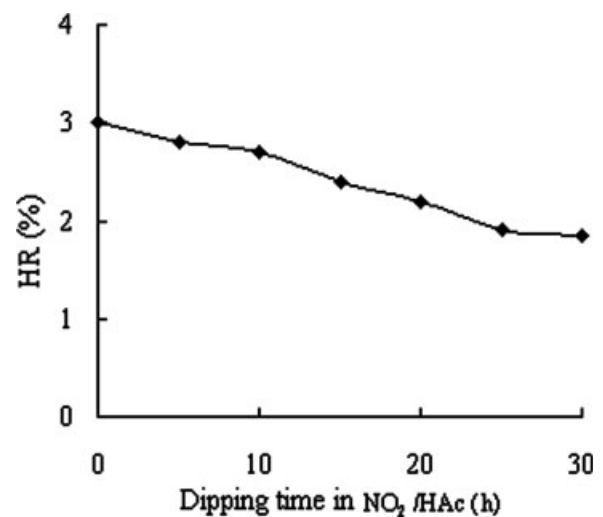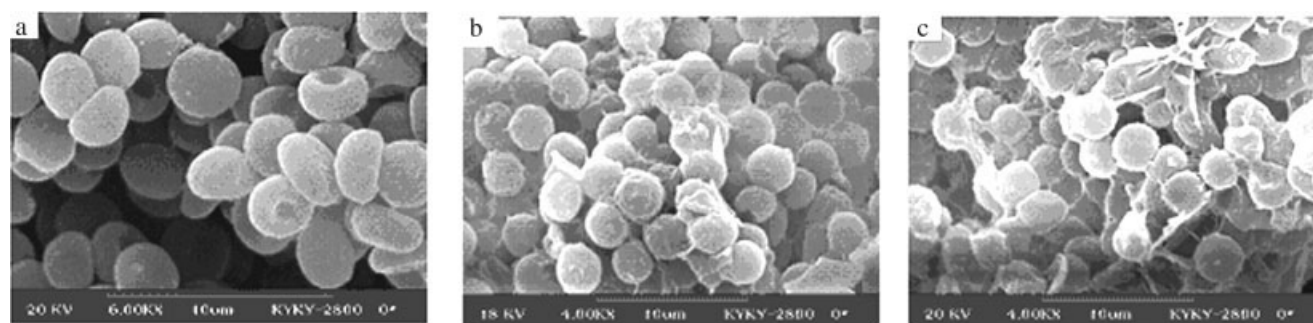

Figure 4 Effects of oxidation degree on swelling ratio of the film.

Figure 6 Effects of the chitosan films with different degree of oxidation on hemolytic ratio (HR).

Figure 7 SEMs of sheep blood cells being incubated with modified chitosan and chitosan films for 4.0 h. (a) Control sheep blood cells; (b) incubated with modified chitosan film; (c) incubated with chitosan film.

tensile strength. At the beginning of the oxidation, only the surface chitosan molecules of the film were oxidized by NO_2, and the inner molecules were unable to take part in the reaction; thus, the inner structure of the film displayed no obvious changes. Figure 3 shows that, initially, the breaking strength of the film decreased slowly with the increased dipping time. With the increased dipping time in the system, the amounts of water produced in the oxidation process increased gradually and could not be completely absorbed by the glacial acetic acid, which gave rise to the formation of nitric acid on the film surface. Nitric acid could easily induce the outer or inner chitosan molecules of the film oxidized degradation and damaged the inner microfiber structure. The breaking strength of the film then decreased rapidly after being dipped in the system for 10.0 h.

Swelling ratio

After oxidation, the abundance of carboxyl groups on the film surface led to increased hydrophilicity of the film. In addition, the fold conformation of 6-carboxychitosan molecules was able to weaken the intermolecular hydrogen bond[11] in the film. These two factors permitted the water molecules to permeate the modified chitosan films more easily than they were able to permeate the chitosan films. Figure 4 shows that, initially, the swelling ratio of the film increased rapidly with an increased dipping time in glacial acetic acid saturated with NO_2 gas. With a dipping time of > 10.0 h in the system, the swelling ratio of the film began to decrease. This was because the side reaction, i.e., the oxidative degradation reaction, in the oxidation process made the chitosan molecules in the films decompose to small ones and the small chitosan molecules might dissolve in water during the immersion process.

Blood compatibility of the films

The blood compatibility of the films was estimated by their BCI and HR. As for the modified chitosan films,

the degree of oxidation of the film increased with increased dipping time in the system. The relationship between BCI or HR and the degree of oxidization of modified film could be demonstrated by the BCI or HR variety with the dipping time in saturated NO_2– glacial acetic acid solution, as shown in Figures 5 and 6. The BCI value increased as the degree of oxidization of the films was raised (Fig. 5). This was because when the modified films contacted the blood (pH 7.35–7.45), the —COOH groups on the membrane surface converted into —COO^- groups. Thus, the abundance of negative charges on the membrane surface might prevent the film from adsorbing blood cells because of the electrostatic repulsion between the chitosan membrane surface and the blood cells, thereby avoiding the coagulation of blood cells. Although both chitosan and modified chitosan membranes have low HR values, it was obvious that the HR values of modified membranes decreased as the degree of oxidization increased (Fig. 6). This occurred because the chitosan membrane carried a trace of positive charges on its surface due to amino protonation. When the membrane contacted blood, the electrostatic attraction between the chitosan membrane surface and the erythrocyte membrane containing anionic glycoproteins induced curvature of the erythrocyte membrane, ultimately leading to the rupture and release of hemoglobin. As for the modified chitosan membranes, because of the abundance of negative charges on its surface, the attraction between the modified chitosan membrane surface and the erythrocyte membrane could be decreased or avoided; the same effect was observed with the hemolysis. This result was clearly also visualized by SEM (Fig. 7). After incubation with chitosan or modified chitosan films for 4.0 h, blood cells were damaged more seriously by chitosan film than by modified film.

CONCLUSION

Dipping chitosan films into saturated NO_2–glacial acetic acid solution permitted the hydromethyl groups on the surface to be oxidized to the carboxyl

groups, and led to improved blood compatibility of the films. During the oxidation process, with an increased dipping time of the films in the system, the tensile strength of the films decreased slowly at first, and then rapidly 14 h later. The modified chitosan films could be swelled in water more easily than was possible for the chitosan films.

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