Preparation of Water-Soluble Chitosan Derivatives and Their Antibacterial Activity

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ABSTRACT: Carboxymethyl chitosan sodium (CMCTS) was synthesized by chitosan and chloroacetic acid under an alkali catalyst. Acrylic acid sodium salt and methylacrylic acid sodium salt were grafted onto CMCTS to obtain copolymers with good water solubility. The graft reaction was carried out at 70°C for 2 h, and ammonium persulfate was used as an initiator. The structure changes of chitosan and its derivatives were investigated by the FTIR. The antibacterial activity of chitosan derivatives against *Staphylococcus aureus* and *Escherichia coli* were explored by the viable cell counting method. © 2002 Wiley Periodicals, Inc. J Appl Polym Sci 85: 1357–1361, 2002

Key words: graft copolymers; carboxymethyl chitosan; antibacterial activity; FTIR; structure

INTRODUCTION

Chitin is one of the most important natural polysaccharides, and it is widely found in the shells of arthropods (crabs, shrimp, etc.). Chitosan is its principal derivative, and it exists in the cell walls of fungi and algae. These substances have many applications in drug delivery systems, hemodialysis, artificial skin, and wound repairing materials because of their good biocompatibility, biodegradation, and nontoxicity.[1–3]

Chemical modification is helpful in improving the water solubility of chitosan and its derivatives, thus widening their applications.[4–8] Graft copolymerization is anticipated to be a promising approach because it can provide a wide variety of molecular designs. Many articles have been published on graft copolymerization onto chitin or chitosan.[9–21] However, there are very little reports about graft copolymerization onto the derivatives of chitin or chitosan.[22–25] In this study, acrylic acid sodium salt (AASS) and methylacrylic acid sodium salt (MAASS) were grafted onto carboxymethyl chitosan sodium (CMCTS) to obtain copolymers with good water solubility. Their antibacterial activities were explored by the viable cell counting method for possible application in wound repairing materials.

EXPERIMENTAL

Materials

The chitosan used in this study is a commercial material purchased from Zhejiang Yuhuan Biochemical Co. Ltd. Its degree of deacetylation is 97% as determined by ^{1}H-NMR, and its viscosity molecular weight is 8.8×10^5. After being dissolved in dilute acetic acid and neutralized by NaOH solution, it was then purified in a Soxhlet

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apparatus by refluxing in alcohol for 24 h and dried at 60°C under a vacuum for 48 h. Ammonium persulfate (APS) is an analytical grade reagent that was used as an initiator. The AASS and MASS were obtained by neutralization after the acids were redistilled under a vacuum. All other reagents were analytical grade and used as received.

Staphylococcus aureus and *Escherichia coli* were supplied by the Microbiology Laboratory of Zhejiang University.

Preparation of CMCTS

The CMCTS was prepared according to a similar method described by Zhang et al.[26] Purified chitosan (4.0 g) was added to 40.0 g of NaOH solution (50%) and put into a refrigerator at −18°C overnight for alkalization. After the excessive alkali solution was extruded, the chitosan was put into a 100-mL reactor. Isopropyl alcohol (20.0 mL) was added and chloroacetic acid (5.0 g) dissolved in 20.0 mL of isopropyl alcohol was added into the system dropwise. Then the mixture was refluxed at 65°C under extensive stirring. After an additional 2 h the reaction was stopped and the solvent was discarded. The mixture was neutralized and precipitated in acetone. The product was carefully washed successively by absolute alcohol and 75% alcohol, then dried at 60°C under a vacuum.

Graft Copolymerization

The CMCTS (0.20 g) and a predetermined amount of monomer were added into a 100-mL reactor and stirred for 30 min under a nitrogen atmosphere with heating to 70°C; then 0.10 mmol APS dissolved in 10.0 mL of H_2O was slowly added into the reactor to initiate the graft polymerization. The reaction products were precipitated in acetone, filtrated, washed with acetone, and dried at 60°C under a vacuum. The homopolymers were extracted in a Soxhlet apparatus by refluxing in methanol for 24 h and dried at 60°C under a vacuum for 48 h. The grafting percentage (G) was calculated according to the following equation:

$$G = [(W_2 - W_1)/W_1] \times 100 \quad (1)$$

where W_1 represents the weight of CMCTS and W_2 represents the products after refluxing by methanol.

Characterization of Chitosan Derivatives

The degree of substitution of CMCTS (0.45) was determined from elemental analysis carried out on a CE instruments apparatus (model EA 1110, ThermoQuest Italia S.P.A). The structure changes of chitosan and its derivatives were investigated by FTIR (Nicolet 470).

Evaluation of Antibacterial Activity

Freeze-dried ampoules of *S. aureus* (SP) and *E. coli* (SP) were opened, and a loopful of culture was spread as single colonies on nutrient agar and incubated at 37°C for 24 h. A representative colony was picked off with a wire loop and placed in 5 mL of nutrient broth (1% peptone, 0.5% NaCl, 0.3% beef extract, pH 7.4), which was then incubated overnight at 37°C. The cultures of *E. coli* and *S. aureus* containing $\sim 10^7$ cells/mL were prepared and used for the antibacterial tests by appropriately diluting them with sterile 0.85% NaCl solution.

The solutions of chitosan derivatives with different concentrations were prepared in distilled water and then sterilized at 121°C for 30 min. Exposure of bacterial cells to the copolymers was started when 0.5 mL of the bacterial culture was added to 4.5 mL of the copolymer solution that was preequilibrated at 37°C. At various contact times, decimal serial dilutions were prepared by taking 0.1-mL portions into 9.9 mL of sterile 0.85% NaCl solution and mixing. From these dilutions, 0.2-mL portions were removed and quickly spread on the nutrient agar. The plates were incubated at 37°C and the colonies were counted after 24 and 48 h. The counting was done in triplicate each time.[27] The initial cell concentration was determined by a similar spread plate method.

RESULTS AND DISCUSSION

The grafting yield of the chitosan derivatives was determined by the changes in weight before and after the graft reaction and is summarized in Table I.

The graft copolymerization of CMCTS with AASS and MAASS was identified by FTIR spectroscopy. As shown in Figure 1, the IR spectrum of chitosan (curve a) shows peaks assigned to the saccharide structure at 1152, 1082, 1028, and 897 cm^{-1} and a strong amino characteristic peak at

Table I Graft Polymerization of Maleic Acid onto CMCTS at 70°C

Copolymers	Monomers Concn (mol/L)	Grafting (%)	Water Solubility
CMCTS-*g*-AASS	1.2	875	Water soluble
CMCTS-*g*-MAASS	1.2	933	Water soluble

Reaction conditions: 0.20 g CMCTS and 0.4 m*M* APS for 2 h.

around 3420 cm^{-1}. The peaks at 1640 and 1325 cm^{-1} are assigned to amide I and III bands, respectively. The peak at 1421 cm^{-1} is the joint contribution of bend vibrations of OH and CH. In the IR spectrum of CMCTS (Fig. 1, curve b) the strong peaks at 1596 and 1411 cm^{-1} could be assigned to the respective asymmetry and symmetry stretch vibration of COO^-. Also, the C—O adsorption peak of the secondary hydroxyl group becomes stronger and moves to 1070 cm^{-1}. The results indicate that the substitution occurs at the C_6 position. In the IR spectra of CMCTS-*g*-MAASS (Fig. 1, curve c), there is a characteristic absorption band of carboxylate found at 1545 cm^{-1}. In the IR spectra of CMCTS-*g*-AASS (Fig. 1, curve d), a characteristic absorption band of carboxylate is found at 1561 cm^{-1}. The peaks at 1410 and 1322 cm^{-1} are the characteristic peaks of poly(acrylic acid sodium). In the IR spectra of the two copolymers, the characteristic absorption peaks of polysaccharide at around 1100 cm^{-1} are not so clear because of the high grafting percentage.

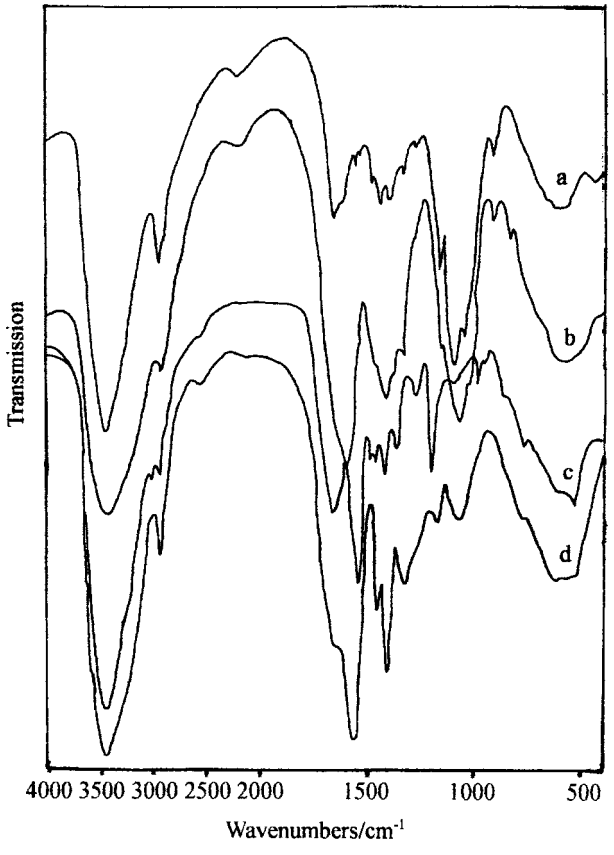

Figure 1 FTIR spectra of CTS (curve a), CMCTS (curve b), CMCTS-*g*-MASS (curve c), and CMCTS-*g*-ASS (curve d).

The antibacterial activity of CMCTS-*g*-AASS and CMCTS-*g*-MAASS was explored by the viable cell counting method. The *S. aureus* and *E. coli* bacteria were selected as test cells because they are the most frequent bacteria in wound infection and representative Gram positive and Gram negative bacteria, respectively. Three plates were counted to obtain an average value for the viable cells. Figure 2 shows plots of the log (survivors) versus exposure time for CMCTS-*g*-AASS with *S. aureus* [Fig. 2(a)] and *E. coli* [Fig. 2(b)]. About 10^7 cells/mL of *S. aureus* and *E. coli* were exposed to 10 and 1 μg/mL, respectively, of CMCTS-*g*-AASS in sterile distilled water. As shown in the figure, the antibacterial action is obvious at two concentrations. More than 99.98% of *S. aureus* and 99.99% of *E. coli* were killed within 30 min of contact even at 1 μg/mL, which shows that this chitosan derivative has a high rate of killing cells and high antibacterial activity. After 30 min the cell survivors decline slowly or have a small increase, especially the *E. coli* at 1 μg/mL. This means that CMCTS-*g*-AASS has low bacteriostatic action at low concentration. The bactericidal action is principal in the initial contact of the copolymer and bacteria. After 30 min the cell survivors do not decline with the increase in contact time, which indicates the optimal bactericidal time is within 30 min. Figure 3 shows the effect of CMCTS-*g*-MAASS against *S. aureus* [Fig. 3(a)] and *E. coli* [Fig. 3(b)]. More than 99.98 and 99.97% of *S. aureus* were killed within 30 min at concentrations of 10 and 1 μg/mL, respectively. The concentration dependence of the effect of

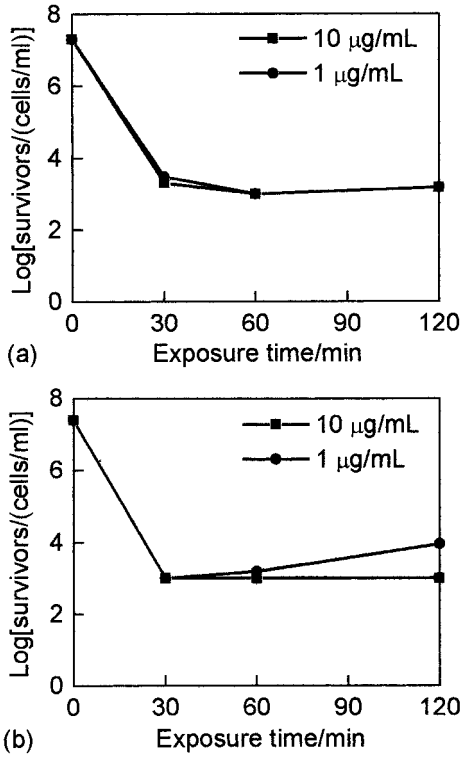

Figure 2 Plots of log (survivors) versus the exposure time for CMCTS-*g*-AASS against (a) *Staphylococcus aureus* and (b) *Escherichia coli*.

CMCTS-*g*-MAASS on *S. aureus* is obvious and adheres to the similar rule that the viable cells of *S. aureus* decrease with the increase in the contact time. There are fewer survivors of cells at a high concentration of the copolymer and with the increase in contact time of the copolymer and cells. All of the *E. coli* were killed within 30 min of the contact of the cells and CMCTS-*g*-MAASS at 10 μg/mL, while there are still some viable cells at 1 μg/mL.

The CMCTS-*g*-MAASS is more effective than CMCTS-AASS, especially against *E. coli*, which has a relatively complicated cell wall with a bilayer structure. It has a thin membrane containing less peptidoglycan than that of *S. aureus*, and thus has a relatively loose network structure. Thus, it is easily attacked by the copolymer. Its outer membrane contains lipopolysaccharides, lipids, and proteins associated with hydrophobic interaction. The hydrophobicity of the outer membrane is easily attached by CMCTS-*g*-MAASS that has a relatively strong hydrophobicity because of its methylene groups in the polymer chains.

The antibacterial mechanism of chitosan is generally considered to ascribe to the cationic nature of chitosan under an acidic condition. This chitosan derivative is mainly anionic nature under neutral conditions. There have been no reports on the antibacterial mechanism of water-soluble anionic chitosan derivatives with high molecular weight. Chitosan has been reported to bind a range of heavy metals and trace elements. The coordination capability of chitosan is related to the content of free NH_2 groups. The degree of protonation of NH_2 in chitosan is constant at a given pH value.[28] When the pH value is high, the degree of protonation of NH_2 groups is low. Because the antibacterial test is explored in sterile distilled water, the amino group is free and has strong coordination ability. Based on the above facts, a possible reason might be ascribed to the strong coordination capability of chitosan toward metals.

The antibacterial activities of this chitosan derivative can also be closely correlated to the formation of hydrophobic microareas. At pH 7 the degree of protonation of NH_2 is very low and thus the repulsion of NH_3^+ is weak, so the strong intermolecular and intramolecular hydrogen bond results in the formation of hydrophobic microareas

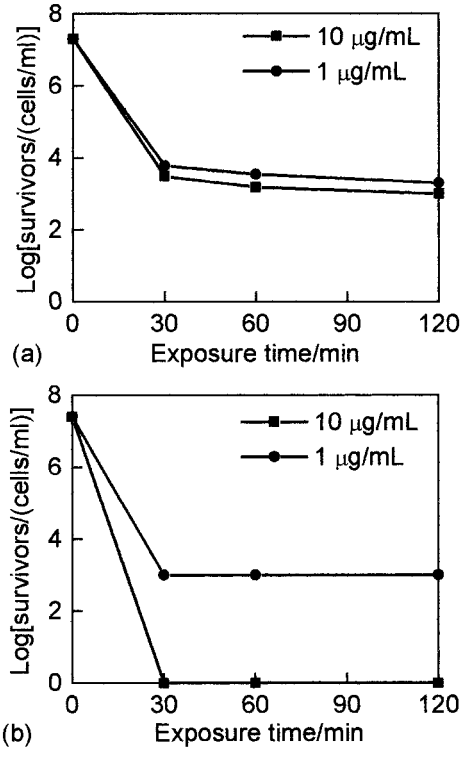

Figure 3 Plots of log (survivors) versus the exposure time for CMCTS-*g*-MAASS against (a) *Staphylococcus aureus* and (b) *Escherichia coli*.

in the polymer chains.[28] At the same time, the carboxyl groups in the polymer chains are strongly hydrophilic. Therefore, the polymer chains have hydrophobic and hydrophilic parts. This amphiphilic structure provides structure affinity between the cell walls of the bacteria and the chitosan derivatives. On the other hand, carboxyl groups having strong polarity can react with phospholipids in cell membranes and the nonpolar parts of the derivatives can insert the hydrophobic areas. The cell membranes will be broken because of the strong electrostatic interaction and result in the death of cells. The membrane destruction mechanism can be proved by transmission electron microscopy, which is currently being investigated in our laboratories.

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