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Preparation and antibacterial activity of a water-soluble chitosan derivative

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Abstract

Hydroxypropyl chitosan (HPCTS) was synthesized from chitosan and propylene epoxide under alkali condition. It was characterized by Fourier-transform infrared (FTIR), NMR, and elemental analysis. To obtain multiple-derivated chitosan, maleic acid sodium (MAS) was grafted onto HPCTS at 70 °C for 2 h in an aqueous solution using ammonium persulfate as an initiator. The grafted copolymer was confirmed by FTIR. At high grafting percentage, good water solubility at various pH values will be obtained. Antibacterial activities of the derivatives against *Staphylococcus aureus* and *Escherichia coli* were explored by the cut plug method and the viable cell counting method in sterile distilled water. More than 99.9% of *S. aureus* and *E. coli* were killed within 30 min of contact with the derivative HPCTS-*g*-MAS 3 at the concentration of 100 ng ml^{-1}. The action was more effective against *S. aureus* than *E. coli*, which could be attributed to the fact that the latter has a complicated bilayer cell structure. The mechanism of action should be related to the fact that the derivative has strong coordination capability and its amphiphilic structure.

Keywords: Hydroxypropyl chitosan; Multiple-derivated chitosan; Maleic acid sodium; Antibacterial activity; Viable cell counting method

1. Introduction

Marine polysaccharide drugs have attracted much attention (Faulkner, 2001). Chitosan, one of the most important marine polysaccharides, has many peculiar biological activities such as immunity, norcholesterol, and antibacterial, and thus has prospective applications in the fields of medicine and biotechnology (Felse & Panda, 1999; Kumar, 2000).

Chitosan has poor solubility, which has limited its applications as a polysaccharide drug. Chemical modification has been attempted to improve water solubility and bioactivities of chitosan. Regioselective reaction will be helpful to obtain chitosan derivatives with special structure or unique functions such as anti-HIV-1 activity (Kurita, Shimada, Nishiyama, Shimojoh, & Nishimura, 1998; Nishimura et al., 1998). However, the reaction course is complicated and the isolation of products is difficult. Among various modification methods, graft copolymerization is anticipated to be a promising approach because it can be easily carried out and provide a wide variety of molecular designs.

According to the structure–activity relationship, multiple derivation of chitosan is quite significant in view of preparing polysaccharide-based advanced materials with multifunction. The study of multifunctional chitosan derivatives will be favorable to find new kinds of polysaccharide drugs with multifunctions such as antibacterial, antioxidant, antitumor activities and so on. Interestingly, many chitosan derivatives have been found to have antibacterial activity (Muzzarelli et al., 1990; Jung, Kim, Choi, Lee, & Kim, 1999; Kim, Choi, Chun, & Choi, 1997; Kochkina & Chirkov, 2000; Liu, Guan, Yang, Li, & Yao, 2001).

In this paper, multiple-derivated chitosan (HPCTS-*g*-MAS) was prepared by etherification of chitosan with propylene epoxide followed by the graft copolymerization of maleic acid sodium (MAS). Here, the antibacterial activities against *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were explored by the cut plug method and the viable cell counting method for possible applications in wound repairing materials, antibacterial fibers, and food preservation.

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2. Experimental

2.1. Materials

Chitosan is a commercial material supplied by Zhejiang Yuhuan Biochemical Co. Ltd (China). It has deacetylation degree of 97%, calculated from ^{1}H NMR, and viscosity average molecular weight of 8.8×10^5. After dissolved and precipitated several times, it was purified in a Soxhlet apparatus by refluxing in alcohol for 24 h, then dried at 60 °C under vacuum for 48 h. Ammonium persulfate (APS) is an analytical grade reagent and used as an initiator. MAS was obtained by the neutralization of maleic acid (analytical grade). All other reagents are analytical grade and used without further purification.

S. aureus and *E. coli*, supplied by Microbiology Laboratory of Zhejiang University, were selected as test cells because they are the most frequent bacteria in the wound infection and representative gram-positive and gram-negative bacteria, respectively. The two bacteria were incubated at 37 °C for 24 h on a nutrient agar plate before use.

2.2. Preparation of hydroxypropyl chitosan

Hydroxypropyl chitosan (HPCTS) was synthesized from chitosan and propylene epoxide under alkali condition. Purified chitosan (3.0 g) was added into 30.0 g 50 wt% NaOH solution and put into a refrigerator at −18 °C for alkalization. Alkali chitosan and isopropyl alcohol (30.0 ml) were mixed and stirred for 1 h at 40 °C. Then 30.0 ml propylene epoxide was added, and refluxed 2 h at 60 °C with continuous stirring. The reaction mixture was adjusted to pH 7.0 by adding 1:1 (v/v) HCl, filtrated, and the obtained product was repeatedly washed by acetone and 95% (v/v) alcohol, then dried under vacuum at 60 °C for 48 h.

2.3. Graft copolymerization

A small amount of HPCTS (0.20 g), a predetermined amount of MAS, and 15.0 ml of deionized water were charged into a three-necked round-bottomed flask in a water bath at 70 °C. Nitrogen gas was bubbled for 30 min to remove the dissolved oxygen under stirring. 0.10 mmol APS dissolved in 10.0 ml H_2O was slowly added into the reactor to initiate the graft polymerization. Reaction products were precipitated in acetone, filtrated, washed with acetone, and dried at 60 °C under vacuum. Homopolymers were extracted in a Soxhlet apparatus by refluxing in alcohol for 24 h, and dried at 60 °C under vacuum for 48 h. The graft parameters were calculated in the following manner.

$$\text{Grafting percentage (G\%)} = [(W_3 - W_1)/W_1] \times 100 \quad (1)$$

$$\text{Grafting efficiency (GE\%)} = [(W_3 - W_1)/(W_2 - W_1)] \times 100 \quad (2)$$

$$\text{Total conversion (TC\%)} = [(W_2 - W_1)/W_4] \times 100 \quad (3)$$

where W_1, W_2, W_3, and W_4 denote the weight of HPCTS, weight of graft copolymerization product, weight of graft copolymer, and weight of monomer, respectively.

2.4. Characterization of chitosan derivatives

Elemental analysis was performed in a CE instruments apparatus Mod. EA 1110 (ThermoQuest Italia S. P. A). ^{1}H NMR spectrum was measured on a Bruker DMX 500 spectrometer at 70 °C. HPCTS was dissolved in D_2O, which contained a small amount of CF_3COOH. ^{1}H chemical shifts were expressed in ppm downfield from the signal for sodium 4,4-dimethyl-4-silapenture sulfonate (DSS) as an internal reference. A Fourier-transform infrared (FTIR) spectrometer (Nicolet 470) was employed to confirm the structures of chitosan derivatives. Molecular weights of graft copolymers were determined by gel permeation chromatography (GPC) with poly(ethyl oxide) as standard on a Waters-208 apparatus. (column, Ultrahydrogel 500 and 1000; eluent, H_2O; flow rate, 0.8 mg ml^{-1}; column temperature, 32 °C; concentration of samples, 2 mg ml^{-1}).

2.5. Evaluation of antibacterial activity

The antibacterial spectrum of chitosan and its derivatives was determined against the test bacteria on powdery samples by the cut plug method on nutrient agar (peptone 1%, NaCl 0.5%, beef extract 0.3%, agar 2%, pH 7.4) (Pridham et al., 1956). The assay plates were seeded with the test bacteria, after the solidification the wells were made and filled with 10 mg powdery sample. The plates were incubated at 37 °C for 24 and 48 h, respectively, after which the diameters of inhibition zones were measured.

The derivative HPCTS-*g*-MAS 3 with good water solubility was further assayed by viable cell counting method to quantify the inhibitory effect. A loopful of each culture was spread, respectively, to give 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 (peptone 1%, NaCl 0.5%, beef extract 0.3%, pH 7.4), which was then incubated overnight at 37 °C. By appropriately diluting with sterile distilled water, the culture of *E. coli* and *S. aureus* containing $\sim 10^8$ cells per ml were prepared and used for antibacterial test.

The solution of HPCTS-*g*-MAS with different concentration were prepared in distilled water and then sterilized at 121 °C for 30 min. Exposure of bacterial cells to the copolymer was started when 0.5 ml of the bacterial culture was added to 4.5 ml of the copolymer solution which was pre-equillibrated at 37 °C. At various contact times (30, 60, 90, and 120 min), decimal serial dilutions were prepared by taking 0.1 ml portions into 9.9 ml of sterile distilled water

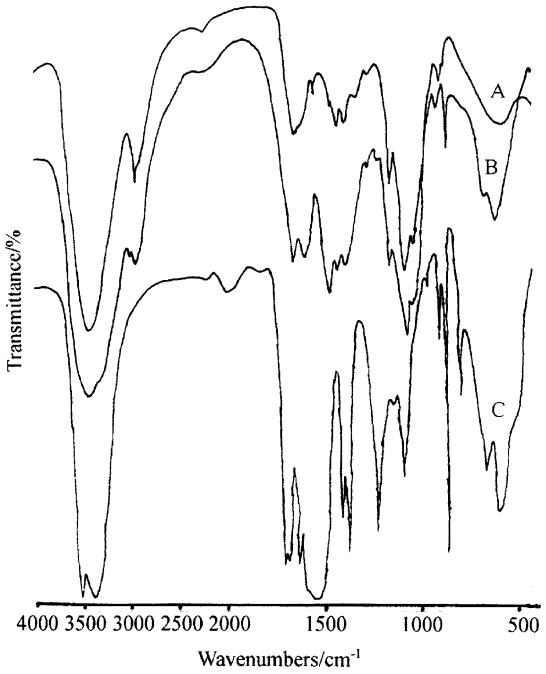

Fig. 1. FTIR spectra of (A) chitosan, (B) HPCTS, and (C) HPCTS-*g*-MAS 3.

and mixing intensively. From this dilutions 0.2 ml portions were, respectively, 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, respectively. Three parallel plates were used for counting the viable cells and the counting was done in triplicate each time. The initial cell concentration was enumerated by the similar spread plate method.

3. Results and discussion

3.1. Preparation and characterization of chitosan derivatives

The degree of substitution of HPCTS was calculated by the elemental analysis data: Anal. Calcd for $[C_6H_{10}NO_4(C_3H_7O)_{0.93}(H)_{0.07}\cdot 1.6H_2O]_n$: C, 43.11; N, 5.72; H, 6.94. Found: C, 43.23; N, 5.74; H, 6.98. The deacetylation degree of HPCTS was taken as 100%, because the deacetylation followed the course of alkalization and etherification. In the ^{1}H NMR spectroscopy, the protons of the hydroxypropyl moiety successively absorb at $\delta = 3.10$, 5.05 and 3.80 ppm.

Structure changes of chitosan and its derivatives were confirmed by FTIR spectroscopy (Fig. 1). The IR spectrum of chitosan shows peaks assigned to the saccharide structure at 1152, 1082, 1028, and 897 cm^{-1}, and a strong amino characteristic peak at around 3420, 1655 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 vibration of OH and CH. In the IR spectrum of HPCTS, the strong peak at 1460 cm^{-1} could be assigned to the asymmetry deformation of CH_3. And the C–O adsorption peak of secondary hydroxyl group becomes stronger and moves to 1067 cm^{-1}. The intensity of the primary alcohol at around 1025 cm^{-1}, due to C–O stretching vibration, becomes much smaller than in chitosan. The new peak that appears at 2970 cm^{-1} indicates the incorporation of the hydroxypropyl moiety. The results indicate that the substitution occurs at C_6 position. The copolymers all have peaks at 1700 cm^{-1} and characteristic broad bands of carboxylate at 1560–1520 cm^{-1} (here only HPCTS-*g*-MAS 3 was shown). As expected, it is difficult to find characteristic peaks of saccharide unit in the FTIR spectra of the grafted copolymer because of high grafting percentage.

The substituting degree of HPCTS is 0.93, but it has poor water solubility and can only swell in deionized water or under acidic condition, which perhaps should be attributed to its low substituting degree and the high molecular weight of chitosan. Compared with chitosan, HPCTS has higher reactivity for the graft copolymerization (shown in Table 1). There is a lower G%, GE%, and TC% when MAS was grafted onto chitosan. The incorporation of hydroxypropyl group into chitosan will decrease effectively intermolecular hydrogen bond; increase the accessibility and homogenization of graft copolymerization. CTS-*g*-MAS prepared under the similar reaction condition has poor water solubility, which is not suitable for the above antibacterial test.

The grafting parameters of chitosan derivatives, determined by changes in weight before and after the graft reaction, were summarized in Table 2. The grafting percentage increases with the increase in concentration of the monomer. As expected, the grafted copolymer has better water solubility at higher G%. Table 3 shows the average molecular weight of graft copolymers. With the increase of G%, molecular weights increase.

Table 1
Graft copolymerization of chitosan and HPCTS (reaction conditions: CTS, HPCTS: 0.2 g; MAS: 1.2 mol/l; APS: 0.4 mM; 70 °C; 2 h)

Graft copolymer	Percentage of grafting (%)	Efficiency of grafting (%)	Total conversion (%)	Water solubility[a]
CTS-*g*-MAS	867	61.9	43.8	Poor water solubility
HPCTS-*g*-MAS	1633	84.5	98.6	Good water solubility

[a] 10 mg copolymer was dissolved in 20 ml deionized water and put for 2 days.

Table 2
Graft copolymerization of MAS onto HPCTS (reaction conditions: HPCTS: 0.2 g; APS: 0.4 mM; 70 °C; 2 h)

Copolymer	Concentration of monomer (mol l^{-1})	Percentage of grafting (G%)	Efficiency of grafting (GE%)	Total conversion (TC%)	Water solubility[a]
HPCTS-MAS 1	0.6	560	81.9	69.7	Water-insoluble[b]
HPCTS-MAS 2	1.2	1633	84.5	98.6	Water-soluble
HPCTS-MAS 3	1.8	1720	63.7	90.0	Water-soluble

[a] 10 mg copolymer was dissolved in 20 ml deionized water and put for 2 days. The pH values were adjusted by dilute HCl or NaOH solution to evaluate solubility of copolymers under different pH values.
[b] HPCTS-MAS 1 swelled in deionized water and turned into a hydrogel. It swelled under acidic condition, and was dissolved when pH > 12.

3.2. Antibacterial activity assay

Antibacterial activities of chitosan and its derivatives against *S. aureus* and *E. coli* were explored by the cut plug method and the viable cell counting methods. The capability of chitosan derivatives to inhibit the growth of the tested bacteria on solid media is shown in Table 4. The results show that chitosan and HPCTS have no effect against the test bacteria, which should be owe to their poor water solubility and their high molecular weight (Liu et al., 2001). The derivative HPCTS-*g*-MAS 1 has small diameters of inhibition zone, which should be mainly attributed to the fact that it can only swell in the deionized water. HPCTS-*g*-MAS 2 and HPCTS-*g*-MAS 3 have almost the same diameters of inhibition, and the results show that the antibacterial activity against *E. coli* is more effective than *S. aureus*.

Antibacterial activities of the copolymer at different concentrations against *S. aureus* and *E. coli* were explored by the viable cell counting method described earlier. HPCTS-*g*-MAS 3 with good water solubility was selected for antibacterial test. Fig. 2(a)–(c) show plots of log (survivors) verus exposure time for the copolymer against *S. aureus* and *E. coli* at different concentrations. About 10^8 cells per ml of *S. aureus* and *E. coli* were exposed to 10, 1 μg ml^{-1}, and 100 ng ml^{-1} of the copolymer in sterile distilled water, respectively. As shown in the figures, the effect of the copolymer is obvious at three different concentrations. The viable cells decrease with the increase in contact time of the copolymer and cells. More than 99.9% of *S. aureus* and *E. coli* were killed within 30 min of contact even at the lowest concentration of 100 ng ml^{-1}, which shows that this copolymer has high rate of killing cells and high antibacterial activity. Moreover, the bactericidal action is principal in the initial contact of the copolymer and the bacteria. After 30 min the survivors of cells decline slowly with the increase in contact time, which indicates the bacteriostatic action is main. Concentration dependence of bactericidal action is not so obvious, perhaps should be attributed to the large molecular weight of the graft copolymer ($M_n = 40{,}000$, shown in Table 3) and the fact that all the three concentrations are lower than its minimum bactericide concentration.

Table 3
GPC results of grafted copolymers

Copolymer	M_n	M_w	M_z	M_{z+1}	Polydispersity
HPCTS-MAS 1	29,000	53,000	114,000	196,000	1.83
HPCTS-MAS 2	30,000	60,000	136,000	240,000	1.96
HPCTS-MAS 3	40,000	74,000	154,000	258,000	1.83

Effect of the copolymer on *E. coli* is not so effective as that on *S. aureus*, which should be attributed to their different cell walls. *S. aureus*, a typical gram-positive bacterium, the cell wall of which is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allow foreign molecules to come into the cell without difficulty. But *E. coli*, a typical gram-negative bacterium, the cell wall of which is made up of a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein, and phospholipid. Because of the bilayer structure, the outer membrane is a potential barrier against foreign molecules with high molecular weight. Therefore, the copolymer has different effects on the two bacteria.

The antibacterial mechanism of chitosan is generally considered due to the amino group at the C-2 position of the glucosamine residue, that is, the cationic nature of chitosan at acidic condition. This chitosan derivative is mainly anionic nature at neutral condition, so the adsorption and binding of cationic group are not so effective to explain its antibacterial mechanism. Chitosan has been reported to bind a range of heavy metals and trace elements (Roller & Covill, 1999). The degree of protonation of NH_2 in chitosan

Table 4
Diameters of inhibition zones (mm) against *E. coli* and *S. aureus* (–: this is no obvious inhibition zone in the agar plates)

Bacteria	CTS	HPCTS	HPCTS-*g*-MAS 1	HPCTS-*g*-MAS 2	HPCTS-*g*-MAS 3
E. coli	–	–	6.5	8	8
S. aureus	–	–	10	12	12

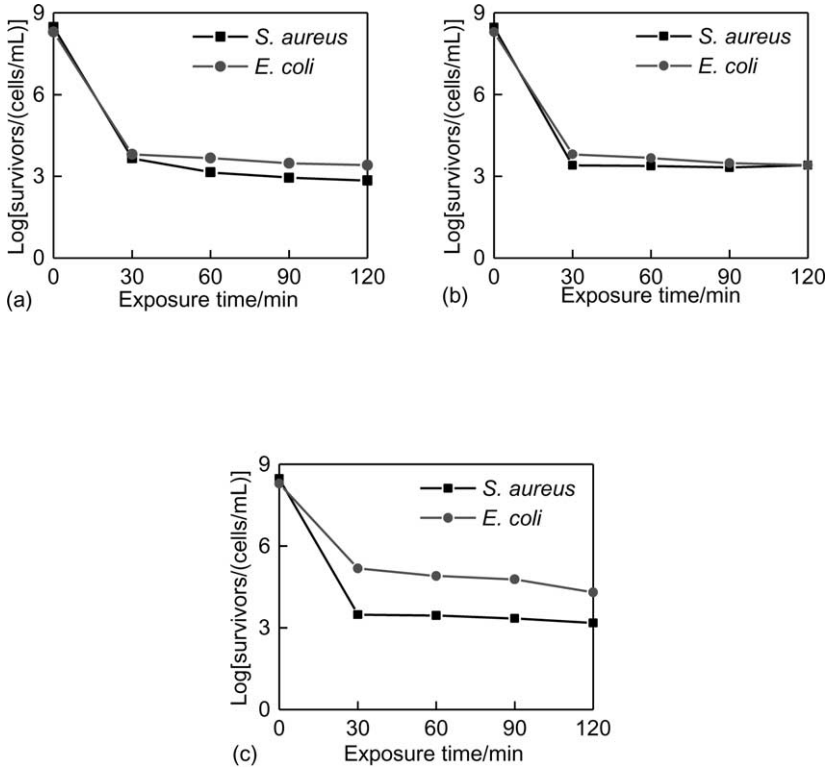

Fig. 2. Plots of log (survivors) versus exposure time for HPCTS-*g*-MAS 3 against *S. aureus* and *E. coli* at different concentrations: (a): 10 μg; (b): 1 μg; (c): 100 ng.

is constant when the pH value is given (Chen, Zhang, & Guo, 2000). The higher is the pH value, the lower of degree of protonation of NH_2 is. Since the antibacterial test was 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 attributed to the strong coordination capability of chitosan towards metals and the fact that maleic acid has strong complex ability.

Antibacterial activities of this chitosan derivative can also be closely related to the formation of hydrophobic micro-area. At pH 7, the degree of protonation of NH_2 is very low, that is, the repulsion of NH_3^+ is weak, so the strong intermolecular and intramolecular hydrogen bond results in the formation of hydrophobic micro-area in polymer chain (Chen et al., 2000). At the same time, the carboxyl group in polymer chain is 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 derivative.

4. Conclusions

Multiple-derivation of chitosan was carried out through etherification reaction and graft copolymerization. The derivatives with high grafting percentage have good water solubility. Compared with chitosan and HPCTS, the derivatives show good inhibition effects against *S. aureus* and *E. coli*, which should be attributed to their different modes of action. Both the cut plug method and the viable cell counting method show that the multiple-derivated chitosans were more effective against *S. aureus* than *E. coli*. Considering its potential antioxidant and antitumor activity (Xie, Xu, & Liu, 2001), the multiple-derivated chitosan will have potential applications in biomedicine as marine polysaccharide drug.

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