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Synthesis and characterization of chitosan derivatives with dual-antibacterial functional groups

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ABSTRACT

With the aim to discover chitosan derivatives with enhanced antibacterial activity and good water solubility compared with natural chitosan, a novel *O*-quaternary ammonium *N*-acyl thiourea chitosan (OQCATUCS) bearing double antibacterial groups with different degrees of substitution has been synthesized. The derivative was characterized by FTIR, ^{13}C NMR, elemental analysis, XRD, TGA and zeta potential analysis. Water solubility was also investigated. The antimicrobial activities of chitosan and its derivatives were investigated by assessing the mortality rates of *Staphyloccocus aureus*, *Escherichia coli*, *Aspergillus niger*, *Pseudomonas aeruginosa* and *Bacillus subtilis*. The order of antibacterial activities was *O*-quaternary ammonium *N*-acyl thiourea chitosan (OQCATUCS) > *O*-quaternary ammonium chitosan (OQCS) > chitosan (CS). The zeta potential and antibacterial results indicated that the introduced quaternary ammonium and thiourea groups increased the positive charge of chitosan derivative, thereby enhanced its antibacterial activity. The mechanism of chitosan derivatives against *E. coli* and *S. aureus* was evaluated via analyzing integrity of cell membranes and transmission electron microscopy data. These results demonstrated that OQCATUCS killed the bacteria via disrupting the cell membrane.

1. Introduction

Chitin is a natural polymer that is close behind cellulose in terms of abundance. It is obtained from the exoskeletons of crustaceans such as shrimps and crabs, from some fungi, and from insect cell walls. Chitosan is the deacetylated derivative of chitin, composed of glucosamine, known as 2-amino-2-deoxy-(1 → 4)-β-D-glucopyranose [1]. Based on its biological properties, chitosan derivatives have been applied in a variety of research areas, such as agriculture [2], medicine [3], environment [4] and food industry [5]. In recent years, chitosan and its derivatives have attracted considerable attention owing to their antimicrobial and antifungal activities [6,7]. Despite its unique antimicrobial properties, the poor solubility of chitosan at neutral pH limits its applications. Therefore, much effort has been focused on producing functional derivatives of chitosan with increased solubility in water. These chitosan derivatives included quaternary ammonium salts of chitosan [8], ethylamine-hydroxyethyl chitosan [9], *N*-alkylated chitosan [10], *N*-acylated chitosan [11,12] and *p*-aminobenzoyl chitosan ester [13]. Many chitosan derivatives have higher antimicrobial activity than native chitosan. However, the antimicrobial activity of these materials is still lower than that of the antimicrobial agents currently used. Therefore, synthesizing new derivatives that contain both antibacterial and water-soluble groups may facilitate the use of chitosan as antibacterial material.

Thiourea derivatives have strong antifungal activity that is comparable to that of the common antifungal antibiotic ketoconazole [14]. Eweis et al. prepared a benzoyl thiourea derivative of chitosan and showed that its antifungal activity is much greater than that of native chitosan [15]. Mohamed et al. prepared three different acyl thiourea derivatives of chitosan (acetyl, chloroacetyl, and benzoyl) and indicated that they have a significant inhibitory effect on the investigated fungi at concentrations of 5–1000 μg/mL [16].

There are two types of reactive functional groups present in chitosan (amino group and hydroxyl group) that can be used to chemically alter its properties under mild conditions. Recently, Zhao [17] reported that the chitosan derivatives bearing double functional groups had higher growth suppression against *Staphyloccocus aureus* and *Escherichia coli* compared to corresponding single functional group. In this study, we synthesized the water-soluble chitosan derivatives bearing double functional groups. The C_2—NH_2 of chitosan was first protected through formation of Schiff base by benzaldehyde. The C_6—OH was grafted with ETA to introduce the permanent positively charged quaternary ammonium

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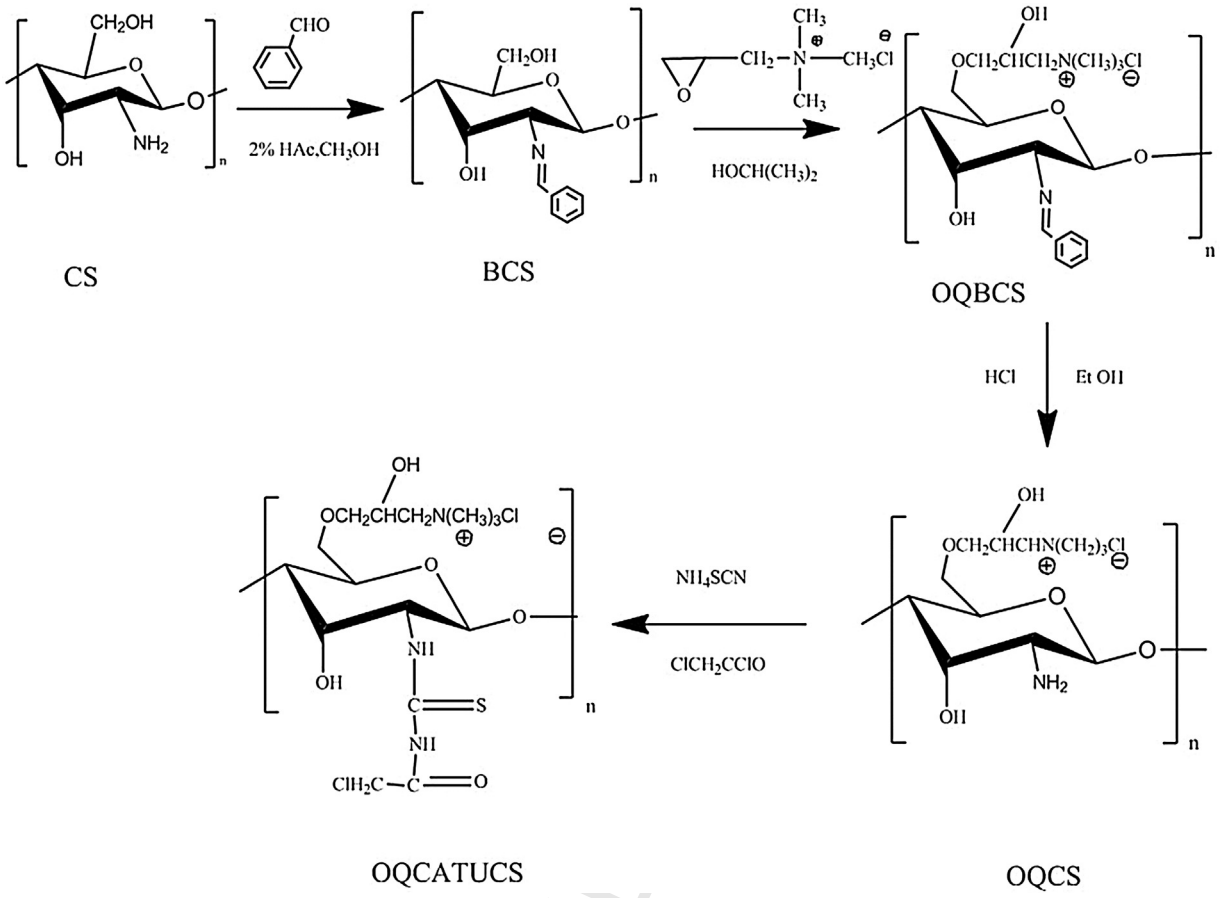

Scheme 1. Synthesis of *O*-quaternary ammonium-*N*-acyl thiourea chitosan.

group, which enhanced the water solubility and the antibacterial activity over the entire pH range. By the deprotection of C_2—NH_2, then the amino group of OQCS was reacted with chloroacetyl thiourea, which also increased the electropositivity charge of OQCS and enhanced the antimicrobial activity toward different bacteria. Therefore *O*-quaternary ammonium *N*-acyl thiourea chitosan was successfully synthesized and its antibacterial activity against *S. aureus*, *E. coli*, *Aspergillus niger*, *Pseudomonas aeruginosa* and *Bacillus subtilis* were investigated. Moreover, we discussed the synergistic antibacterial mechanism of *S. aureus* Gram-positive and *E. coli* Gram-negative bacteria via analyzing integrity of cell membranes and transmission electron microscopy data. The synthesis route and structures of the chitosan derivatives are depicted in Scheme 1.

2. Materials and methods

2.1. Materials

Chitosan with a degree of deacetylation of 90% and a molecular weight of 5.0×10^4 was purchased from Zhejiang Haidebei Biochemical Corp, China. 2,3-Epoxypropyltrimethyl ammonium chloride (ETA) was purchased from Dongying Guofeng Fine Chemical Co., Ltd. (Shandong, China). All other chemicals and reagents were analytical grade supplied by Guangzhou Chemical Reagent Company. *S. aureus* (ATCC6538), *E. coli* (ATCC8739), *A. niger* (ATCC16404), *P. aeruginosa* (ATCC9027) and *B. subtilis* (ATCC6633) were provided by the Guangdong Institute of Microbiology.

2.2. Synthesis of chitosan derivatives

2.2.1. Synthesis of Schiff's base of chitosan (BCS)

Chitosan shiff base (*N*-benzylidene-chitosan) was prepared according to the literature [13]. Chitosan (2.000 g, 18.6 mmol pyranose) was dissolved in 100 mL solution of 2% (w/v) acetic acid and diluted with 120 mL of methanol over 45 min for swelling of chitosan. Then benzaldehyde (19.71 g, 186 mmol) dissolved in 100 mL methanol was added to the resulting solution and the mixture was stirred at 60 °C for 5 h. Then the solution was poured into substantial anhydrous ethanol to remove the unreacted benzaldehyde. The precipitate was washed three times with anhydrous ethanol and the product was dried at 50 °C for 24 h.

2.2.2. Preparation of O-quaternary ammonium N-benzylidene chitosan (OQBCS)

O-Quaternary ammonium *N*-benzylidene (OQBCS) was prepared by grafting quaternary ammonium groups onto chitosan according to the method reported previously [18]. Chitosan schiff base (1.500 g, 6.15 mmol pyranose) and ETA (9.345 g, 61.5 mmol) were dispersed in 100 mL of isopropanol and the mixture was stirred at 70 °C for 30 h. The reaction mixture was precipitated using acetone and washed with acetone and ethanol solution (95%), filtered and dried at 50 °C under vacuum for 24 h.

2.2.3. Synthesis of O-quaternary ammonium chitosan (OQCS)

O-Quaternary ammonium *N*-benzylidene chitosan (OQBCS) was dispersed in the mixture of anhydrous ethanol and 0.25 mol/L HCl for 24 h. The solution was adjusted to neutrality with aqueous 1%