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Homogeneous synthesis and characterization of quaternized chitin in NaOH/urea aqueous solution

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ABSTRACT

Water-soluble and white quaternized chitin (QC) was homogeneously synthesized by stirring transparent chitin solution (2%) in 8 wt%NaOH/4 wt% urea aqueous solution containing 2,3-Epoxypropyltrimethylammonium Chloride (EPTMAC) at 10 °C for 24 h. The structure and properties of quaternized chitin were characterized by FT-IR, XRD, ^{1}H NMR, GPC, element analysis and ζ-potential. The results indicate that quaternary groups were successfully incorporated onto chitin backbones and the degree of substitution (DS) of quaternary groups can be easily adjusted by changing the molar ratio of chitin unit to EPTMAC. Additionally, quaternized chitin shows better antibacterial activity against *Escherichia coli* and *Staphylococcus aureus* as compared with chitosan. Thus, this work provides a simply and "green" method to functionalize chitin and the resulting quaternized chitin may have potential applications in environmental, food and biomedical fields.

1. Introduction

Recently, our lab has developed a new and environment-friendly solvent (i.e. NaOH/urea) to dissolve chitin (Hu et al., 2007). We have shown that chitin from various sources can be dissolved in 8% NaOH/4% urea by using freeze/thaw cycles (Li et al., 2010). Fibers (Pillai, Paul, & Sharma, 2009) and hydrogels (Chang, Chen, & Zhang, 2011) from chitin with good mechanical strength and excellent biocompatibility were fabricated by using this new solvent.

Herein, we show that chitin can be homogenously functionalized with quaternary groups in NaOH/urea aqueous solution in a facile method. The introduction of quaternary groups into polysaccharide molecules can greatly improve its water solubility and impart additional biological activities to the matrix (Jia et al., 2001). For instance, quaternized chitosan shows better antibacterial activities than chitosan; quaternized cellulose has enhanced DNA delivery efficiency than cellulose itself (Song, Sun, Zhang, Zhou, & Zhang, 2008). Only β-chitin has been successfully quaternized by heterogeneous reaction with 3-chloro-2-hydroxypropyltrimethylammonium chloride (CTA) in 2-propanol (Chen, Wu, Pu, Zheng, Shi, & Huang, 2010). Scope of this work is to demonstrate for the first time that shrimp α-chitin undergoes homogeneous quaternization and water-soluble product can be obtained. Chitin powder was firstly dissolved in NaOH/urea solvent and quaternary groups were introduced by adding 2,3-Epoxypropyltrimethylammonium Chloride to the chitin solution. This homogeneous reaction can take place at low temperature. The structure of quaternized chitin was studied by various techniques and its antibacterial property was evaluated by disk diffusion method. We believe this simple and environment-friendly approach is important for chitin functionalization and quaternized chitin obtained in this work can find its application in environmental, food and biomedical fields.

2. Experimental

2.1. Materials

Chitin powder was purchased from Jinke Chitin Co., Ltd. (Zhejiang, China). The degree of acetylation (DA) was determined by elemental analysis to be 0.98 and the molecular weight (M_w) was 5.0×10^5 Da (Chang et al., 2011). Chitosan was purchased from Sigma with a deacetylation degree of 85% and a molecular weight of 200 kDa. 2,3-Epoxypropyltrimethylammonium Chloride (EPTMAC) was purchased from Adamas Reagent Co., Ltd. (Swiss). All other reagents were of analytical grade and were used without further purification.

2.2. Preparation of quaternized chitin in NaOH/urea aqueous solution

Chitin solution was prepared according to our previous work (Hu et al., 2007). Chitin powder (4.0 g) was suspended in 200 g

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8 wt% NaOH/4 wt% urea aqueous solution. The resulting mixture was stored at −20 °C and stirred twice over 36 h. After it was thawed to room temperature, transparent chitin solution (2.0 wt%) was obtained.

Quaternized chitin was homogeneously synthesized by one-pot reaction. In a typical assay, EPTMAC (6.705 g) was added to chitin solution (50 g) and the resulting mixture was stirred at 10 °C for 24 h. Then, it was neutralized with HCl aqueous solution. Small amount of insoluble parts in quaternized chitin solution were removed by filtration and centrifugation at 6500 × *g* for 10 min. After dialysis against distilled water for 7 days (50,000–60,000 cut-off, $AgNO_3$ (0.1 M) was used to check the complete removal of Cl^-), white quaternized chitin powder was obtained by freeze-dry technique. Quaternized chitins coded as QC1, QC2, and QC3 were made by changing the molar ratio of EPTMAC to chitin units as 4:1, 7:1, and 9:1.

2.3. Characterization of quaternized chitin

Fourier transform infrared spectra (FT-IR) of quaternized chitin were recorded by method of KBr pellets on a Nicolet5700 Fourier transform infrared spectrometer.

The DS of quaternization was determined by titrating the amount of Cl^- ions with $AgNO_3$ solution (Li, Du, Wu, & Zhan, 2004). DS is calculated as Eq. (1):

$$\mathrm{DS} = \frac{V \times c/1000}{V \times c/1000 + (W_1 - W_2)/203} \quad (1)$$

where c (mol/L) is the concentration of $AgNO_3$ solution, V (mL) is the volume of $AgNO_3$ solution, W_1 (g) is the weight of quaternized chitin, $W_2 = Vc \times 354.5/1000$. Here 354.5 is the molar mass of QC unit.

The degrees of deacetylation (DD) of quaternized chitin were obtained by the results of elemental analysis (Vario III, Elementar, Germany) and DS values. The DD values were calculated as Eq. (2).

$$\frac{C}{N} = \frac{[6 + (1 - \mathrm{DD}) \times 2 + \mathrm{DS} \times 6] \times 12}{(1 + \mathrm{DS}) \times 14} \quad (2)$$

The zeta potentials of quaternized chitin were performed on a Nano-ZS ZEN3600 (Malvern Instruments, UK) at 25 °C. Before measurement, quaternized chitin was dissolved in distilled water to prepare test solution (1 mg/mL) and then filtered using millipore filter (0.22 μm).

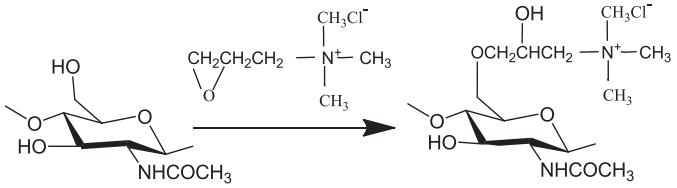
Weight-average molecular weights (M_w) of quaternized chitin were measured by gel permeation chromatography (GPC, TSP-P100, USA). The eluent was 0.2 M CH_3COOH/0.1 M CH_3COONa and the flow rate was maintained at 1.0 mL/min.

The ^1H nuclear magnetic resonance (^1H NMR) was carried out on a Varian INOVA-600 spectrometer. A certain amount of quaternized chitin was dissolved in D_2O to prepare a 5 wt% solution. Chemical shifts were given in ppm using tetramethylsilane (TMS) as an internal reference.

X-ray diffraction (XRD) test was performed on an XRD diffractometer (D8-Advance, Bruker). The XRD patterns with Cu K_α radiation (λ = 0.154 nm) at 40 kV and 50 mA were recorded in the region of 2θ from 5° to 40°.

2.4. Estimation of water solubility

The water solubility of quaternized chitin at various pHs was determined by turbidity measurement (Gonil et al., 2011). Aqueous solution of quaternized chitin (1 mg/mL or 5 mg/mL) was prepared by dissolving quaternized chitin in deionized water. Either HCl solution (0.1 or 1 M) or NaOH solution (0.1 or 1 M) was slowly added to adjust the pH. The transmittance of the solutions at different pHs was recorded on a UNICO UV-2000 Spectrophotometer at 600 nm.



Scheme 1. Possible process for the quaternization of chitin.

2.5. Antimicrobial activity

Gram-positive bacteria (*Staphylococcus aureus*) and Gram-negative bacteria (*Escherichia coli*) were selected to evaluate the antimicrobial activity of quaternized chitin. The disk diffusion method (Muzzarelli, Tarsi, Filippini, Giovanetti, Biagini, & Varaldo, 1990; Li, Shi, Wang, & Du, 2011) was used in this test. 500 mL nutrient medium (beef extract 2.5 g, peptone 5 g, agar 10 g, NaCl 2.5 g, pH 7.0) was autoclaved at 126 °C for 30 min. Microorganism suspension was diluted to 10^6 cfu/mL, and 50 μL of the microorganism suspension was added onto the agar plates containing 15 mL nutrient medium. After the homogeneous dispersion of microorganism suspension, paper disks containing test solution (sterile water, 1 wt% chitosan, 1 wt% quaternized chitin) were pasted on the agar plates. The microscope was used to observe and measure the inhibition zones after incubation at 37 °C for 24 h.

3. Results and discussion

3.1. Synthesis of quaternized chitin

Scheme 1 shows the homogeneous quaternization of chitin dissolved in NaOH/urea aqueous solution by adding EPTMAC as etherifying agent. It is reported (Roberts, 1992) that EPTMAC mainly reacts with the amino groups (–NH_2) in an acidic medium, whereas it reacts with the hydroxyl groups (–OH) under alkaline conditions. The NaOH/urea solvent system provides an alkaline condition which benefits the reaction between EPTMAC and the hydroxyl groups on chitin. Besides, acetyl groups in chitin cover most of the amino groups. It is reasonable to predict that the substitution occurs at the C-6 hydroxyl groups in the NaOH/urea solvent. Quaternized chitins with DS from 0.25 to 0.42 were prepared by changing the molar ratio of EPTMAC to chitin unit. The reaction conditions for quaternization of chitin are illustrated in Table 1. During the process, the solution was kept transparent and remained completely homogeneous. The DD value of the QC sample was reduced compared with the original chitin. The DS value of the quaternized chitin enhanced from 0.25 to 0.42 with increasing addition of EPTMAC. The zeta potential increased from +25.4 mV to +35.6 mV, which may be attributed to more quaternary ammonium salts introduced onto chitin backbones when DS is higher. The molecular weight (M_w) of quaternized chitin samples decreased from 166 kDa to 127 kDa with an increasing of the DS values from 0.25 to 0.42. Degradation of the chitin during the reaction might cause this phenomenon.

3.2. Structure of quaternized chitin

The structure of quaternized chitin was firstly studied by FT-IR. Fig. 1 shows the FT-IR spectra of the original chitin and the quaternized chitin samples (QC2 and QC3). The amide I band was splitted into two peaks which appear at 1658 cm^{-1} and 1620 cm^{-1} in the FT-IR spectra of the original chitin. This indicates that the chitin was in the form of α (Jang, Kong, Jeong, Lee, & Nah, 2004). The peaks at 1560 cm^{-1} and 1316 cm^{-1} corresponded to the amide II and III band. Compared with the spectra of the original chitin, a new peak

Table 1
Conditions and results of the homogeneous quaternization of chitin using 2,3-Epoxypropyltrimethylammonium Chloride in NaOH/urea aqueous solution.

Sample	Molar ratio[a]	Time (h)	Temperature (°C)	C (%)	N (%)	DS	DD (%)	ζ (mV)	M_w (kDa)
QC1	4	24	10	37.45	6.01	0.25	20.64	+25.4	166
QC2	7	24	10	40.96	6.70	0.35	23.57	+30.2	143
QC3	9	24	10	40.30	6.76	0.42	32.19	+35.6	127

[a] The molar ratio of 2,3-Epoxypropyltrimethylammonium Chloride to chitin unit.

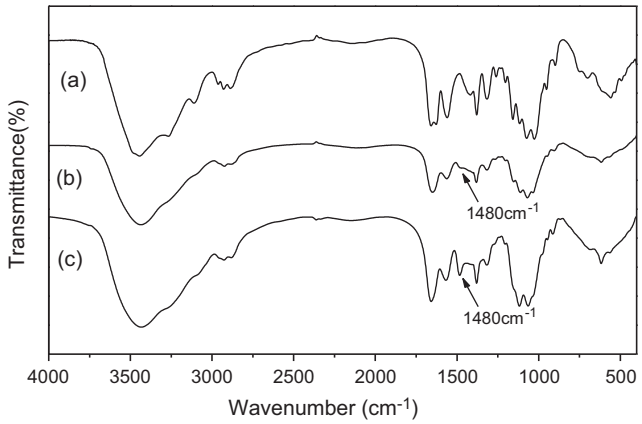


Fig. 1. FT-IR spectra of original chitin (a) and quaternized chitins with DS 0.25 (b) and DS 0.42 (c), respectively.

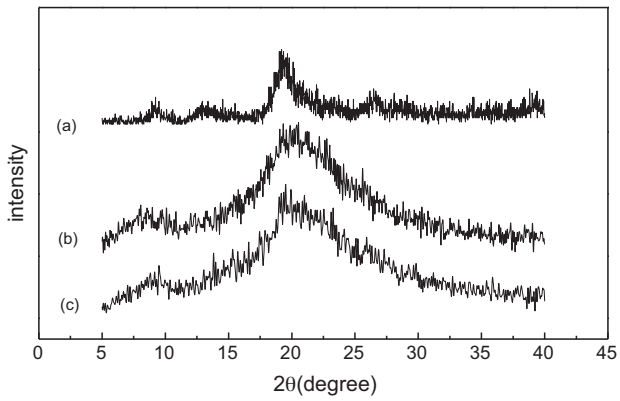


Fig. 3. X-ray diffraction patterns of original chitin (a) and quaternized chitins with DS 0.25 (b) and DS 0.42 (c), respectively.

appears at 1480 cm^{-1} in QC2 and QC3, which was attributed to the methyl groups of ammonium (Nam, Kim, & Ko, 1999). This indicates introduction of quaternized substituents onto chitin chains. It is notable that the adsorption at 1480 cm^{-1} in QC3 is stronger than that in QC2, which means higher substitution happens in QC3. This observation is consistent with the titration result that QC3 has higher DS than QC2.

The structure of quaternized chitin was further characterized by ^{1}H NMR. Fig. 2 shows the ^{1}H NMR spectrum of QC3. The peak at δ = 1.98 ppm was assigned to the –$COCH_3$, suggesting that the QC samples retain most of acetamido. The peak at δ = 3.15 ppm was assigned to the methyl groups in the quaternary ammonium salt group (Lim & Hudson, 2004). The ^{1}H NMR spectrum further proves the substitution of quaternary ammonium salts onto the chitin backbone.

Fig. 3 shows the XRD patterns of original chitin, QC1 and QC2. There were five peaks at 2θ = 9.3°, 12.7°, 19.3°, 23.3° and 26.4° in the XRD pattern of chitin powder which also indicates the chitin was in the form of α (Wada & Saito, 2001). However, the disappearance of five characteristic peaks in the XRD patterns of QC samples demonstrated that during the dissolution and quaternization process the chitin transit from a crystalline structure to an amorphous state. This illustrated that the intra and inter-molecular hydrogen bonds of chitin were broken during dissolution, leading to the loss of crystallinity, and that the quaternized reaction induced further destruction of the crystalline structure (Chang et al., 2011).

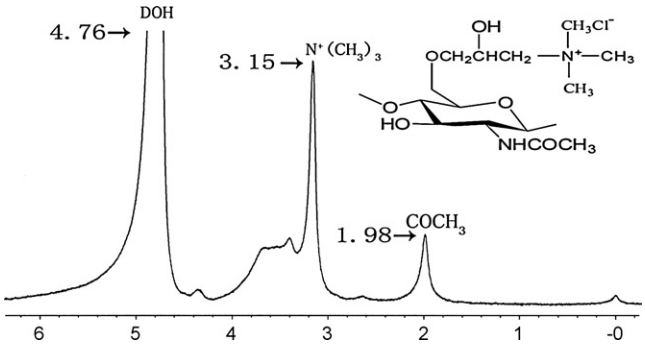


Fig. 2. ^{1}H NMR spectrum of quaternized chitin with DS 0.42.

3.3. Water solubility

Quaternization of chitin can greatly improve its water solubility. Fig. 4 exhibits the solubility of quaternized chitin (QC1 and QC3) with different concentration at different pH values. The results show good water solubility of prepared quaternized chitin in a wide pH range. The water solubilities of QC1 and QC3 decreased when the concentration changed from 1 mg/mL to 5 mg/mL. This may be ascribed to the intermolecular interactions such as van der Waals forces increased. The water solubilities of QC1 and QC3 were higher at lower pH. The ionic strength might be a cause for this phenomenon (Kubota, Tatsumoto, Sano, & Toya, 2000). We also found that quaternized chitin with a higher DS showed better water solubility in whole pH range. This can be explained by the fact that the chain of quaternized chitin was more flexible with a higher DS value (Xu, Xin, Li, Huang, & Zhou, 2010).

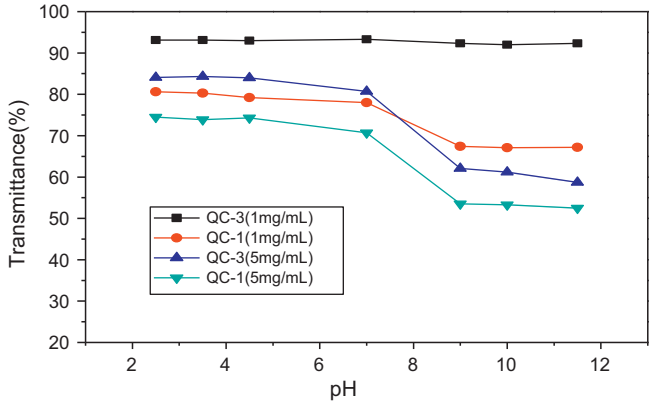


Fig. 4. The pH dependence of water solubility of quaternized chitins with DS 0.25 and DS 0.42 at 1 mg/mL and 5 mg/mL.

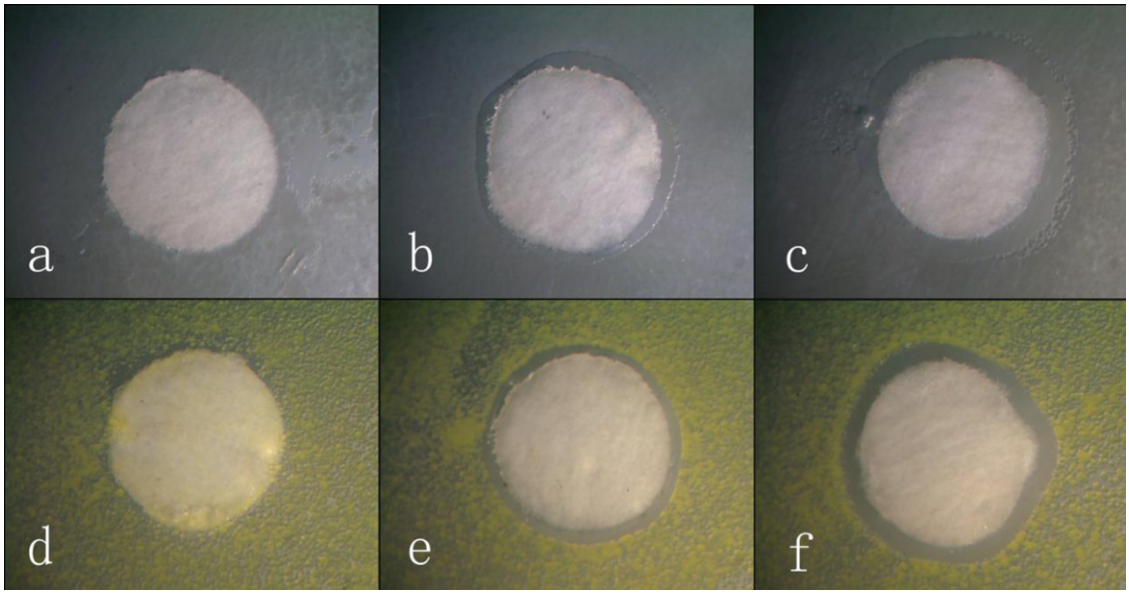


Fig. 5. Antimicrobial activities of chitosan and quaternized chitin with DS 0.42 against *E. coli* and *S. aureus*. Images (a–c) are paper disks containing sterile water, 1 wt% chitosan, 1 wt% quaternized chitin with DS 0.42, respectively against *E. coli*; Images (d–f) correspond to paper disks containing sterile water, 1 wt% chitosan, 1 wt% quaternized chitin with DS 0.42, respectively against *S. aureus*.

3.4. Evaluation of antimicrobial activity

Fig. 5 shows the antimicrobial activities of the chitosan (1 wt%) and QC3 (1 wt%) against *E. coli* and *S. aureus* using disk diffusion method. It can be found that there was no inhibition zone for the filter paper containing sterile water (Fig. 4a and d). The appearance of inhibition zone around filter paper treated with chitosan is consistent with the fact that chitosan can inhibit the growth of *E. coli* and *S. aureus* (Fig. 4b and e). Apparently, the inhibition area of QC3 was larger than that of chitosan against the two microbes, suggesting that QC3 exhibited better antimicrobial activity against both *E. coli* and *S. aureus* than chitosan (Fig. 4c and f). The antimicrobial activity of polycationic chitosan derivatives towards Gram-negative bacteria is due to the amino groups interact with the predominantly anionic components on the bacterial surface and change the membrane permeability (Liu, Du, Wang, & Sun, 2004) whereas the main mechanism for the antibacterial activity of chitosan against *S. aureus* may be attributed to the formation of a film over the surface of the cell membrane preventing the nutrients from entering the cell (Tsai & Su, 1999). Quaternized chitin may have the similar antimicrobial mechanism. After quaternization, the chitin became a water-soluble polyelectrolyte with a high charge density. The negatively charged surface of the bacteria cell is the target site of the polycation (Avadi et al., 2004; Jia et al., 2001). Quaternized chitin can interact and form polyelectrolyte complexes with acidic polymers produced at the bacterial cell surface, which inhibits the growth of the tested bacteria.

4. Conclusions

In this work, we have demonstrated that shrimp α-chitin can be functionalized with quaternary groups in a homogeneous reaction. This one-pot reaction can take place at low temperature with no organic solvent involved. FT-IR, ^{1}H NMR, element analysis and ζ-potential give the evidence of the presence of quaternary groups on chitin backbones. The degree of substitution (DS) can be adjusted from 0.25 to 0.42 by changing the molar ratio of EPTMAC to chitin. Importantly, this simple modification enlists chitin with water solubility and antibacterial activity. It is anticipated that more chitin derivatives besides quaternized chitin can be obtained by homogeneous reaction and NaOH/urea solvent will certainly provide a convenient means to functionalize chitin.

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