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ORIGINAL PAPER

Synthesis, characterization and antibacterial activity of quaternized *N*,*O*-(2-carboxyethyl) chitosan

Zhao-sheng Cai · Zhan-qian Song · Chun-sheng Yang · Shi-bin Shang · Yan-bai Yin

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Abstract *N*,*O*-(2-carboxyethyl)chitosan (*N*,*O*-2-CEC) was prepared from chitosan with 3-chloropropionic acid as modifying agent and NaOH as catalyst. Different quaternary ammonium groups were introduced into *N*,*O*-2-CEC by the reaction between *N*,*O*-2-CEC and different 2,3-epoxypropyl trialkyl ammonium chlorides in the presence of 25% NaOH aqueous solution, and obtained different quaternized *N*,*O*-2-carboxyethyl chitosans (QCECs). Structures of QCECs were characterized by FT-IR, ^{1}HNMR and gel permeation chromatography (GPC). Antimicrobial activity of QCECs was evaluated against a gram-negative bacterium *Escherichia coli* and a gram-positive bacterium *Staphylococcus aureus*. Compared with *N*,*O*-2-CEC and quaternized chitosans, the QCECs had much stronger antimicrobial activity, which increased with increasing chain length of the alkyl in the quaternary ammonium groups. The presence of benzyl in quaternary ammonium groups could endow QCECs with much better antimicrobial activity.

Keywords *N*,*O*-(2-carboxyethyl)chitosan · Quaternized *N*,*O*-2-carboxyethyl chitosans · Synthesis · Characterization · Antimicrobial activity · 2,3-Epoxypropyl trialkyl ammonium chloride

Z.-s. Cai · Z.-q. Song (✉) · S.-b. Shang · Y.-b. Yin
Institute of Chemical Industry of Forest Products, CAF, 210042 Nanjing, Jiangsu, People's Republic of China
e-mail: zqsong@public1.ptt.js.cn

Z.-s. Cai · Z.-q. Song · S.-b. Shang · Y.-b. Yin
Key and Open Laboratory on Forest Chemical Engineering, SFA, 210042 Nanjing, Jiangsu, People's Republic of China

Z.-s. Cai · C.-s. Yang
Department of Chemical and Biological Engineering, Yancheng Institute of Technology, 224003 Yancheng, Jiangsu, People's Republic of China

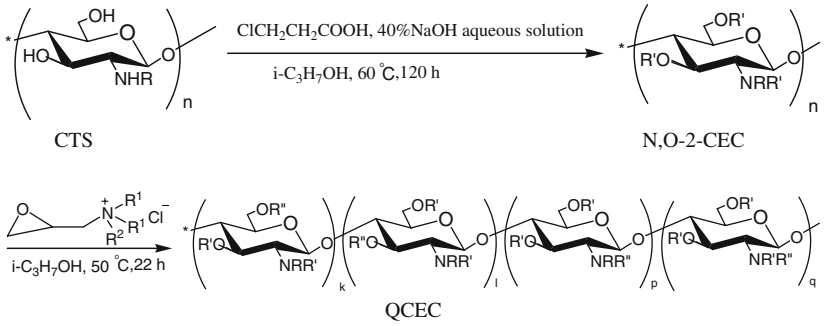
Introduction

Chitosan (CTS) is a linear polysaccharide composed of *N*-glucosamine and *N*-acetyl-glucosamine units, in which the number of *N*-glucosamine units exceeds 50% [1]. It can be produced by the partial deacetylation of chitin, which is estimated to be produced annually almost as much as cellulose and is the most abundant natural amino polysaccharide [2, 3]. Chitosan has found several of applications being employed either alone or in blending with other natural polymers (starch, gelatin, alginates, etc.) in the food and pharmaceutical industries due to its excellent chemical, physical, and biological properties, such as biocompatibility, biodegradability, non-toxicity, adsorptive properties, film-forming ability, and antimicrobial activity [4–9]. However, the lower solubility of chitosan in water solutions with higher pH (>6.0) prevents CTS from being applied in its original form [10]. For this reason, much effort has been applied to the development of suitable procedures for the preparation of water-soluble derivatives of chitosan [11–13]. Carboxyalkylation, a useful technique to modify the physic-chemical properties of chitosan, becomes one of the most promising methods [14–17].

N,*O*-(2-carboxyethyl)chitosan (*N*,*O*-2-CEC), one of carboxyalkylation derivatives of chitosan, can be obtained by the reaction between chitosan and 3-halopropionic acid (such as 3-chloropropionic acid, 3-bromopropanoic acid, and 3-iodopropionic acid) in the presence of sodium hydroxide or sodium hydrocarbonate [10, 18]. It shows good solubility in a wide range of pH. In addition, *N*,*O*-2-CEC also shows many excellent properties, for example, the antioxidant and antimutagenic activity[10], the ability to control drug release and to chelated metal ion [19, 20], the properties to form hydrogel and to pose membrane, etc. [21, 22]. Among them, the antimicrobial activity may be the most important one.

Numerous studies have been done to investigate bactericidal activities of chitosan and its derivatives, and several mechanisms have been proposed[23–27]: (1) the cationic nature of chitosan and its derivatives helps them to bind with sialic acid in phospholipids and then restrains the movement of microbiological substances; (2) oligomeric chitosan or the derivatives of chitosan with low molecular weight and good solubility can easily penetrate into the cells of microorganisms and prevent the growth of cells by preventing the transformation of DNA into RNA; (3) the trace metal cations required for the microorganism's growth are selectively chelated by the chitosan or its derivatives, therefore the microbial proliferation could be inhibited; (4) interaction between positively charged chitosan derivative and negatively charged microbial cell membranes leads to the leakage of proteinaceous and other intracellular constituents.

Negative charges on the carboxyl group in *N*,*O*-2-CEC make it being regarded as an excellent chelating host for metal cation substrate and enhance its antimicrobial activity. But the negatively charged property of cell membranes would result in the *N*,*O*-2-CEC not binding with microbial cell membranes very well under neutral and basic conditions, and leading to the decrease of its antibacterial activity under non-acidic condition. In order to overcome this defect, it is necessary to improve the positive charge of *N*,*O*-2-CEC. Among the methods to improve the positive charge



Scheme 1 Synthesis of carboxyethyl chitosan and quaternized carboxyethyl chitosan. R=H or $COCH_3$, R′=H or CH_2CH_2COOH, R″=H or $CH_2CH(OH)CH_2NR^1R^1R^2Cl$. QCEC1: $R^1=R^2=CH_3$, QCEC2: $R^1=R^2=CH_2CH_3$, QCEC3: $R^1=R^2=CH_2CH_2CH_3$, QCEC4: $R^1=R^2=CH_2CH_2CH_2CH_3$, QCEC5: $R^1=CH_3$, $R^2=CH_2C_6H_5$

of chitosan derivative, quaternizing modification is usually regarded as a simple and efficient way [28–30].

The present work described the preparation of quaternized *N*,*O*-2-CEC (QCECs), in which carboxyethyl group and quaternary ammonium group were both introduced into chitosan molecular chain. Antimicrobial activities of QCECs were evaluated against a gram-positive bacterium *Staphylococcus aureus (S. aureus)* and a gram-negative bacterium *Escherichia coli (E. coli)* and compared with *N*,*O*-2-CEC, quaternized chitosans (QCTSs), and chitosan. The reaction process for the preparation of *N*,*O*-2-CEC and its quaternized derivatives were shown in Scheme 1.

Experimental

Materials

Chitosan was purchased from Sinophar. Chemical Reagent Co. Ltd, its degree of deacetylation was determined to be 92.3% by potentiometric titration and its viscosity molecular weight (M_v) was 3.13×10^5. The aqueous solutions of 2,3-epoxypropyl trialkyl ammonium chloride were prepared in our laboratory as described literature [31], their mass concentrations were measured by potential titration to be ca 500 g/L. Sodium hydroxide was purchased from the third factory of chemical reagent in Tianjin, excellent grade. *S. aureus* and *E. coli* were supplied by Microbiology Laboratory of Nanjing University of Technology and were inoculated on a gel containing 1% peptone, 2% agar, 3% meat extract and 0.5% NaCl before use. All other chemicals were of reagent grade and used without purification as received.

Preparation of *N*,*O*-2-CEC

Chitosan (9.84 *g*), 2-propanol (100.0 mL) and NaOH aqueous solution (36.0 g, mass concentration is 40.0%) were added to a four-neck bottle. The mixture was heated to 45.0 °C in water bath and was alkalized for 2.0 h under stirring. Then 3-

chloropropionic acid (19.54 g, 180 mmol) dissolved in 2-propanol (ca 60 mL) was added dropwise to the mixture and allowed to react at 60.0°C for 5 days. The mixture was filtered and the filter reside was dissolved by distilled water (ca 300 mL). The pH of above blending was adjusted to ca 8.0 with HCl solution (mass concentration is ca 10.0%) and filtered. Absolute alcohol (480 mL) was added to the filter juice under stirring and the mixture was separated through centrifugal settling. The resultant dreg was washed by CH_3OH aqueous solution (ca 120 mL, mass concentration is ca 85.0%) for three times, then by absolute ethanol (90.0 mL) for three times and filtered. The solid was dried by vacuum drying apparatus under 60 °C and 0.1 MPa vacuum tightness for 12 h and obtained *N*,*O*-2-CEC in 87.2% yields. It was put into vacuum drier for use later.

Quaternization of *N*,*O*-2-CEC and CTS with 2,3-epoxypropyl trialkyl ammonium chlorides

Dried *N*,*O*-2-CEC (2.32 g), 2-propanol (30.0 mL) and NaOH aqueous solution (8.35 g, mass concentration is 25.0%) were added into a four-neck bottle. The mixture was heated to 45.0 °C in water bath under stirring and alkalized for 1.0 h. Then the aqueous solution of 2,3-epoxypropyl trialkyl ammonium chloride (40 mmol) was added dropwise to the mixture and allowed to react at 50.0 °C for 22.0 h under continuous stirring. The mixture was filtered and the filter reside was dissolved by distilled water (ca 60 mL). The pH of blending was adjusted to ca 7.0 by HCl solution (mass concentration is ca 10.0%) and filtered. Absolute alcohol (90 mL) was added to filter juice under stirring and the mixture was separated through centrifugal settling. The other treatment procedure was similar to that of *N*,*O*-2-CEC except CH_3OH aqueous solution was ca 40.0 mL and absolute ethanol was ca 30.0 mL, and the quaternized *N*,*O*-2-CEC (QCECs) was obtained.

The chitosan (1.64 g) was dispersed in distilled water (100 mL). The pH of mixture was adjusted to ca 2.0 by HCl solution (mass concentration is ca 10.0%) under stirring until the CTS dissolved completely. Then the pH of CTS solution was adjusted to ca 9.0 by hartshorn (mass concentration is ca 5.0%) in order to precipitate the CTS from the solution. The precipitate was collected by filtration, and washed with distilled water to ca pH 7.0. The incompact or swollen chitosan obtained above was dispersed in distilled water (100 mL) with stirring, and the pH of mixture was adjusted to ca 9.0. The aqueous solution of 2,3-epoxypropyl trialkyl ammonium chloride (40 mmol) was added dropwise to the mixture and allowed to react at 80.0 °C for 24 h under continuous stirring. The reaction mixture was condensed by vacuum distillation in order to evaporate ca 70 mL H_2O. The residual solution was poured into absolute alcohol (120 mL) and formed precipitate. The precipitate was collected by filtration and was washed with acetone for three times. The product of quaternized CTSs (QCTSs) was obtained by drying the precipitate at 60 °C under vacuum.

The 2,3-epoxypropyl trialkyl ammonium chlorides that were utilized as grafting agent involved 2,3-epoxypropyl trimethyl ammonium chloride, 2,3-epoxypropyl triethyl ammonium chloride, 2,3-epoxypropyl tripropyl ammonium chloride, 2,3-epoxypropyl tributyl ammonium chloride and 2,3-epoxypropyl dimethyl benzyl ammonium chloride, respectively.

Characterization

Fourier transform infrared (FT-IR) spectra were recorded with KBr pellets on a Nicolet Nexux FT-IR 670 spectrometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air. ^1H NMR spectra were obtained with Bruker AV-300 spectrometer at 300.13 MHz at 30 ± 0.5 °C. All the ^1H NMR spectra were measured in D_2O solution and the samples were dissolved in a 5 mm diameter tube at a concentration of ca 20 mg/mL.

The substitution degree of carboxyethylation (DS of CE) of *N*,*O*-2-CEC was estimated by potentiometric titration according to literature [32]. Weight-average molecular weights (M_w) of the samples were measured by GPC according to literature [32]. The substitution degree of quaternization (DS of QA) of QCECs was determined by potentiometry according to literature [33].

Evaluation of antimicrobial activity

The agar plate method was used to determine the minimum inhibitory concentration (MIC) of CTS, *N*,*O*-2-CEC, QCTSs, and QCECs. The samples were prepared at a concentration of 0.05% (*w*/*v*), then they were autoclaved at 121 °C for 30 min. Duplicate twofold serial dilutions of each sample were added to nutrient broth (beef extract 5 g, peptone 10 g to 1,000 mL distilled water, pH 7.0) for final concentration of 0.025, 0.0125, 0.00625, 0.00313, and 0.00156%. Some samples were prepared by the similar method except the final concentrations were 0.00125, 0.00063 and 0.00031%, respectively. The culture of each bacterium was diluted by sterile distilled water to ca 100 CFU/mL. A loop of each suspension was inoculated on nutrient medium with sample or control added. After inoculation, the plates were incubated at 37 °C for 48 h, and the colonies were counted and the MIC values were obtained.

The MIC was considered to be the lowest concentration that completely inhibited against on agar plates comparing, disregarding a single colony or a faint haze caused by the inoculum [34].

Results and discussions

Identification of resonance in the spectra

The FT-IR spectra of CTS, *N*,*O*-2-CEC and QCEC1 to QCEC5 were presented in Fig. 1. The absorption bands at 1,656, 1,595, 1,323, 1,381 cm^{-1} in the spectrum of CTS were assigned to primary, secondary, and tertiary amides and $–CH_3$ bend vibration. Both characteristic peaks for CTS at 3,359 and 1,070 cm^{-1} could be attributed to the O–H and C–O vibration, respectively. Two strong peaks at 1,569 and 1,411 cm^{-1} in *N*,*O*-2-CEC spectrum, 1,571 and 1,409 cm^{-1} in QCEC1 spectrum, 1,569 and 1,410 cm^{-1} in QCEC2 spectrum, 1,572 and 1,413 cm^{-1} in QCEC3 spectrum, 1,571 and 1,411 cm^{-1} in QCEC4 spectrum and 1,570 and 1,414 cm^{-1} in QCEC5 spectrum were ascribed to the asymmetrical and symmetrical stretching of $-COO^-$ group. In the spectrum of *N*,*O*-2-CEC, the C–O stretching

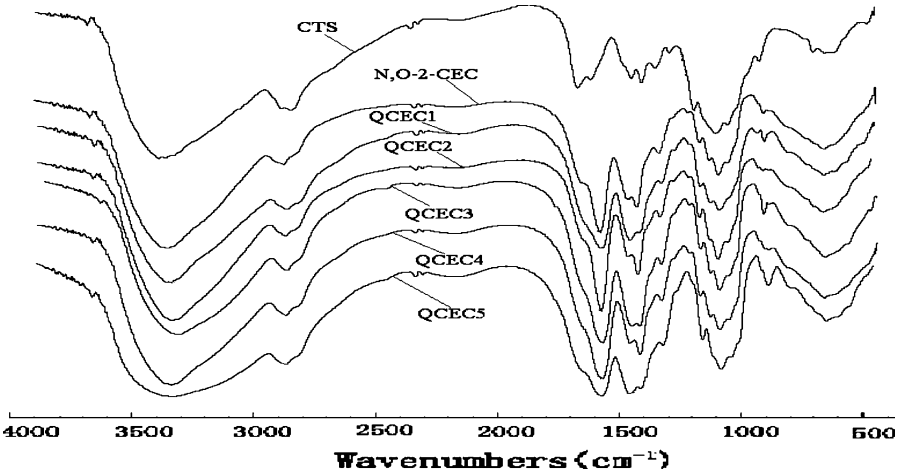


Fig. 1 FT-IR spectra of CTS, *N,O*-2-CEC, QCEC1, QCEC2, QCEC3, QCEC4, and QCEC5. QCEC1, QCEC2, QCEC3, QCEC4, and QCEC5 were the products of *N,O*-2-CEC modified by 2,3-epoxypropyl trimethyl ammonium chloride, 2,3-epoxypropyl triethyl ammonium chloride, 2,3-epoxypropyl tripropyl ammonium chloride, 2,3-epoxypropyl tributyl ammonium chloride and 2,3-epoxypropyl dimethyl benzyl ammonium chloride, respectively

band at 1,032 cm^{-1} corresponding to the primary hydroxyl group of CTS became weakening, which confirmed a high carboxyethylation of C-6. In contrast, a new peak (1,457, 1,455, 1,460, 1,461, and 1,453 cm^{-1} for QCEC1 ~5, respectively) appeared and it implied that the methyl of the quaternary ammonium group had been introduced into *N,O*-2-CEC.

The signals at 2.43–2.59 and 3.56 ppm in the ^1H NMR spectrum of *N,O*-2-CEC were assigned to the hydrogen of C-2 and C-3 and methylene of carboxyethyl group substituted on C-6 and C-3, respectively. The resonance of 2-substituted carboxyethyl-protons (-NCH_2CH_2COOD) of chitosan occurred in the spectral region of 2.27 and 2.90 ppm. Compared with *N,O*-2-CEC, these resonances also occurred in the ^1H NMR spectrum of QCEC1-5. There were some new resonances for QCECs. For QCEC1, a stronger resonance appeared in 3.16 ppm, which could be attributed to the methyl-protons of quaternary ammonium group. The adsorbing peak at ca 3.64 ppm, which attributed to the methyl-protons of hydropropyl, became stronger than that of *N,O*-2-CEC for QCEC1-5, it indicated that the quaternary ammonium group had been introduced onto *N,O*-2-CEC. A signal at 6.98 ppm in the ^1H NMR spectrum of QCEC-5 could be attributed to the protons on phenyl of quaternary ammonium group (Figs. 2, 3, 4, 5, 6, 7).

Antimicrobial activity of CTS, *N,O*-2-CEC, QCTSs and QCECs

MIC value of CTS, *N,O*-2-CEC, QCTSs and QCECs were listed in Table 1. The results showed that CTS had the lowest antimicrobial activity against the tested bacteria under the experimental condition primarily due to its poor solubility in the neutral condition. The antibacterial activities of *N,O*-2-CEC, QCTSs, and QCECs

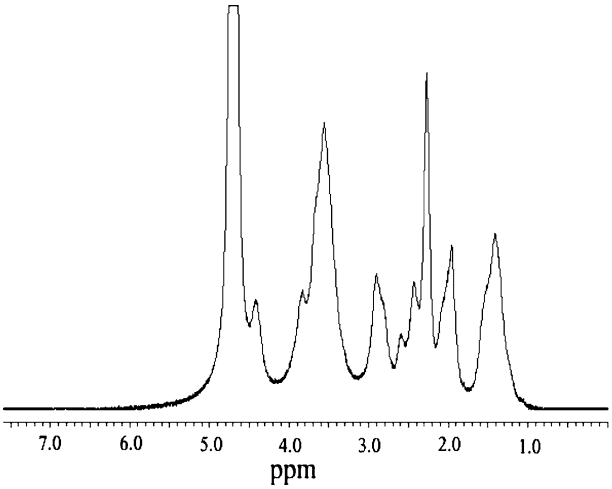


Fig. 2 ^{1}H NMR spectrum of *N*,*O*-2-CEC

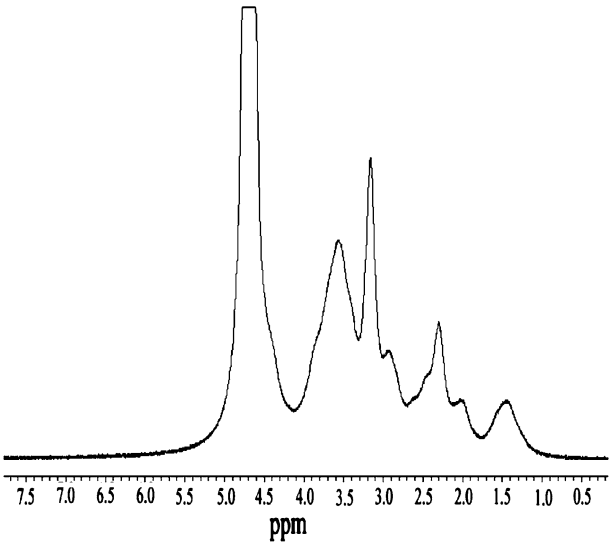


Fig. 3 ^{1}H NMR spectrum of QCEC1

against *S. aureus* were much better than *E. coli*. It could be explained by differences in structures of cell walls between *S. aureus* and *E. coli* [35]. *S. aureus* is a typical gram-positive bacterium, its cell wall 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 the *E. coli* is a typical gram-negative bacterium, its cell wall is made up of a thin membrane of peptide

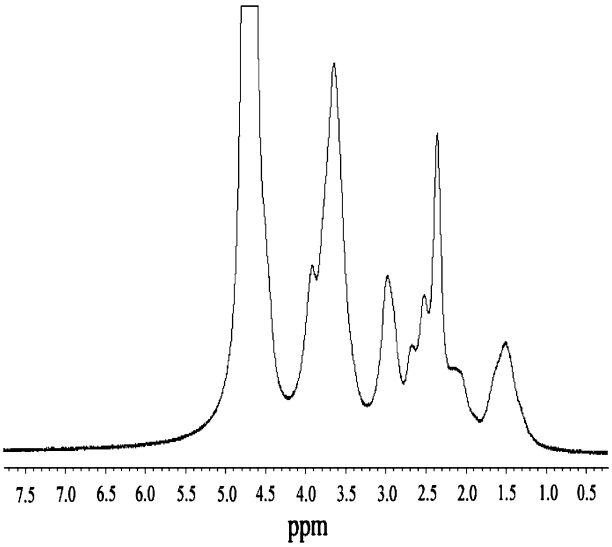


Fig. 4 ^{1}H NMR spectrum of QCEC2

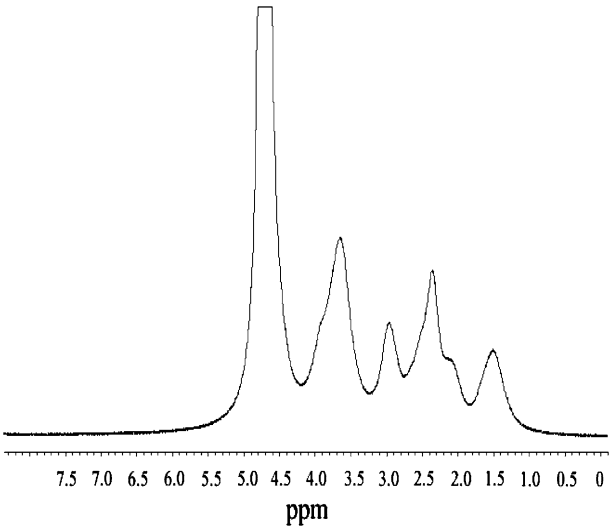


Fig. 5 ^{1}H NMR spectrum of QCEC3

polyglycogen and an outer membrane constitute of lipopolysaccharide, lipoprotein, and phospholipids, so it is difficult for the foreign molecules to penetrate into the cell of *E. coli*.

The QCECs may display its antimicrobial activity through binding with sialic acid in phospholipids, chelating the trace metal and adsorbing on microbial cell membranes. The carboxyl groups could endow QCECs with a capacity to

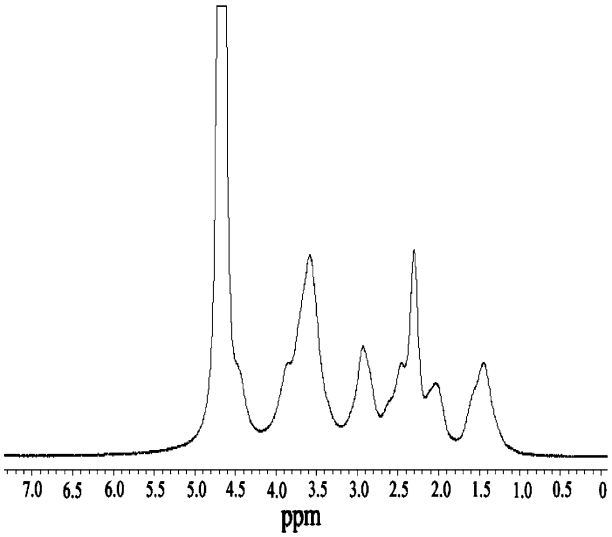


Fig. 6 ^{1}H NMR spectrum of QCEC4

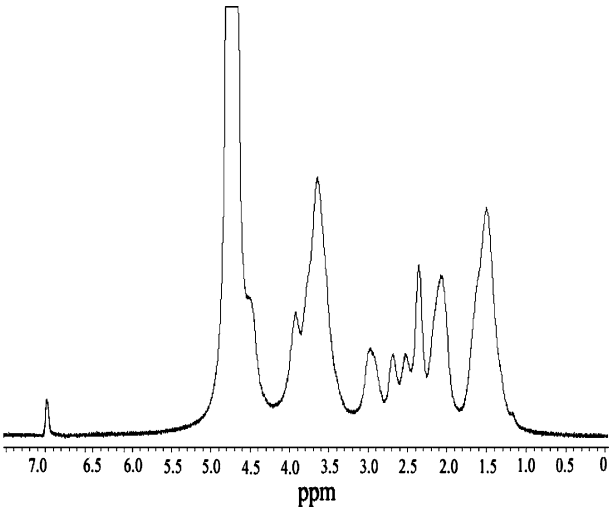


Fig. 7 ^{1}H NMR spectrum of QCEC5

selectively chelated trace metal cations, and the cationic quaternary group could ensure QCECs to associate with anions on the bacteria wall easily, suppress its biosynthesis, disrupt the mass transport across the wall, and accelerate the death of bacteria [36], the antimicrobial activity of QCECs against the tested bacteria was much stronger than that of *N*,*O*-2-CEC and QCTSs.

The antibacterial activities of QCECs were in the descending order of QCEC4 > QCEC3 > QCEC2 > QCEC1, which indicated that the increase of chain length of the alkyl in the quaternary ammonium groups could improve the antibacterial activity of QCECs. This result showed that the antibacterial activities of QCECs were different from that of chitosan derivative reported by Sadeghi and others [37]. The reason may concerned with the following factors: (1)the structure of QCECs is different from trimethyl chitosan (TMC) and triethyl chitosan (TEC); (2) the increase of chain length of the alkyl in the quaternary ammonium groups is advantageous for the adsorption of QCECs on the cell wall of bacteria and the ability of QCECs to bind with sialic acid in phospholipids; (3)when QCECs is utilized as antibacterial agent, there may exist a synergistic effect between its carboxymethyl group and its quaternary ammonium group.

The presence of benzyl in the quaternary group could improve the binding capability of QCEC with bacteria and restrained the movement of microbiological substances; therefore, QCEC5 showed the strongest antimicrobial activity among all samples tested in Table 1.

Table 1 The MIC value of CTS, *N,O*-2-CEC, QCECs, and QCTSs

Sample	DS of CE	DS of QA	M ($\times 10^5$)	MIC for *S. aureus* (μg/mL)	MIC for *E. coli* (μg/mL)
CTS	0	0	3.13	>500	>500
N,O-2-CEC	0.72	0	3.47	31.3	62.5
QCTS1	0	0.86	3.93	31.3	62.5
QCTS2	0	0.83	4.13	31.3	31.3
QCTS3	0	0.78	4.32	31.3	31.3
QCTS4	0	0.71	4.48	15.6	31.3
QCTS5	0	0.76	4.19	15.6	31.3
QCEC1	0.72	0.59	4.09	31.3	31.3
QCEC2	0.72	0.57	4.36	15.6	31.3
QCEC3	0.72	0.54	4.57	12.5	15.6
QCEC4	0.72	0.51	4.75	6.3	12.5
QCEC5	0.72	0.55	4.54	6.3	6.3

DS of CE and DS of QA represented the substitution degree of carboxyethylation and the substitution degree of quaternary ammonium group for chitosan derivatives, respectively

QCEC1, QCEC2, QCEC3, QCEC4, and QCEC5 were the product of *N,O*-2-CEC modified by 2,3-epoxypropyl trimethyl ammonium chloride, 2,3-epoxypropyl triethyl ammonium chloride, 2,3-epoxypropyl tripropyl ammonium chloride, 2,3-epoxypropyl tributyl ammonium chloride and 2,3-epoxypropyl dimethyl benzyl ammonium chloride, respectively

QCTS1, QCTS2, QCTS3, QCTS4 and QCTS5 were the product of CTS modified by 2,3-epoxypropyl trimethyl ammonium chloride, 2,3-epoxypropyl triethyl ammonium chloride, 2,3-epoxypropyl tripropyl ammonium chloride, 2,3-epoxypropyl tributyl ammonium chloride and 2,3-epoxypropyl dimethyl benzyl ammonium chloride, respectively

M was weight-average molecular weights (M_w) of samples except CTS was viscosity molecular weight (M_v)

Conclusion

Quaternized *N*,*O*-2-carboxyethyl chitosans (QCECs) were prepared by the reaction between chitosan and 3-chloropropionic acid, followed by modification with 2,3-epoxypropyl trialkyl ammonium chlorides, including 2,3-epoxypropyl trimethyl ammonium chloride, 2,3-epoxypropyl triethyl ammonium chloride, 2,3-epoxypropyl tripropyl ammonium chloride, 2,3-epoxypropyl tributyl ammonium chloride and 2,3-epoxypropyl dimethyl benzyl ammonium chloride, as grafting agents, respectively. Compared with the CTS, QCTSs and *N*,*O*-2-CEC, QCECs showed much better inhibitive capability against both *S. aureus* and *E. coli*, it indicated that there was a synergistic effect between carboxymethyl group and quaternary ammonium group on the antibacterial activity. The length of alkyl chain or the presence of benzyl group in the quaternary group could improve the antibacterial activity of QCECs greatly.

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