Synthesis of 2-Hydroxypropyl Dimethylbenzylammonium *N,O*-(2-carboxyethyl) Chitosan Chloride and Its Antibacterial Activity

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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 binding-acid agent. 2-Hydroxypropyl dimethylbenzylammonium *N,O*-(2-carboxyethyl) chitosan chloride (HPDMBA-CEC) was obtained by the reaction of *N,O*-2-CEC with glycidyl dimethyl benzyl ammonium chloride (GDMBA) using NaOH as catalyst. The structures of chitosan derivatives were characterized by FTIR, ^{1}H NMR, and gel permeation chromatography. The antimicrobial activity of HPDMBA-CEC was evaluated against a Gram-negative bacterium *Escherichia coli (E.coli)* and a Gram-positive bacterium *Staphylococcus aureus (S. aureus)*. Compared with CTS, *N,O*-2-CEC, and HPDMBA-CTS, HPDMBA-CEC had much stronger antimicrobial activity, and this activity increased with increasing substitution degree of quaternary ammonium group (DQ). When the substitution degree of carboxyethylation (DS of CE) was 0.72 and DQ was 0.60, the minimum inhibitory concentrations (MICs) of HPDMBA-CEC were 3.1 and 6.3 μg/mL against *S. aureus* and *E. coli*, respectively. © 2008 Wiley Periodicals, Inc. J Appl Polym Sci 111: 3010–3015, 2009

Key words: biological applications of polymers; polysaccharides; chemical modification; synthesis; water-soluble polymers

INTRODUCTION

Chitosan (CTS) is a natural linear biopolymer consisting of β-(1,4)-2-acetamido-2-deoxy-D-glucose (GlcNAc) and β-(1,4)-2-amino-2-deoxy-D-glucose (GlcN). Several applications of CTS have been found in the food and pharmaceutical industries mainly because of its excellent properties such as biocompatibility, biodegradability, nontoxicity, adsorptive properties, film-forming ability, antimicrobial activity, etc.[1–6] However, the limited solubility of chitosan in water solution with pH higher than ca. 6.0 render CTS inapplicable in its pure nonderivatized form.[7] For this reason, much effort has been made to develop suitable procedures for the preparation of water-soluble chitosan derivatives.[8–10] Among various methods for the chemical modification of chitosan, carboxyalkylation is one of the most attractive methods.[11–14]

N,O-(2-carboxyethyl)chitosan (*N,O*-2-CEC), which is soluble under acidic, neutral, and basic conditions, is the product resulted from the chemical modification of chitosan with 3-halopropionic acid as modifying agent in the presence of sodium hydroxide or sodium hydrocarbonate.[7,15] *N,O*-2-CEC has many excellent properties such as antioxidant and antimutagenic activity,[13] the ability to control drug release and to chelated metal ion,[16,17] the properties to form hydrogel and to pose membrane, etc.[18,19] Among all of these properties, the antimicrobial activity may be the most important one.

Because of the negative charges of the carboxyl groups, *N,O*-2-CEC could be regarded as an excellent chelating host for metal cation substrate, which is necessary for the microorganism's growth, and shows better antimicrobial activity than chitosan. However, the negatively charged property of microbial cell membranes leads to the fact that *N,O*-2-CEC cannot bind with microbial cell membranes very well under neutral and basic conditions and

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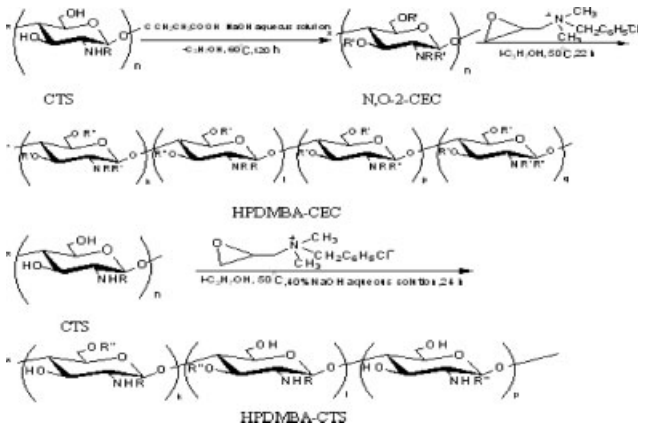

Scheme 1 Synthesis of *N,O*-2-CEC, HPDMBA-CEC, and HPDMBA-CTS. R=H or $COCH_3$; R′=H or CH_2CH_2COOH; R″=H or $CH_2CH(OH)CH_2N(CH_3)_2CH_2C_6H_5Cl$.

therefore, the antibacterial activity of *N,O*-2-CEC would be impaired under nonacidic condition. To overcome this defect, it is necessary for *N,O*-2-CEC to improve its positive charge. Among the methods to improve the positive charge of chitosan derivative, quaternary modification is usually regarded as a simple and efficient way.[20–22]

This article reported the preparation of 2-hydroxypropyl dimethylbenzylammonium *N,O*-2-CEC chloride (HPDMBA-CEC) in which both carboxyethyl group and quaternary ammonium group were introduced into chitosan molecular chain, and the antimicrobial activities of HPDMBA-CEC against a Gram-positive bacterium *Staphylococcus aureus (S. aureus)* and a Gram-negative bacterium Escherichia coli (E. coli) were evaluated by agar plate method. To explain the antimicrobial activities of HPDMBA-CEC clearly, the antimicrobial activities of *N,O*-2-CEC, 2-hydroxypropyl dimethylbenzyl ammonium chitosan chloride (HPDMBA-CTS), and CTS were also investigated in our research. The preparation of *N,O*-2-CEC, HPDMBA-CTS, and HPDMBA-CEC is presented in Scheme 1.

EXPERIMENTAL

Materials

Chitosan was purchased from Sinopharm Chemical Reagent (Shanghai, People's Republic of China), and 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 solution of glycidyl dimethyl benzyl ammonium chloride (GDMBA) was prepared in our laboratory by the similar method illustrated in literature,[23] and its mass concentration was measured by potential titration to be ca. 500 g/L. Sodium hydroxide was purchased from the third factory of chemical reagent in Tianjin (Tianjin, People's Republic of China), excellent grade. A Gram-positive bacterium *S. aureus* and a Gram-negative bacterium *E.coli* were provided by the Microbiology Laboratory of Nanjing University of Technology (Nanjing, People's Republic of China), and they were inoculated on a gel containing 1% peptone, 2% agar, 3% meat extract, and 0.5% NaCl. All other chemicals were of reagent grade and used without purification as received.

Preparation of N,O-2-CEC and HPDMBA-CTS

CTS (9.84 g), 100.0 mL of 2-propanol and 36.0 g of NaOH aqueous solution (mass concentration, 40.0%) were added into a four-necked bottle. The mixture was heated to 45.0°C using water bath and was alkalized for 2.0 h under stirring. Then 3-chloropropionic acid (19.54 g, 180 mmol) dissolved in ca. 60 mL 2-propanol was added into the bottle dropwise in case the temperature was higher than 60.0°C. When the 3-chloropropionic acid was added completely, the temperature of mixture was elevated to 60.0°C and allowed to react for 5 days at this temperature. The mixture was filtered, and the filtered residue was dissolved in ca. 300 mL distilled water. The pH of blending was adjusted to ca. 8.0 with HCl solution (mass concentration, 10.0%) and filtered. Absolute alcohol (480 mL) was added into the filtrate under stirring, and the mixture was separated through centrifugal settling. The resultant dreg was washed by 120.0 mL CH_3OH aqueous solution (mass concentration, ca. 85.0%) for three times, and then by 90.0 mL absolute ethanol for three times and filtered. The solid was dried by vacuum-constant temperature drying apparatus under 60°C and 0.1 MPa vacuum tightness for 12 h. It was put into vacuum drier for later use. The yield of *N,O*-2-CEC was 87.2%.

CTS (3.28 g) was alkalized as the preparation procedure of *N,O*-2-CEC except 2-propanol was 50.0 mL and NaOH aqueous solution was 10.0 g. Then, GDMBA aqueous solution (80 mmol) was added into the bottle dropwise in case the temperature was higher than 45.0°C. When the GDMBA was added completely, the temperature of blending was elevated to 45.0°C and allowed to react at this temperature for 24 h under stirring. The mixture was filtered, and the filtered residue was dissolved in ca. 60 mL distilled water. The pH of the blending was adjusted to ca. 7.0 with HCl solution (mass concentration, 10.0%) and filtered. Absolute alcohol (90.0 mL) was added into the filtrate under stirring, and the mixture was separated through centrifugal settling. The resultant dreg was treated like the preparation procedure of *N,O*-2-CEC. The HPDMBA-CTS was obtained in 81.7% yield.

Quaternization of N,O-2-CEC with GDMBA

Dried *N,O*-2-CEC (2.32 g), 30.0 mL of 2-propanol, and 8.35 g of NaOH aqueous solution (mass concentration, 25.0%) were added into a four-necked bottle. The mixture was heated to 45.0°C in water bath under stirring and was alkalized for 1.0 h. Then, calculated aqueous solution of GDMBA was added into the bottle dropwise in case the temperature was higher than 50.0°C. When the GDMBA was added completely, the temperature of blending was elevated to 50.0°C and allowed to react for 22.0 h at this temperature. The mixture was filtered, and the filtered residue was dissolved in ca. 60 mL distilled water. The pH of the solution was adjusted to ca. 7.0 with HCl solution (mass concentration, 10.0%) and filtered. Absolute alcohol (90 mL) was added into the filtrate under stirring, and the mixture was separated through centrifugal settling. The resultant dreg was treated like the procedure of *N,O*-2-CEC, and the HPDMBA-CEC was obtained.

Characterization

Fourier transform infrared (FTIR) spectra were recorded with KBr pellets on a Nicolet Nexux FTIR 670 spectrometer. Sixteen scans at a resolution of 4 cm^{-1} were averaged and referenced against air. ^1H NMR spectra of CTS, *N,O*-2-CEC, HPDMBA-CEC, and HPDMBA-CTS were obtained with Bruker AV-300 spectrometer at 30°C ± 0.5°C, respectively. All the ^1H NMR spectra were measured in D_2O solution, except for the CTS which was in D_2O-TFA solution, and the sample was 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 and HPDMBA-CECs were estimated by alkalimetry. Samples were dissolved in 0.100 mol/L hydrochloric acid (50 mL) and titrated with 0.100 mol/L sodium hydroxide. The alkalimetric curves were constructed by recording pH vs. V_{NaOH}.

Weight-average molecular weights (M_w) of samples were measured by gel permeation chromatography with poly(ethyl oxide) as standard on a Water-208 apparatus (column: Ultrahydrogel 500 and 1000; eluent: water; flow rate: 1.0 mL/min; column temperature: 32°C; concentration of samples: 0.8 mg/mL.)

The degree of substitution of the quaternization group (DQ) of HPDMBA-CECs and HPDMBA-CTS were determined by the potentiometry. Briefly, samples were dissolved in double-distilled water (50 mL) and titrated with 0.100 mol/L aqueous silver nitrate using silver electrode as working electrode and calomel electrode as reference electrode. The titrating curves were constructed by recording κ vs. V_{AgNO3}.

Evaluation of antimicrobial activity of CTS, N, O-2-CEC, HPDMBA-CTS, and HPDMBA-CEC

The minimum inhibition concentrations (MICs) of CTS, *N,O*-2-CEC, HPDMBA-CTS, HPDMBA-CEC, and ofloxacin were determined by similar agar plate method illustrated in literature,[24] except the initial concentration of the samples were 500 μg/mL, the final concentrations were 250, 125, 62.5, 31.3, 15.6, 12.5, 6.3, 3.1, 1.56, 1.0, 0.5, 0.25, and 0.125 μg/mL. The culture of each bacterium was diluted by sterile-distilled water to ca. 100 CFU/mL, and the inoculated plates were incubated at 37°C for 48 h.

The minimum inhibitory concentration (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.[24]

RESULTS AND DISCUSSION

Identification of resonance in the spectra

The FTIR spectra of CTS, *N,O*-2-CEC, HPDMBA-CTS, and HPDMBA-CEC are presented in Figure 1. The absorption bands at 1655, 1593, 1317, 1380 cm^{-1} in the spectrum of CTS were assigned to primary, secondary and tertiary amides, and CH_3 bending

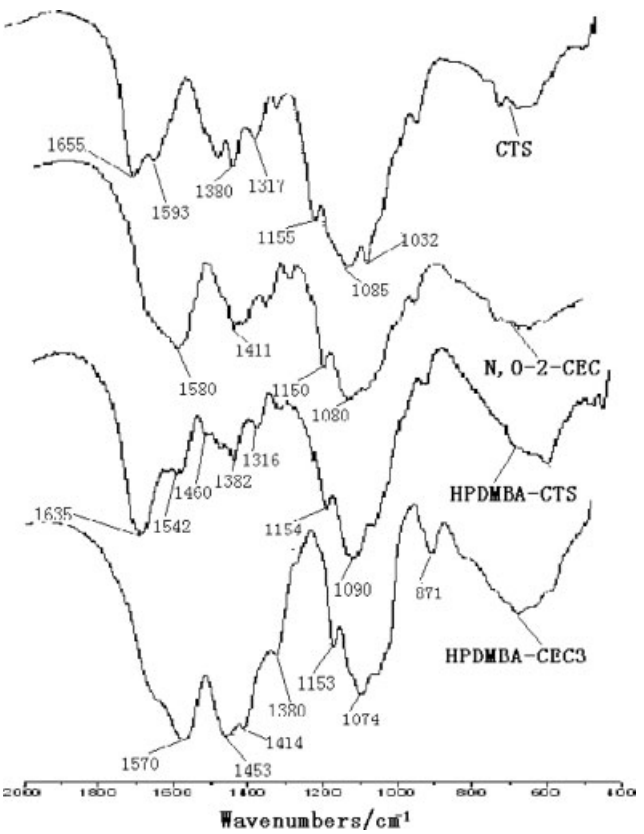

Figure 1 FTIR spectra of CTS, *N,O*-2-CEC, HPDMBA-CEC3, and HPDMBA-CTS3.

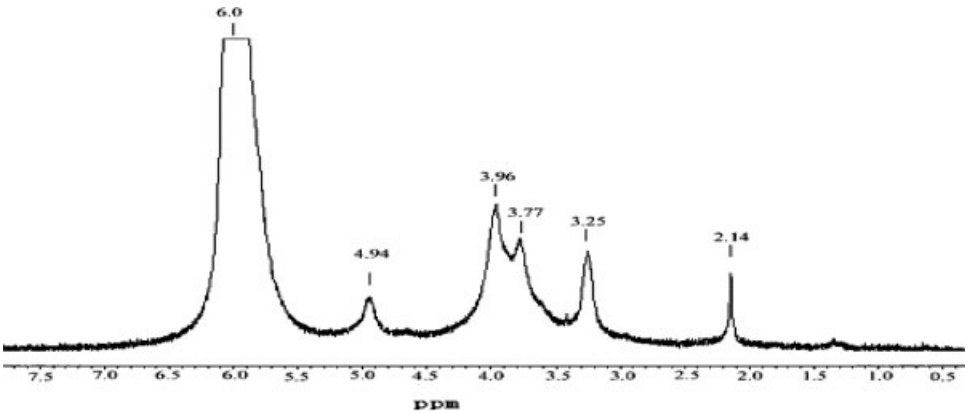

Figure 2 ^{1}H NMR spectrum of chitosan.

vibration. Characteristic peaks for CTS at 1085 cm^{-1} could be attributed to the C—O vibration. Two strong peaks at 1580 and 1411 cm^{-1} in *N,O*-2-CEC spectrum and 1570 and 1414 cm^{-1} in HPDMBA-CEC3 spectrum were ascribed to the asymmetrical and symmetrical stretching of $-COO^-$ group. In the spectrum of *N,O*-2-CEC, the C—O stretching band at 1032 cm^{-1} corresponding to the primary hydroxyl group of CTS became weakening, which confirmed a high carboxyethylation of OH-6. In contrast, a new peak appeared at 1453 cm^{-1} for HPDMBA-CEC3 and 1460 cm^{-1} for HPDMBA-CTS attributed to the methyl of the quaternary ammonium group that has been introduced into N,O-2-CEC and CTS.

Figures 2–5 show the ^{1}H NMR spectra of CTS, *N,O*-2-CEC, HPDMBA-CTS, and HPDMBA-CEC3, respectively. The ^{1}H NMR spectrum of CTS showed signals at 3.25 and 2.14 ppm that were assigned to the proton of C-2 of GlcN and acetyl of GlcNAc unit, 4.94 and 3.4–4.5 ppm that were assigned to the hydrogen of C-1 and C-3 to C-6 of GlcN and GlcNAc, respectively. The ^{1}H NMR spectrum of N,O-2-CEC showed signals at 2.43–2.59 and 3.56 ppm that were assigned to the hydrogen of C-2 and C-3 methylene of 6-substituted and 3-substituted carboxyethyl group, respectively. The resonance of 2-substituted carboxyethyl-protons ($-NCH_2CH_2$ COOD) of chitosan occurred in the spectral region of 2.27 and 2.90 ppm. The ^{1}H NMR spectrum of *N,O*-2-CEC showed signals at 4.42, 1.95, and 1.41 ppm that were assigned to the hydrogen of C-1, proton of carboxyethyl, and N-acetyl, respectively. Compared with *N,O*-2-CEC, these resonances also occurred in the ^{1}H NMR spectrum of HPDMBA-CEC3. However, there were some new resonances for HPDMBA-CEC3. A signal at 6.98 ppm in the ^{1}H NMR spectrum of HPDMBA-CEC3 could be attributed to the protons on phenyl of quaternary ammonium group. Two resonances appeared in 2.64 and 4.50 ppm, which could be attributed to the methylene protons of quaternary ammonium group. The adsorbing peak at ca. 3.60 ppm, which could be attributed to the methylene protons of hydropropyl, became stronger than that of *N,O*-2-CEC. All these indicated that the quaternary ammonium group had

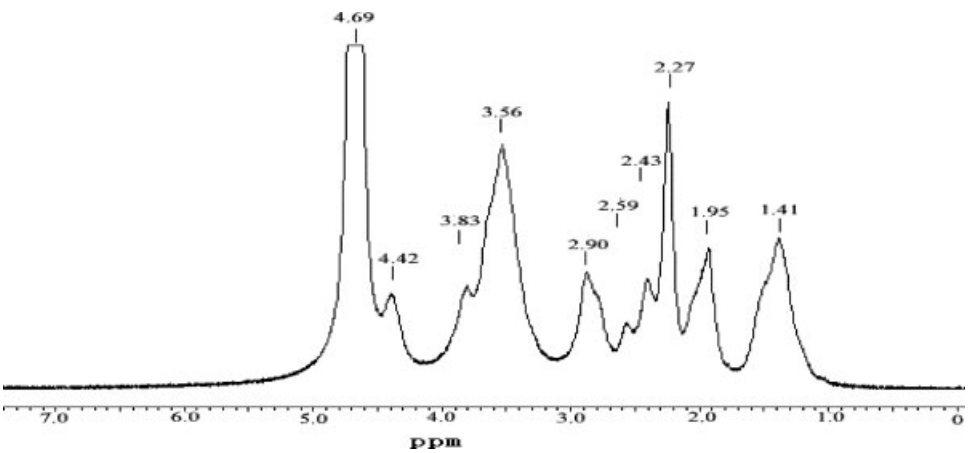

Figure 3 ^{1}H NMR spectrum of *N,O*-2-CEC.

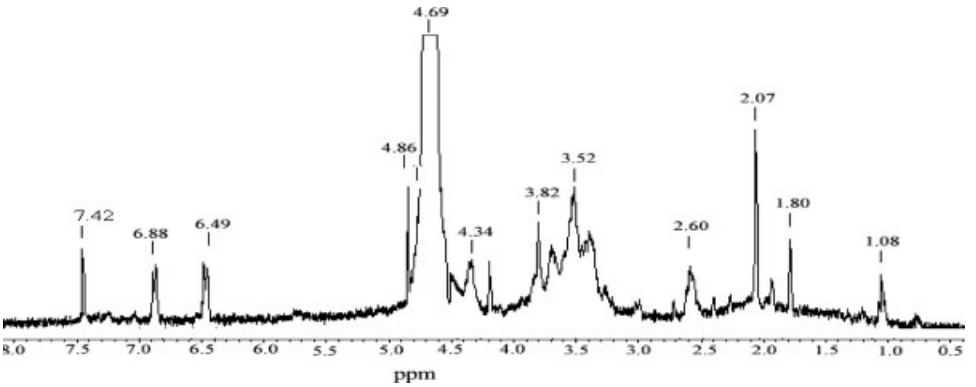

Figure 4 ^{1}H NMR spectrum of HPDMBA-CTS.

been introduced into *N,O*-2-CEC. The resonances at 6.49, 6.88, 7.42, and 4.34 ppm in the ^{1}H NMR spectrum of HPDMBA-CTS were assigned to the hydrogen of methylidyne and methylene of benzyl, respectively. The adsorbing peak at ca. 2.62, 3.30, 3.64, and 4.20 ppm could be attributed to the methylene protons and methylidyne protons of hydropropyl of quaternary ammonium group.

Antimicrobial activity of CTS, N,O-2-CEC, HPDMBA-CTS, and HPDMBA-CECs

The MICs of CTS, *N,O*-2-CEC, HPDMBA-CTS, and HPDMBA-CECs against *S. aureus* and *E. coli* are shown in Table I.

The results of antimicrobial experiments showed that CTS had little antimicrobial activity against the tested bacteria under the experimental conditions, it is owed to the poor solubility in neutral condition. The antibacterial activities of *N,O*-2-CEC, HPDMBA-CTS, and HPDMBA-CECs against *S. aureus* were higher than *E. coli*. The reason resulted in this difference may be concerned with the different structure of cell walls between *S. aureus* and *E. coli*.[25] *S. aureus* is a typical Gram-positive bacterium and its cell wall is fully composed of peptide polyglycogen. The peptidoglycan layer is composed of networks with plenty of pores, which allows foreign molecules to come into the cell without difficulty. On the opposition, the *E. coli* is a typical Gram-negative bacterium and its cell wall is made up of a thin membrane of peptide polyglycogen and an outer membrane constitute of lipopolysaccharide, lipoprotein, and phospholipids. Because of the bilayer structure of cell wall, it is difficult for the foreign molecules to enter into the cell of *E. coli*.

Introduction of quaternary group could improve the cationic property of CTS and CEC, promote the derivative of chitosan to associate with anions on the bacteria cell wall easily, suppress microbial biosynthesis, disrupt the mass transport across the wall, and accelerate the death of bacteria[26]; therefore, the antimicrobial activities of HPDMBA-CTS and HPDMBA-CECs against the tested bacteria were better than that of *N,O*-2-CEC. Since the trace metal cations, which are necessary for the microorganism's

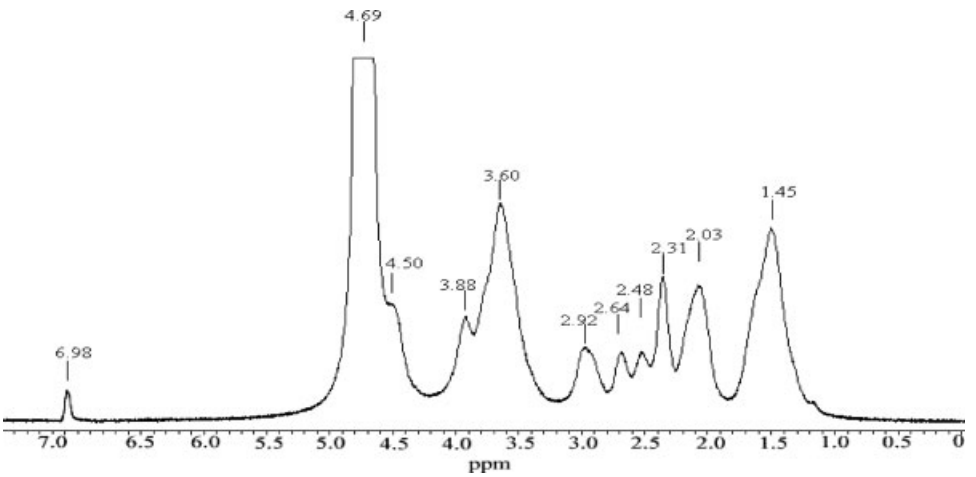

Figure 5 ^{1}H NMR spectrum of HPDMBA-CEC3.

TABLE I
The MIC Value of CTS, N,O-2-CEC, HPDMBA-CTS, HPDMBA-CECs, and Ofloxacin Against *S. aureus* and *E. coli*

Sample	DS of CE	DQ	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
HPDMBA-CTS	0	0.79	4.36	15.6	31.3
HPDMBA-CEC1	0.72	0.37	4.03	12.5	15.6
HPDMBA-CEC2	0.72	0.48	4.34	6.3	12.5
HPDMBA-CEC3	0.72	0.55	4.54	6.3	6.3
HPDMBA-CEC4	0.72	0.60	4.68	3.1	6.3
Ofloxacin[a]	0	0		0.125	0.50

HPDMBA-CEC1, HPDMBA-CEC2, HPDMBA-CEC3, and HPDMBA-CEC4 were the product of N,O-2-CEC modified by 20, 30, 40, and 50 mmol EPDMBA per 2.32 g N,O-2-CTS, respectively. M is the weight-average molecular weights (M_w) of samples and CTS is the viscosity molecular weight (M_v).

[a] The molecular weight of ofloxacin is 361.38 g/mol.

growth, could be selectively chelated by the carboxyethyl group of HPDMBA-CEC, and there was a synergistic effect between carboxyethyl group and quaternary ammonium group, the antimicrobial activities of HPDMBA-CECs were stronger than that of HPDMBA-CTS. The increase of DQ could promote the adsorption of HPDMBA-CECs on the anionic cell wall of bacteria and endow the HPDMBA-CECs with much stronger antimicrobial activity.

Compared with ofloxacin, the antibacterial activity of HPDMBA-CEC is lower, but its antibacterial activity is higher than that of the chitosan derivatives reported by others.[8,10,23–25] Furthermore, the biocompatibility, biodegradability, and nontoxicity of HPDMBA-CEC may be the predominance for its application in biomaterial field.

CONCLUSION

N,O-2-CEC was prepared with chitosan and 3-chloropropionic acid, which served as the modifying agent in the presence of 40% NaOH aqueous solution. Then, HPDMBA-CEC was obtained by the modification of *N,O*-2-CEC with GDMBA as the grafting agent. Compared with CTS, *N,O*-2-CEC, and HPDMBA-CTS, HPDMBA-CEC showed much better inhibition effects against *S. aureus* and *E. coli*, and its antibacterial activity increased with the increase of the substitution degree of the quaternization group (DQ). These characters ensure that HPDMBA-CEC has more extensive applications in the field of antibacterial material.

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