Synthesis of 2-Hydroxypropyl Dimethylbenzyl Ammonium *N,O*-Carboxymethyl Chitosan Chloride and Its Antibacterial Activity

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Summary: *N,O*-carboxymethyl chitosan (*N,O*-CMC) was prepared from chitosan with chloroacetic acid as modifying agent and NaOH as binding-acid agent. 2-Hydroxypropyl dimethyl benzyl ammonium *N,O*-carboxymethyl chitosan chloride (HPDMBA-N,O-CMC) was obtained by the reaction between *N,O*-CMC and glycidyl dimethyl benzyl ammonium chloride (GDMBA) using NaOH as catalyst. The structures of chitosan derivatives were characterized by FT-IR, ^{1}H NMR and gel permeation chromatography (GPC). The antimicrobial activity of HPDMBA-N,O-CMC 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*-CMC and 2-hydroxypropyl dimethylbenzyl ammonium chitosan chloride (HPDMBA-CTS), HPDMBA-N,O-CMC had much stronger antimicrobial activity . When the substitution degree of carboxymethylation (DS of CM) was 0.856 and the substitution degree of quaternary ammonium group (DQ) was 0.573, the minimum inhibitory concentrations (MICs) of HPDMBA-CMC were 6.3 and 12.5 μg/mL against *S. aureus* and *E. coli*, respectively.

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). CTS has been widely applied in the food and pharmaceutical industries due to its many excellent properties such as biocompatibility, biodegradability, nontoxicity, adsorptive properties, film-forming ability, antimicrobial activity, etc[1-6]. However, CTS in its nonderivatized form is hardly soluble in water at pH higher than *ca* 6.0, which limits its further application [7]. In this regard, intense attention has been paid to the development of suitable methods to prepare water-soluble chitosan derivatives [8-10]. Among them, carboxyalkylation is one of the most attractive methods [11-14].

N,O-carboxymethyl chitosan (*N,O*-CMC), which is soluble under acidic, neutral and basic conditions, is the product obtained from the chemical modification of chitosan with chloroacetic acid as modifying agent in the presence of sodium hydroxide [7]. *N,O*-CMC has many excellent properties such as antibacterial activity [2,3,8,9], forming hydrogel[8-10] and flocculating properties[1,7,15], etc. Among all of these properties, the antimicrobial activity may be the most important one. Due to negative charges of the carboxyl groups, *N,O*-CMC could act as an excellent chelating host for metal cation that is necessary for the microorganism's growth, and could show better antimicrobial activity than chitosan. On the other hand, the negatively-charged microbial cell membranes impedes the binding of *N,O*-CMC under neutral and basic conditions, thus impairing its antibacterial activity under non-acidic condition. In this end, it is necessary to introduce positively-charged groups into *N,O*-CMC. Among the reported methods, the quaternary modification of CTS and its derivatives is usually a simple and efficient way [16-18].

We herein reported the preparation of 2-hydroxypropyl dimethylbenzylammonium *N,O*-carboxymethyl chitosan chloride (HPDMBA-N,O-CMC) in which both carboxymethyl group and quaternary ammonium group were introduced into chitosan molecular chain. The antimicrobial activities of HPDMBA-N,O-CMC against a Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and a Gram-negative bacterium *Escherichia coli* (*E. coli*) were evaluated by agar plate method. Control experiments using *N,O*-CMC, 2-hydroxypropyl dimethylbenzyl ammonium chitosan chloride (HPDMBA-CTS) and CTS were conducted. The preparation of *N,O*-CMC, HPDMBA-CTS and HPDMBA-N,O-CMC were presented as Scheme 1.

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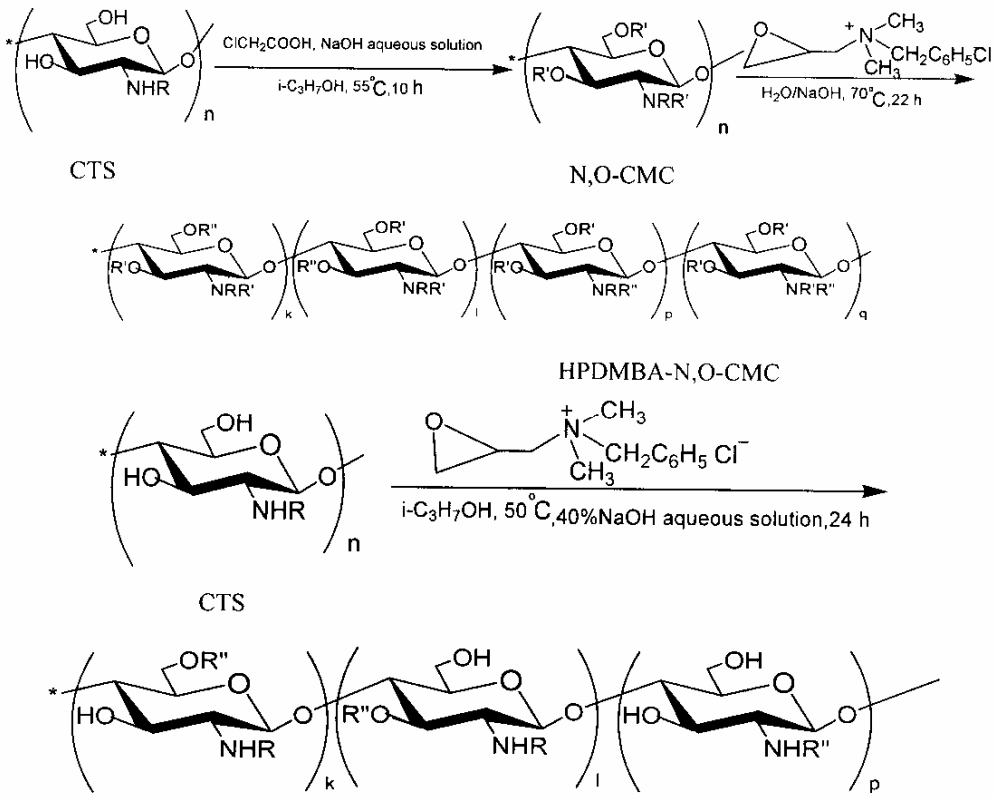

Scheme 1: Synthesis of *N,O*-CMC, HPDMBA-*N,O*-CMC and HPDMBA-CTS
R = H or $COCH_3$; R′ = H or CH_2COOH; R″ = H or $CH_2CH(OH)CH_2N(CH_3)_2CH_2C_6H_5Cl$.

Results and Discussion

Identification of Resonance in the Spectra

The FT-IR spectra of CTS, *N,O*-CMC, HPDMBA-CTS and HPDMBA-N,O-CMC were presented in Fig. 1. The absorption at 1655, 1593, 1317, 1380 cm^{-1} in the spectrum of CTS were assigned to the bending vibration of primary, secondary and tertiary amides, and $-CH_3$. Characteristic peaks for CTS at 1085 cm^{-1} could be attributed to the C-O vibration. Two strong peaks at 1601 and 1417 cm^{-1} in *N,O*-CMC spectrum, and 1602 and 1417 cm^{-1} in HPDMBA-N,O-CMC spectrum were ascribed to the asymmetrical and symmetrical stretching of $-COO^-$ group. In the spectrum of *N,O*-CMC, the C-O stretching band at 1032 cm^{-1} corresponding to the primary hydroxyl group of CTS became weak, which confirmed a high carboxymethylation of -OH at C6 position. In contrast, a new peak appeared at 1480 cm^{-1} for HPDMBA-N,O-CMC and 1460 cm^{-1} for HPDMBA-CTS were attributed to the $-CH_3$ of the quaternary ammonium group that was pre-introduced into N,O-CMC and CTS.

The ^1H NMR spectra of CTS, *N,O*-CMC, HPDMBA-CTS and HPDMBA-N,O-CMC were shown in Figs. 2-5, respectively. As for CTS, the signals at 3.25 and 2.14 ppm were assigned to the hydrogen of C-2 of GlcN and acetyl of GlcNAc unit, and 4.94 and 3.4-4.5 ppm to the hydrogen of C-1 and C-3 to C-6 of GlcN and GlcNAc, respectively. The resonance of 6-substituted and 3-substituted carboxymethyl-protons($-OCH_2COOD$) in chitosan appeared in the spectral region of 4.2-4.6 ppm and 4.6-4.7 *ppm*, respectively[19]. The resonance of 2-substituted carboxymethyl-protons ($-NCH_2COOD$) of chitosan occurred in the spectral region of 3.2-3.4 *ppm*[19,20]. In comparison with *N,O*-CMC, some

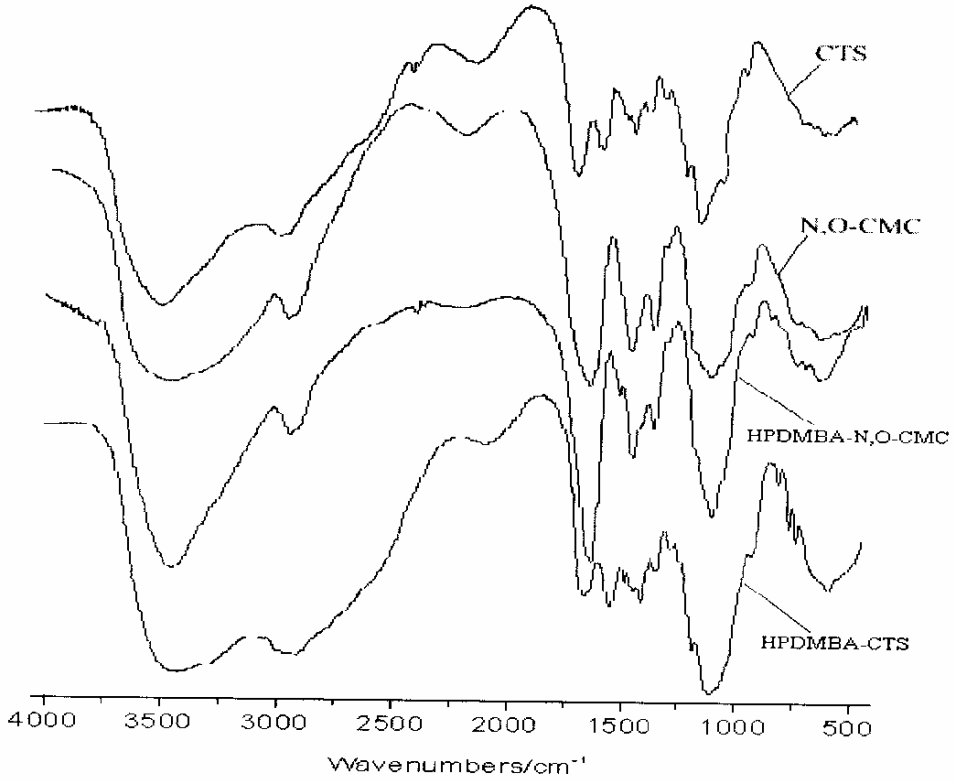

Fig. 1: The FT-IR spectra of CTS, *N,O*-CMC, HPDMBA-CTS and HPDMBA-*N,O*-CMC.

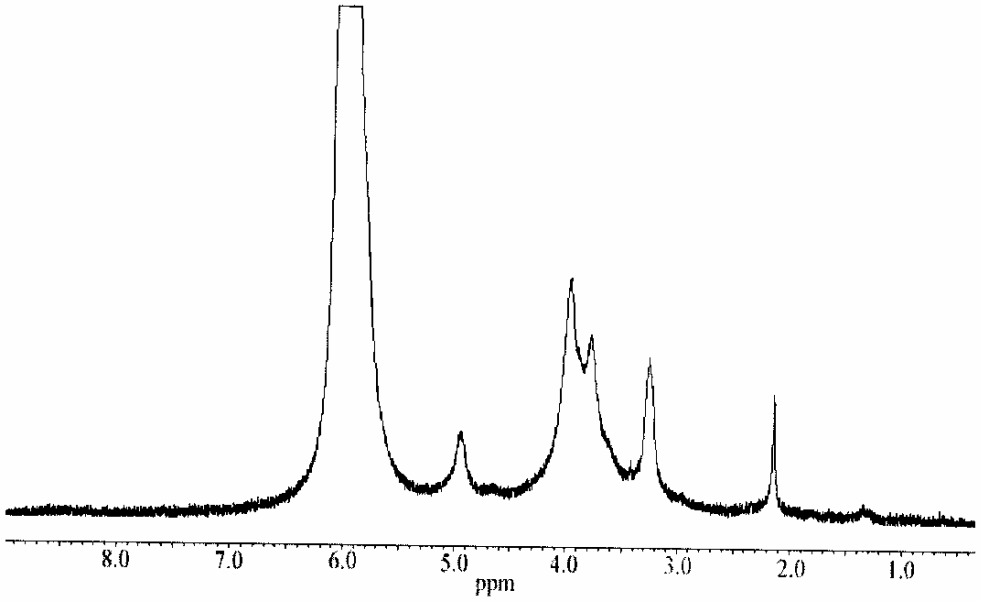

Fig. 2: ^{1}H NMR spectrum of chitosan.

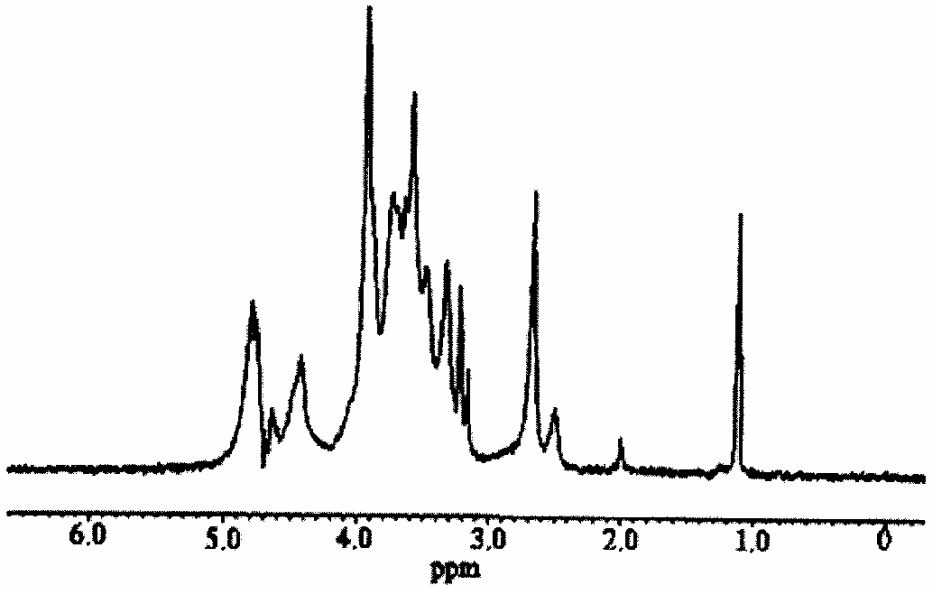

Fig. 3: ^{1}H NMR spectrum of *N,O*-CMC.

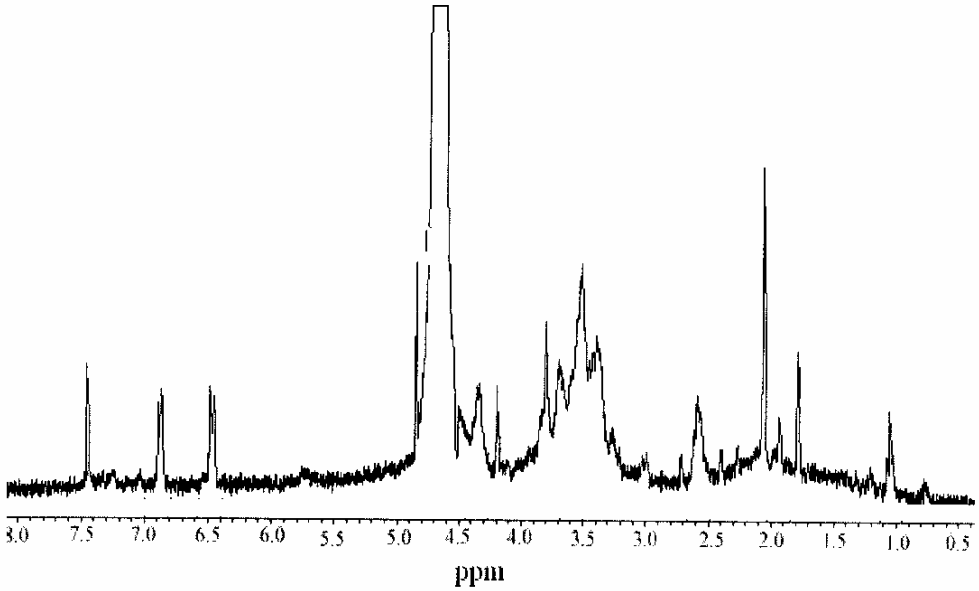

Fig. 4: ^{1}H NMR spectrum of HPDMBA-CTS.

new signals appearred in the ^{1}H NMR spectrum of HPDMBA-N,O-CMC in addition to those occurred in that of *N,O*-CMC. A signal at 7.58 ppm in the ^{1}H NMR spectrum of HPDMBA-N,O-CMC could be attributed to the protons of phenyl of quaternary ammonium group. Two resonances appeared at 2.72 and 4.61 *ppm*, which could be attributed to the methylene-protons of quaternary ammonium group. The peak at *ca* 3.61-3.68 ppm that could be attributed to the methylene-protons of hydropropyl became

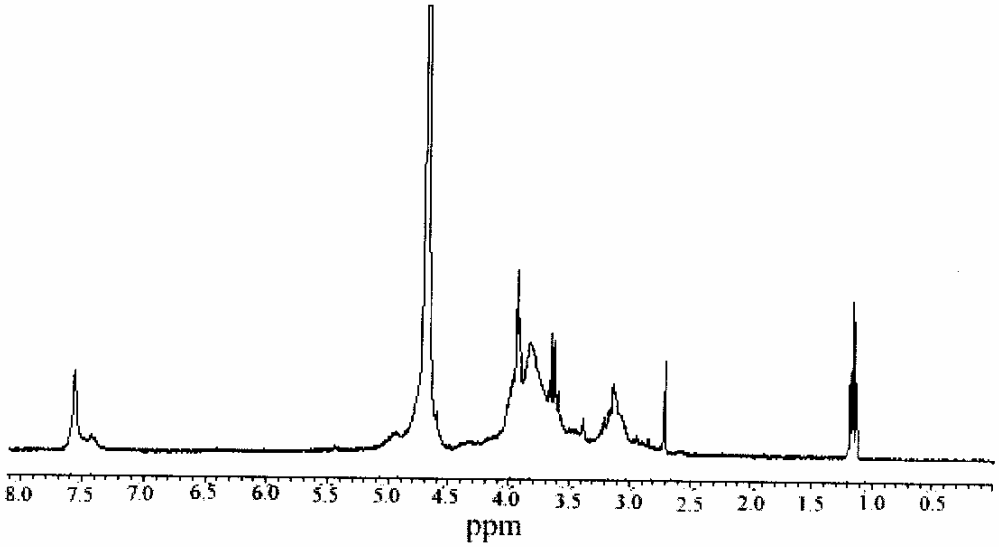

Fig. 5: ^{1}H NMR spectrum of HPDMBA-*N,O*-CMC.

stronger than that of *N,O*-CMC. These results indicated that the quaternary ammonium group has been introduced into *N,O*-CMC. The peaks 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 peaks at *ca* 2.62, 3.30, 3.64 and 4.20 ppm could be attributed to the methylene- and methylidyne-protons of hydropropyl of quaternary ammonium group.

Antimicrobial Activity of CTS, N,O-CMC, HPDMBA-CTS, HPDMBA-N,O-CMC and Ofloxacin

The MICs of CTS, *N,O*-CMC, HPDMBA-CTS, HPDMBA-N,O-CMC and ofloxacin against *S. aureus* and *E. coli* were listed in Table-1.

The results showed that CTS had little antimicrobial activity against the tested bacteria under the experimental conditions, which may be due to its poor solubility in neutral solution. The antibacterial activities of *N,O*-CMC, HPDMBA-CTS and HPDMBA-N,O-CMC against *S. aureus* were higher than that of *E. coli*, which may be related to the different structure of cell walls between *S. aureus* and *E. coli* [21]. *S. aureus* is a typical Gram-positive bacterium and its cell wall is fully composed of peptide polyglycogen. The peptidoglycan layer features networks with plenty of pores, which facilitates foreign molecules to come into the cell. On the contrary, *E. coli* is a typical Gram-negative bacterium and its cell wall is constructed by a thin membrane of peptide polyglycogen and an outer membrane constituted of lipopolysaccharide, lipoprotein and phospholipids. Because of such bilayer structure of cell wall, it is difficult for the foreign molecules to come into the cell of *E. coli*.

Introduction of quaternary group into CTS and N,O-CMC could improve their cationic property, which may promote the derivative of CTS to bind

Table-1: The MIC value of CTS, *N,O*-CMC, HPDMBA-CTS, HPDMBA-CMC and Ofloxacin against *S. aureus* and *E. coli*.

Sample	DS of CM	DQ	M ($\times10^5$)	MIC for *S. aureus*,, μg/mL	MIC for *E. coli*, μg/mL
CTS	0	0	3.13	>250	>250
N,O-CMC	0.856	0	3.12	62.5	62.5
HPDMBA-CTS	0	0.761	4.36	15.6	31.3
HPDMBA-N,O-CMC	0.856	0.573	4.60	6.3	12.5
Ofloxacin	0	0	*	0.125	0.50

DS of CM and DQ represent the substitution degree of carboxymethylation and the substitution degree of quaternary ammonium group for chitosan derivatives, respectively.
M is weight-average molecular weights (Mw) of samples except CTS is viscosity molecular weight (Mv).
*The molecular weight of Ofloxacin is 361.38, g/mol

with the anions on the bacteria cell wall easily. Such binding may suppress microbial biosynthesis, disrupt the mass transport across the wall, and accelerate the death of bacteria [22]. Thus, the quaternized products including HPDMBA-CTS and HPDMBA-N,O-CMC demonstrated higher antimicrobial activity against the tested bacteria than that of *N,O*-CMC. On the other hand, the trace metal cations, which is necessary for the microorganism's growth, could be chelated selectively by the carboxymethyl group of HPDMBA-N,O-CMC. Moreover, a synergistic effect may exist between carboxymethyl group and quaternary ammonium group. These results may thus lead to the stronger antimicrobial activities of HPDMBA-N,O-CMC than that of HPDMBA-CTS.

Compared with other reported CTS derivatives [8, 10, 21, 23, 24], the antibacterial activity of HPDMBA-N,O-CMC is higher, but it is lower than that of ofloxacin However, the well-known advantages of CTS and its derivatives in biocompatibility, biodegradability and nontoxicity may open a wide application space in biomaterial field.

Experimental

Materials and Methods

Chitosan was purchased from Sinopharm. Chemical Reagent Co., Ltd., its deacetylation degree was determined to be 92.3 % by potentiometric titration and its viscosity-average molecular weight (Mv) was 3.13×10^5. The aqueous solution of glycidyl dimethyl benzyl ammonium chloride (GDMBA) was prepared in our laboratory by a similar method [23], its mass concentration was measured by potential titration to be *ca* 500g/L. Sodium hydroxide was purchased from the third factory of chemical reagent in Tianjin, excellent grade. A Gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and a *Gram-negative* bacterium *Escherichia coli* (*E. coli*) were provided by Microbiology Laboratory of Nanjing University of Technology and 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 further purification.

Fourier transform infrared (FT-IR) spectra were recorded on a Nicolet Nexus FT-IR 670 spectrometer in KBr pellets. 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 30 ± 0.5 °C. All the ^1H NMR spectra were measured in D_2O solution except that CTS 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.

Preparation of N,O-CMC and HPDMBA-CTS

CTS (9.84 g) was added into a four-neck bottle containing 2-propanol (100.0 mL) and NaOH aqueous solution (36.0 g, mass concentration, 40.0 %), and alkalized at 45 °C for 2.0 h in water bath under stirring. Then, chloroacetic acid (17.01 g, 180 mmol) dissolved in *ca* 17 mL of distilled water was added into the bottle dropwise in case the temperature was higher than 50 °C. The mixture was allowed to react at 55 °C for 10 h, and then filtered. The reside was dissolved in *ca* 300 mL of distilled water. The pH of blending was adjusted to *ca* 8.0 with aqueous HCl solution (mass concentration, 10.0 %) and filtered. Absolute ethanol (480 mL) was added into the filtrate under stirring. The resultant precipitate was separated through centrifugal settling, and washed by aqueous methanol solution (120.0 mL, mass concentration, *ca* 85.0 %) and then absolute ethanol (90.0 mL) for three times, respectively. The obtained solid was characterized as N,O-CMC (See characterization section). It was dried under vacuum at 60 °C and stored in vacuum prior to use.

CTS (3.28 g) was alkalized similar to the above procedure of *N,O*-CMC in 50.0 mL of 2-propanol and 10.0 g of NaOH aqueous solution. Then, aqueous GDMBA solution (80 mmol) was added into the mixture dropwise in case the temperature was higher than 45 °C. After complete addition, the temperature was elevated to 50 °C and allowed to react for 24 h under stirring. The mixture was filtered and the reside was dissolved in *ca* 60 mL of distilled water. The pH of the blending was adjusted to *ca* 7.0 with aqueous HCl solution (mass concentration, 10.0 %) and filtered. The other treatment procedure was similar to that of *N,O*-CMC. The obtained solid was characterized as HPDMBA-CTS (See characterization section). It was stored in vacuum prior to use.

Quaternization of N,O-CMC Using GDMBA as Modifying Agent

Dried *N,O*-CMC (4.72*g*) and distilled water (55.0 mL) were added into a four-neck bottle. The mixture was heated to 45 °C in water bath under stirring for 0.50 h. Then, the calculated aqueous solution of GDMBA was added into the bottle

dropwise in case the temperature was higher than 50 °C. The pH of mixture was adjusted to *ca* 9.0 using NaOH aqueous solution (mass concentration, *ca* 5.0 %). After the mixture was allowed to react at 70 °C for 22.0 h, it was filtered and the filter reside was dissolved in *ca* 60 mL of distilled water. The pH of the solution was adjusted to *ca* 7.0 with HCl solution (mass concentration, *ca* 10.0 %) and then filtered. Absolute ethanol (90 mL) was added into the filtrate under stirring. The resulted solid was collected by centrifugal settling and treated similar to the procedure of *N,O*-CMC. The product obtained from the above method was characterized as HPDMBA-N,O-CMC (See characterization section).

Characterization

The substitution degree of carboxymethylation (DS of CM) of *N,O*-CMC and HPDMBA-N,O-CMC was estimated by alkalimetry. Samples were dissolved in HCl solution (50 mL, 0.100 mol/L) and titrated with 0.100 mol/L of NaOH standard solution. The alkalimetric curves were obtained by plotting pH *vs* V_{NaOH}.

Weight-average molecular weights (Mw) of each sample was measured by gel permeation chromatograph (GPC) on a Water-208 apparatus with poly(ethyl oxide) as standard. The chromatography conditions were as follows. 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-N,O-CMC and HPDMBA-CTS was determined by potentiometry. Briefly, samples were dissolved in double-distilled water (50 mL) and titrated with 0.100 mol/L of aqueous silver nitrate using silver electrode as working electrode and calomel electrode as reference electrode. The titrating curves were obtained by plotting K *vs* V_{AgNO3}.

Evaluation of Antimicrobial Activity of CTS, N,O-CMC, HPDMBA-CTS, HPDMBA-N,O-CMC and Ofloxacin

The minimum inhibition concentrations (MICs) of CTS, *N,O*-CMC, HPDMBA-CTS, HPDMBA-N,O-CMC and ofloxacin were determined by similar agar plate method reported previously [24], except that the initial concentration of the samples were 500 μg/mL, and 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.

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