Synthesis of *N,O*-(2-Carboxyethyl)-Chitosan and its Properties

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Summary: Chitosan (CTS) was modified with 3-chloropropionic acid as modifying agent in 2-propanol medium under strong basic conditions. Different substitution degrees *N,O*-(2-carboxyethyl)-chitosans (*N,O*-2-CECs) were obtained by varying mass ratio of NaOH and 3-chloropropionic acid versus CTS, respectively. The *N,O*-2-CECs were characterized by FT-IR, ^{1}H-NMR, GPC and potentiometric titration and their isoelectric point were determined by using spectrometry method. The antimicrobial activities of *N,O*-2-CECs were evaluated against a *Gram-negative* bacterium *Escherichia coli* (*E. coli*) and a *Gram-positive* bacterium *Staphylococcus aureus* (*S. aureus*). Experimental results showed the isoelectric point of *N,O*-2-CEC decreased from 7.32 to 6.44 with the increase of substitution degree (DS) of carboxyethylation (CE) from 0.28 to 0.72. The minimum inhibition concentration (MIC) of *N,O*-2-CEC for *S. aureus* and *E. coli* also decreased with the increase of DS of CE. When the DS of CE was same, the antimicrobial activity was increased with the decrease of weight-average molecular weights (Mw) of *N,O*-2-CEC up to some extent. The experimental data also demonstrated that *N,O*-2-CECs had a greater antimicrobial activity against *S. aureus* than *E. coli*.

Introduction

Chitosan (CTS) is a polysaccharide consisting of β-(1-4)-2-amino-2-deoxy-D-glucopyranose (GlcN) repeating unit and a small amount of *N*-acetyl-D-glucosamine (GlcNAc) residues and is derived from fully or partially deacetylated chitin [1, 2]. It has three types of reactive functional groups: an amino group at the C-2 position and primary and secondary hydroxyl groups at the C-3 and C-6 positions, respectively [2-4]. Chitosan is a weak base with pKa value about 6.2-7.0 [4, 5]. At relatively low pH (< 6.5), chitosan is positively charged and tends to be soluble in dilute acidic aqueous solutions. But at neutral and alkaline pH values, it tends to lose its charge and may precipitate from solution due to deprotonation of the amino groups [3-5]. For this reason, much more efforts have been applied to the development of suitable procedures for the preparation of water-soluble derivatives of chitosan [6-8]. Among various methods for the modification of chitosan, carboxyalkylation is one of the most attractive methods because it is a useful technique for modifying the chemical and physical properties of chitosan [9-13].

N,O-(2-carboxyethyl)-chitosan (*N,O*-2-CEC) is one of carboxyalkylation derivative of chitosan. It resulted from the modification of chitosan with 3-halopropionic acid (such as 3-chloropropionic acid, 3-bromopropanoic acid and 3-iodopropionic acid) as modifying agent in the presence of sodium hydroxide or sodium hydrogencarbonate and is soluble under acidic, neutral and basic conditions [11, 14]. There are many excellent properties for *N,O*-2-CEC, such as the antioxidant and antimutagenic activity [11], the ability to control drug release and to chelated metal ion [15, 16], the properties to form hydrogel and to pose membrane, etc [17, 18]. Among all of these properties, the antimicrobial activity may be one of the most important properties of *N,O*-2-CEC [19-21]. The present work described the synthesis of *N,O*-(2-carboxyethyl)-chitosan and its solution properties including isoeletric point and antimicrobial activity. The preparation of *N,O*-2-CEC was presented as Scheme 1.

Results and Discussion

Identification of Resonance in the Spectra

Fig. 1 presented the FT-IR spectra of CTS and *N,O*-2-CECs with different DS of CE. The absorption bands at 1655 cm^{-1}, 1593 cm^{-1}, 1317 cm^{-1}

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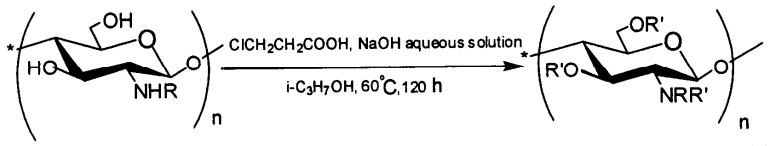

Chitosan *N,O*-(2-carboxyethyl)-chitosan

Scheme-1: Synthesis of *N,O*-(2-carboxyethyl)chitosan, R = H or $COCH_3$, R′ = H or CH_2CH_2COOH.

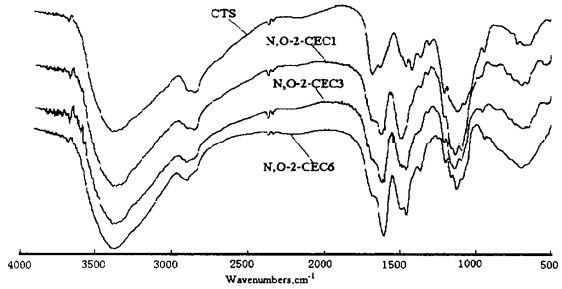

Fig. 1: The FT-IR spectra of CTS and *N,O*-2-CECs.

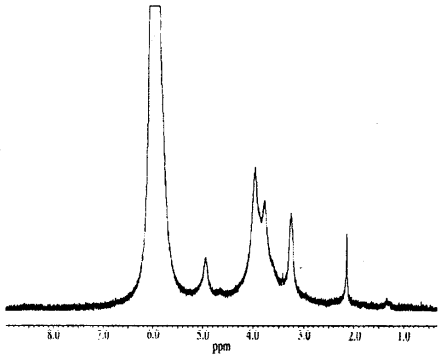

Fig. 2: ^{1}H-NMR spectrum of chitosan.

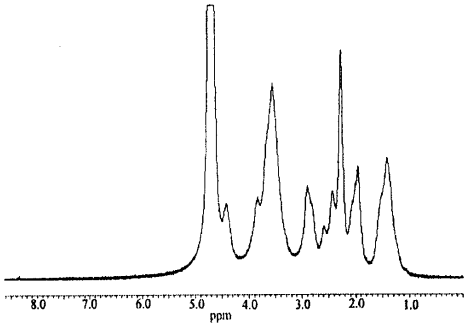

Fig. 3: ^{1}H-NMR spectrum of *N,O*-2-CEC3.

and 1381 cm^{-1} in the spectrum of CTS were assigned to primary, secondary and tertiary amides and $-CH_3$ bend vibration. The characteristic peaks for CTS at 3447 cm^{-1} could be attributed to the N-H, O-H and 1084 cm^{-1} could be attributed to the C-O vibration, respectively. Two strong peaks at 1588 cm^{-1} and 1409 cm^{-1} in *N,O*-2-CEC1 spectrum, 1583 cm^{-1} and 1414 cm^{-1} in *N,O*-2-CEC3 spectrum, 1579 cm^{-1} and 1411 cm^{-1} in *N,O*-2-CEC6 spectrum were ascribed to the asymmetrical and symmetrical stretching of $-COO^-$ group. In the spectrum of *N,O*-2-CECs, the C-O stretching band at *ca* 1030 cm^{-1} corresponding to the primary hydroxyl group of CTS weakened obviously, which confirmed a high carboxyethylation of OH-6.

Fig. 2 and Fig. 3 showed the ^{1}H-NMR spectra of CTS and *N,O*-2-CEC. 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 ($-NCH_2CH_2COOD$) of chitosan occurred in the spectral region of 2.27 ppm and 2.90 ppm.

The Isoelectric Point of N,O-(2-Carboxyethyl)Chitosan

The pHs of isoelectric point (pH_{iep}) of *N,O*-(2-carboxyethyl)-chitosans prepared under different conditions were showed in the Table-1.

It could be seen from Table-1 that the increase of DS of CE from 0.28 to 0.72 could result in the decrease of pH_{iep} of *N,O*-2-CECs from 7.32 to 6.44. When the DS of CE was same (such as *N,O*-2-CEC1 and *N,O*-2-CEC5), the change of M_w had little influence on the pH_{iep}. The DS of CE

Table-1: The pH of isoelectric point of *N,O*-(2-carboxyethyl)-chitosans.

Sample	m_{NaOH} solutions, g	$m_{3\text{-chloropropionic}}$ acids g	DS of CE	Mw($\times10^5$)	pH_{iep}
N,O-2-CEC1	6.0	4.34	0.28	3.10	7.32
N,O-2-CEC2	8.0	4.34	0.39	3.16	7.15
N,O-2-CEC3	10.0	4.34	0.47	3.19	7.01
N,O-2-CEC4	12.0	4.34	0.53	3.22	6.96
N,O-2-CEC5	12.0	2.17	0.28	2.92	7.32
N,O-2-CEC6	12.0	6.51	0.72	3.47	6.44

DS of CE represents the substitution degree of carboxyethylation and Mw is weight-average molecular weights of samples.

increased with the increase of NaOH aqueous solution or 3-chloropropionic acid utilized for the preparation of *N,O*-2-CEC. The reasons that result in these tendencies were as follows. Firstly, the *N,O*-2-CEC had different forms in different pH. When the pH was lower or higher than pH_{iep}, it existed in the form of positive ion or negative ion. When the pH was equal to pH_{iep}, it existed in the form of dipolar ion. Secondly, the increase of DS of CE meant more carboxyethyl group were grafted into chitosan, so it is necessary to form dipolar ion in lower pH for the *N,O*-2-CEC with high DS than that with low DS.

The Antimicrobial Activity of N,O-(2-Carboxy-ethyl)-Chitosan

Table-2 showed the antimicrobial activity of *N,O*-2-CECs with different DS of CE and Mw. The results showed the increase of DS of *N,O*-2-CECs could endow *N,O*-2-CECs with better antimicrobial activity against the tested bacteria under the experimental condition and the decrease of Mw results in the improvement of antimicrobial activities for *N,O*-2-CEC against *S. aureus* and *E. coli* up to some extent. The antibacterial activities of *N,O*-2-CECs against *S. aureus* were more effective than *E. coli*. The reasons resulted in these tendencies could relate with following factors. The increase of DS of CE could improve hydrophilicity of *N,O*-2-CECs, promoted it to adsorb on the cell wall of bacteria and to chelated the trace metal cations, which was necessary for the microorganism's growth, so the movement of microbiological substances would be restrained and the proliferation of the microbial would be inhibited. The decrease of Mw was favorable for *N,O*-2-CECs to penetrate into the cells of microorganisms and prevented the growth of cells by preventing the transformation of DNA into RNA. There is different structure of cell walls between *S. aureus* and *E. coli* [19]. *S. aureus* is a

Taole-2: The MICs value of *N,O*-2-CECs for *S. aureus* and *E. coli*.

Sample	DS of CE	Mw($\times10^5$)	MIC for *S. aureus*, %	MIC for *E. coli*, %
N,O-2-CEC1	0.28	3.10	0.008	0.010
N,O-2-CEC2	0.39	3.16	0.006	0.008
N,O-2-CEC3	0.47	3.19	0.006	0.008
N,O-2-CEC4	0.53	3.22	0.006	0.006
N,O-2-CEC5	0.28	2.92	0.008	0.008
N,O-2-CEC6	0.72	3.47	0.004	0.006

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. On the opposition, the *E. coli* is a typical *gram-negative* bacterium, 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 the cell wall of *E. coli*, there are some difficulties for the foreign molecule to come into its cell.

Experimental

Materials and Methods

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 (Mv) is 3.13×10^5. Sodium hydroxide was purchased from the third factory of chemical reagent in Tianjin with 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 were used without purification as received.

The degree of substitution of carboxy-ethylation (DS of CE) of *N,O*-2-CECs were estimated from potentiometric titration and weight-average molecular weights (Mw) of samples were measured by GPC according to literature [22]. 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. The ^{1}H-NMR spectra of CTS and *N,O*-2-CEC were obtained with Bruker AV-300 spectrometer at 300.13 MHz and 30°C ± 0.5°C and measured in D_2O-TFA solution and D_2O solution, respectively. All the samples were dissolved in a 5 mm diameter tube at a concentration of *ca.* 20 mg/mL.

Preparation of N,O-(2-Carboxyethyl)Chitosan

Chitosan (3.28g), 50.0 mL of 2-propanol and calculated amount of aqueous solution of NaOH (mass concentration, 40 %) were added into a four-neck round bottomed flask. The mixture was heated to 45°C in water bath under continuous stirring and alkalized for 2 h. Then calculated amount of 3-chloropropionic acid was dissolved by 20 mL 2-propanol and added into the bottle dropwise in case the temperature was higher than 60 °C. When the 3-chloropropionic acid was added completely, the temperature was elevated to 60 °C and the reaction was kept at this temperature for five days under continuous stirring. The mixture was filtered and the filter was dissolved in 100 mL distilled water. The pH of the solution was adjusted to *ca* 8.0 with HCl solution (mass concentration, *ca* 10.0 %) and filtered. 160 mL absolute alcohol was added to the filter juice and stirred, then the mixture was separated through centrifugal settling. The resultant dreg was washed by 40.0 mL CH_3OH aqueous solution (mass concentration, *ca* 85.0 %) for three times, then by 30.0 mL absolute ethanol for three times and filtered. The solid was dried by vacuum drying apparatus under 60 °C and 0.1MPa vacuum tightness for 12h and obtained *N,O*-2-CEC. It was put into vacuum drier for use later.

Measurement of Isoelectric Point of Water-Soluble N,O-(2-Carboxyethyl)Chitosan

The pH of isoeletric point of each sample was determined by spectrometric method according to literature [23]. Briefly, series of solution for each sample with different pH from 4.5 to 8.5 were prepared and the increasing step was *ca* 0.2. These solutions were added to a series of test tubes and shaken up, respectively. The light absorbencies of each sample at the wavelengths of 540nm were determined with a spectrophotometer. The pH of the solution with the largest absorbency is just the solution's pH_{iep} value (the pH of isoelectric point).

Evaluation of Antimicrobial Activity Of N,O-(2-Carboxyethyl)-Chitosan

The agar plate method was used to determine the minimum inhibition concentration (MIC) of *N,O*-2-CEC. The samples were prepared at a concentration of 0.2 % (w/v), then they were autoclaved at 121 °C for 30 min. a serial dilutions of each sample were added to nutrient broth (beef extract 5 g, peptone 10 g to 1000 mL distilled water, pH 7.0) for final concentration of 0.02 to 0.002 % decreased in the step of *ca* 0.002 %. 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 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].

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