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Synthesis, characteristic and antibacterial activity of *N*,*N*,*N*-trimethyl chitosan and its carboxymethyl derivatives

Tao Xu[a], Meihua Xin[a], Mingchun Li[a,*], Huili Huang[b], Shengquan Zhou[a]

[a] College of Material Science and Engineering, Huaqiao University, Xiamen 361021, China
[b] College of Chemical Engineering, Huaqiao University, Xiamen 361021, China

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ABSTRACT

O-Methyl free *N*,*N*,*N*-trimethyl chitosan (TMC) was synthesized by treating chitosan with formic acid and formaldehyde firstly, followed by methylation with CH_3I. TMC was further carboxymethylated by monochloroacetic acid to obtain *N*,*N*,*N*-trimethyl-*O*-carboxymethyl chitosan (TMCMC). The products were characterized by FTIR, ^1H NMR, EA and TGA. Their antibacterial activity was investigated against *Staphylococcus aureus* and *Escherichia coli*. The antibacterial activity of TMC decreased as the degree of substitution increased at pH 5.5. But the structure activity relationship was reversed at pH 7.2. TMCMC acted weaker than TMC, and its activity decreased as the degree of carboxymethylation increased. The experimental results showed that the activity of *N*,*N*,*N*-trimethyl amino group was weaker than other non-quaternized amino groups, and carboxymethylation didnot enhance the antibacterial activity directly.

1. Introduction

Mainly obtained from partial deacetylation of the second abundant nature polymer chitin, chitosan, a polysaccharide consisting of β-(1 → 4)-linked 2-amido-2-deoxy-D-glucopyranose and β-(1 → 4)-linked 2-acetamido-2-deoxy-D-glucopyranose, has attracted great attention due to a better understanding of its inherent biological and physicochemical characteristics. Arising from its non-toxicity, biodegradability, biocompatibility, antimicrobial activity, versatile chemical and physical properties, chitosan has been applied in a variety of fields, such as medical applications, biotechnology, textiles, wastewater treatment, cosmetics and agriculture (Kumar, Muzzarelli, Muzzarelli, Sashiwa, & Domb, 2004; Mourya & Inamdar, 2008). However, its poor solubility in aqueous water at pH above 6.5 and most of common-used organic solvents limits its utilizations. As an efficient solution to improve its solubility and antibacterial activity in aqueous water, numerous modifications of chitosan have been reported such as carboxymethylation (Liu, Song, Li, Li, & Yao, 2007), quaternization (Rúnarsson et al., 2007), sugar-modification (Sajomsang, Gonil, & Tantayanon, 2009) and alkylation (Yang, Chou, & Li, 2005). Typically, quaternization is a promising kind of modification due to its multifunction along with favorable solubility at all pH range (Belalia, Grelier, Benaissa, & Coma, 2008; Opanasopit et al., 2009).

N,*N*,*N*-Trimethyl chitosan (TMC), the simplest form of quaternized chitosan, was generally synthesized by chitosan reacting with excess methyliodide in strong alkaline conditions, using *N*-methyl-2-pyrrolidone (NMP) as solvent and sodium iodide as catalyst (Sieval, Thanou, Kotzé, Verhoef, & Brussee, 1998), or synthesized by treating chitosan with appropriate formaldehyde to generate Schiff-base, followed by reaction with a reducing agent and then with methyl halide (Domard, Rinaudo, & Terrassin, 1986; Kim, Choi, Chun, & Choi, 1997). Recently, new methods were employed such as treating chitosan with dimethylsulfate, a less expensive and less poisonous agent (Britto & Assis, 2007). However, almost all the reactions are carried out in strong basic conditions with high temperature, which result in undesirable *O*-methylation. This kind of side reaction is almost uncontrollable and will decrease the solubility of TMC in aqueous medium. Moreover, undesirable *O*-methylation leads to a lot of difficulties when the previous reports were compared. In order to avoid *O*-methylation, an available way is to execute trimethylation with methyl iodide at lower temperature by using DMF/H_2O mixture as solvent instead of NMP and without catalyst (Rúnarsson, Holappa, Jónsdóttir, Steinsson, & Másson, 2008). In this method, the process of methylation was repeated for several times to gain a higher degree of trimethylation (0.88 after four times methylation). Another promising method to obtain *O*-methyl free TMC was performing the reaction by Eschweiler–Clarke reaction firstly, followed by methylation with excessive methyl iodide (Verheul et al., 2008). TMC obtained in this method was not only *O*-methyl free but also without chain scission. So it could be further *O*-modified

* Corresponding author. Tel.: +86 595 2269 0819; fax: +86 595 2269 0917.
E-mail address: mcli@hqu.edu.cn (M. Li).

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conveniently resting on the relative uniform and well characterized structure.

The antibacterial activity of TMC is now attracting great interests. With positive charged *N*-atoms, the antibacterial activity of TMC is superior to chitosan due to permanent quaternary moieties and enhanced solubility (Jia, Shen, & Xu, 2001; Kim et al., 1997; Rúnarsson et al., 2007; Sajomsang, Tantayanon, Tangpasuthadol, & Daly, 2008). Generally, it is believed that chitosan and its derivatives exhibit antibacterial activity due to the formation of complex with cell envelope (Avadi et al., 2004) or interfering gene expression (Liu et al., 2007), and the cationic moiety is always the active site of polycations in both possible mechanisms. Thus with more positive charges, TMC represents stronger antibacterial activity. To date, most of the investigations are based on *O*-methyl TMC. Since alkylation may contribute to the antibacterial activity (Jia et al., 2001), it is possible that final activity is a synergistic effect of quaternization and methylation. By investigating the antibacterial efficiency of *O*-methyl free TMC, researchers figured out that the protonated amino groups contributed to the antibacterial activities rather than trimethylated ones, while *N*-monomethyl amino groups along with *N*,*N*-dimethyl ones functioned the same as a free amino groups (Rúnarsson et al., 2007). Hence, the antibacterial efficiency of trimethylation becomes a controversial issue. Further study on the antibacterial activity of *O*-methyl free TMC is still required.

Carboxyalkylation is another kind of modification of chitosan that could enhance its antibacterial activity and solubility. Though both amino group and hydroxyl group could be carboxyalkylated, here we only focus on *O*-modification. It is reported that the antibacterial efficiency increases in the order of *N*,*O*-carboxymethyl chitosan (*N*,*O*-CMC), chitosan, and *O*-carboxymethyl chitosan (*O*-CMC) (Liu, Guan, Yang, Li, & Yao, 2001). Nevertheless, it is not always the case. While the antibacterial activity of quaternized carboxymethyl chitosan was investigated, there is no clear effect related to the degree of carboxymethylation (Sun, Du, Fan, Chen, & Yang, 2006). In another study, it was deemed that the enhanced antibacterial activity of quaternized *N*,*O*-(2-carboxyethyl) chitosan was a synergetic effect of carboxyalkyl group and quaternary ammonium group (Cai, Song, Yang, Shang, & Yin, 2009). By virtue of discrepancies among different reports, the function of carboxyalkylation on antibacterial activity still requires further investigation.

The purpose of this study was to synthesis *O*-methyl free *N*,*N*,*N*-trimethyl chitosan, and then further carboxymethylate it to obtain *N*,*N*,*N*-trimethyl-*O*-carboxymethyl chitosan (TMCMC) (Fig. 1), and make further efforts to investigate the antibacterial activity of chitosan derivatives against a gram-positive bacterium *Staphylococcus aureus* (*S. aureus*) and a gram-negative bacterium *Escherichia coli* (*E. coli*) at pH 5.5 and pH 7.2 respectively.

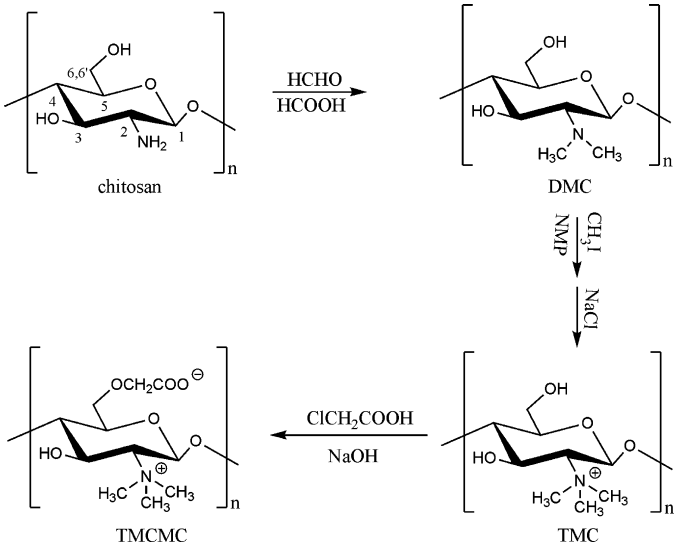

Fig. 1. Synthesis of *N*,*N*,*N*-trimethyl *O*-carboxymethyl chitosan.

2. Materials and methods

2.1. Materials

Chitosan (Mw = 100 kDa, DD = 95.6% according to the manufactory) was purchased from Zhejiang Aoxing Biotechnology Co., Ltd. (China) and refined by dissolving in dilute acetic acid aqueous water and then precipitated by adding NaOH aqueous solution followed by filtration. Methyl iodide (AR) was obtained from Aladdin Reagent Co., Ltd. (China). Other reagents were commercially available and used without further purification. *S. aureus* (ATCC 6538) and *E. coli* (ATCC DH5α), supplied by American Type Culture Collection (ATCC) were used for antibacterial activity test.

2.2. Synthesis of chitosan derivatives

All the following samples, if synthesized at various times, were identified by the reaction time of the last step, in which 'h' indicated hours.

2.2.1. Synthesis of CMC

CMC was synthesized according to previous reports, with some modifications (Muzzarelli, 1988). Briefly, dispersed in 50 mL of 42% (wt%) NaOH solution, 5 g of chitosan was stirred in ice bath for 1 h, followed by adding monochloroacetate dropwise until the final concentration of NaOH was 18%. The reaction lasted for various times at 30 °C. Then the pH was adjusted to 7 with HCl, and 200 mL of 70% ethyl alcohol was added to precipitate the production. The solid was filtered and rinsed with 70–100% ethyl alcohol for three times to dewater and desalt, then vacuum dried at 40 °C for 48 h. Thus we gained Na-form CMC.

In order to obtain H-form CMC, 100 mL of 80% ethyl alcohol aqueous solution was transferred into a beaker, followed by adding 1 g of Na-form CMC and 10 mL of HCl (37%), stirred for 30 min. Then the solid was filtered, rinsed with 70–100% ethyl alcohol to neutral, and vacuum dried (Chen & Park, 2003).

2.2.2. Synthesis of TMC

TMC was synthesized according to former research (Verheul et al., 2008). Briefly, 5 g of chitosan was transferred into a 250 mL round bottom flask prior to adding 15 mL of formic acid, 20 mL of formaldehyde and 90 mL of distilled water, then reacted at 70 °C for 118 h. Subsequently, the solution was evaporated under reduced pressure and 1 mol/L NaOH aqueous solution was used to increase the pH to 12 at which gel formation occurred. This gel was washed with deionized water to remove impurities, then dissolved in dilute HCl aqueous solution (pH 4.0), dialyzed against deionized water for 3 days and lyophilized. Thus *N*,*N*-dimethyl chitosan (DMC) was obtained.

250 mg of DMC was dissolved in 40 mL deionized water prior to adding NaOH to form gel, and then rinsed thoroughly by water and acetone. Afterward DMC was suspended in 50 mL NMP and 2 mL methyl iodide. The dispersion was stirred at 70 °C for the desired time and subsequently dropped in of 1:1 (v/v) ethanol/diethyl ether mixture to precipitate the production. To perform ion exchange, the precipitate was dialyzed against 1% NaCl aqueous solution for 3 days by changing buffer twice a day and deionized water for another 3 days, and then lyophilized to obtain TMC.

2.2.3. Synthesis of TMCMC

1 g of TMC 72 h was added in 20 mL 42% NaOH solution at 30 °C, followed by adding monochloroacetate dropwise until the final concentration of NaOH was 18%. The reaction lasted for various times. Then the pH was adjusted to 7 with HCl. The solution was dialyzed against deionized water for 3 days by changing water once a day, and then lyophilized.

2.3. Characterization

IR spectra were recorded with KBr pellets on a Nicolet NEXUS-470 spectrometer. ^{1}H NMR spectra were measured at 313 K on an INOVA-600 spectrometer, using D_2O as solvent. Elemental analysis was performed with a Vario MICRO elemental analyzer. Thermal-gravimetric analysis was implemented using Universal V2.4F TA thermal analyzer. The analysis was preformed under continuous flow of dry nitrogen gas at a heating rate of 20 °C/min.

The degree of trimethylation for TMC was calculated using the combined integral methods of peak areas in NMR spectra according to the following equation:

$$N,N,N-\text{trimethylation }\% = \frac{[\text{N}(\text{CH}_3)_3]}{[\text{H2, H3, H4, H5, H6, H6}'] \times 6/9} \times 100 \quad (1)$$

where $[\text{N}(\text{CH}_3)_3]$ was the integral of the *N*,*N*,*N*-trimethyl singlet peak (δ = 3.1 ppm) (Rúnarsson et al., 2007). *C*/*N* ratios in EA data were used to evaluate the degree of substitution (DS) of the other chitosan derivatives. The substitution degrees were given in percentage.

2.4. Antibacterial test

Two methods were adopted for investigating the antibacterial activity: determining minimum inhibitory concentration (MIC) in both dilute acidic and weak basic conditions and viable cell account after the treatment with chitosan and its derivatives in acidic conditions.

2.4.1. Determination of MIC

MIC was determined by an agar plate method (Sun et al., 2006) with some modifications. In this method, the samples were prepared at a concentration of 1% (w/v), then autoclaved at 121 °C for 20 min. Duplicate twofold serial dilutions of each sample were added to nutrient broth (pH 5.5 or pH 7.2) for concentration of 0.4%, 0.2%, 0.1%, 0.05%, 0.025%, 0.0125%, 0.00625%, and 0.00313% (w/v). The pH was adjusted by 1% acetic acid or 1% sodium hydroxide. The culture of each bacterium was diluted by sterile distilled water to 10^5–10^6 CFU/mL. A loop of each suspension was inoculated on nutrient medium with sample or control added, and then incubated at 35 °C for 48 h. At last, the colonies were counted to value MIC. The experiments were repeated for three times. MIC was defined as the lowest concentration required inhibiting the growth of bacteria, i.e. the concentration at which no microorganism colony or less than five colonies were visible within 19–38 h (Wang et al., 2009).

2.4.2. Viable cell count at various conditions

Chitosan derivatives were dispersed in 10 mL nutrient broth with the final concentration of 0.1% (wt%) and inoculated with approximately 10^7 CFU/mL of the bacterial strains, and then incubated in ambient air at 35 °C. After 1 h incubation, a 50 μL aliquot or the dilutions were spread on nutrient agar plates, which were incubated at 35 °C for 24 h. Then the numbers of colonies were counted. All the data were the means from at least three parallel experiments with discrepancies among them less than 5%.

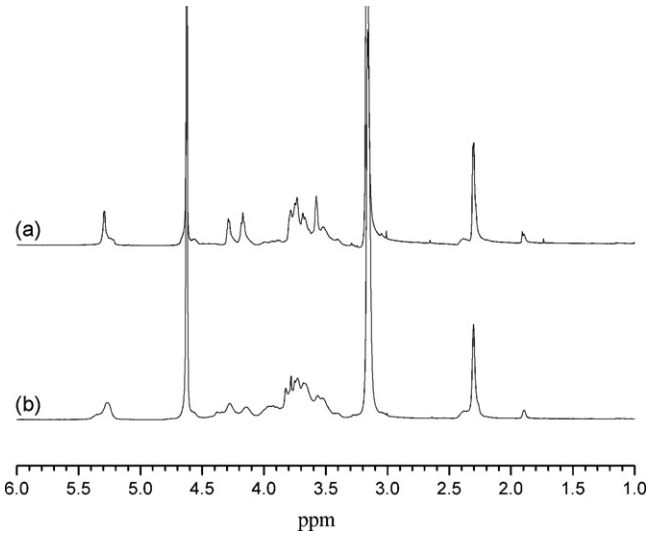

Fig. 2. ^{1}H NMR spectra of (a) TMC 72 h and (b) TMCMC 20 h in D_2O.

3. Result and discussion

3.1. Synthesis and characterization

The ^{1}H NMR (Fig. 2) spectra showed the successful synthesis of *O*-methyl free *N*,*N*,*N*-trimethyl chitosan and *N*,*N*,*N*-trimethyl *O*-carboxymethyl chitosan. In Fig. 2a, the peak at 5.3 ppm was the signal of H1; the peaks from 3.4 ppm to 4.5 ppm were attributed to H2, H6′, H6, H5, H4 and H3 protons of chitosan backbone; the peaks at 3.1 ppm and 2.3 ppm were assigned to hydrogen protons of $-\text{N}(C\text{H}_3)_3$ and $-\text{N}(C\text{H}_3)_2$ respectively; the peak at 1.9 ppm was the signal of $-\text{NCO}C\text{H}_3$ residue. The chemical shifts of both hydrogen in chitosan backbone and hydrogen in *N*-methylation were similar in all the spectra of TMC derivatives. Besides, no peaks from 3.2 ppm to 3.4 ppm (hydrogen protons of C-3 *O*-methylation and C-6 *O*-methylation) were detected, demonstrating the absence of *O*-methylation (Verheul et al., 2008). For TMCMC, the new multi peaks at 3.9 ppm in Fig. 2b was attributed to hydrogen protons of $-\text{OC}H_2\text{COO}^-$, proving carboxymethylation of TMC (Chen & Park, 2003).

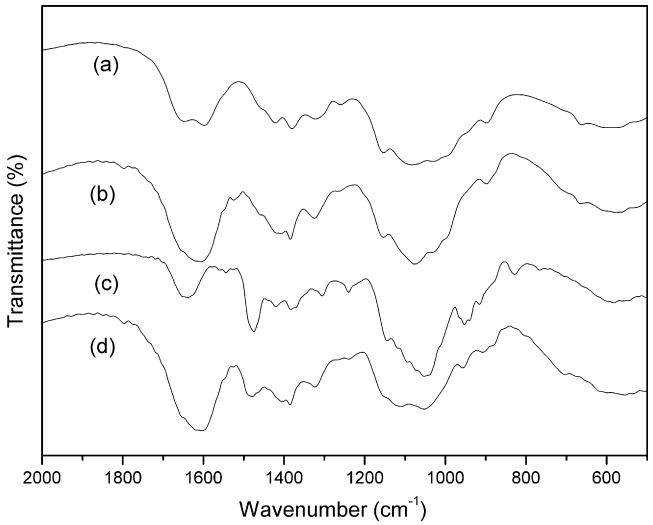

Fig. 3. IR spectra of (a) chitosan, (b) Na-form CMC 20 h, (c) TMC 72 h, and (d) TMCMC 20 h.

Table 1
DS of chitosan derivatives calculated from *C*/*N* ratios in EA data ("—": no substitution).

Chitosan derivatives	DS of *N* (%)	DS of *O* (%)	Structural repeating unit	Calc. (wt%)			Found (wt%)		
				C	N	H	C	N	H
H-from CMC 5 h	—	42.0	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}(C_2H_2O_2)_{0.420}$	44.41	7.47	6.37	34.38	5.79	6.42
H-form CMC 10 h	—	50.7	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}(C_2H_2O_2)_{0.507}$	44.95	7.28	6.35	35.43	5.82	6.36
H-form CMC 20 h	—	58.8	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}(C_2H_2O_2)_{0.588}$	44.26	7.11	6.23	35.68	5.73	6.49
DMC	100.3	—	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}(C_2H_4)_{1.03}$	49.93	7.15	7.77	45.51	6.52	7.67
TMC 72 h	61.2	—	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}(C_2H_4)_{0.344}(C_3H_7)_{0.612}$	51.98	7.04	8.82	41.19	5.58	7.65
TMCMC 5 h	68.9	44.6	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}$ $(C_2H_4)_{0.242}(C_3H_7)_{0.689}(C_2O_2HNa)_{0.446}$	48.68	5.96	7.37	45.84	5.61	7.83
TMCMC 10 h	68.9	52.1	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}$ $(C_2H_4)_{0.242}(C_3H_7)_{0.689}(C_2O_2HNa)_{0.521}$	48.22	5.81	7.22	46.95	5.66	7.71
TMCMC 20 h	68.9	61.5	$(C_6H_{11}O_4N)(C_2H_2O)_{0.044}$ $(C_2H_4)_{0.242}$ $(C_3H_7)_{0.689}$ $(C_2O_2HNa)_{0.615}$	50.54	5.97	7.47	43.23	5.45	7.68

The FTIR spectrum spectra (Fig. 3) also showed the evidence of the reaction together with the results of elemental analysis (Table 1). The FTIR spectrum of chitosan and all its derivatives showed peaks assigned to the saccharide structure at 898 cm^{-1} and 1154 cm^{-1} and a strong amino characteristic peak at around 1614 cm^{-1}. The absorption bands at 1650 cm^{-1} and 1320 cm^{-1} were characteristic peaks of *N*-acetylated chitosan, and attributed to the amide I and III bands respectively. In CMC and TMCMC, the peaks at 1598 cm^{-1} were assigned to carbonyl groups of –COONa owing to *O*-carboxymethylation (Chen & Park, 2003). The new bands at 1490 cm^{-1} in TMC, TMCMC corresponding to an asymmetrical stretching of C–H in the methyl groups, were characteristic of a highly methylated chitosan quaternary salt. It also should be noted that the N–H bending (1614 cm^{-1}) of the primary amine disappeared, which was caused by trimethylation of the primary amine (Rúnarsson et al., 2007).

The degrees of trimethylation for TMCs increased as prolongation of the reaction time, which were 41.2%, 68.9% and 94.7% for 48 h, 72 h and 96 h reaction respectively. These results are in accordance with a previous report (Verheul et al., 2008). It should be noted that DS of dimethylation for DMC was 100.3%, calculated by *C*/*N* ratio in EA data, which meant almost all the free amino groups had been substituted by dimethyl groups. In this way, the degree of trimethylation for TMC 72 h was calculated to be 61.2% by *C*/*N* ratio according to EA data (Table 1). This might be a reasonable method to calculate the degree of trimethylation only if complete substitution of dimethylation was previously certified. Despite this, DS for TMC calculated by the combined integral methods was adopted, for EA was not thought to be as precise as NMR generally. The degree of trimethylation was affected by reaction time, which increased to 94.7%—almost fully trimethylation in consideration of *N*-acetylation—after 96 h reaction. The degree of carboxymethylation was calculated by *C*/*N* ratios on the grounds that the hydrogen atoms of carboxymethyl group were overlapped with hydrogen atoms of chitosan backbone in ^1H NMR spectra. The degree of carboxymethylation was also affected by reaction time. It increased from 42.0% to 58.8% for CMC and from 44.6% to 61.5% for TMCMC when reaction time of carboxymethylation was prolonged from 5 h to 20 h (Table 1).

3.2. Thermal stability

The TGA thermograms of chitosan and its derivatives were shown in Fig. 4. It showed that thermogram of chitosan and its derivatives had two stages of weight loss. The first one started at about 80 °C with 5–12% weight loss due to the loss of adsorbed and bound water. In contrast with chitosan, its derivatives represented more weight loss at this stage because carboxymethylation and quaternization enhanced its hydrophilicity. The second stage registered at nearly 260 °C for chitosan, 220 °C for TMC and CMC, 210 °C for TMCMC, due to the decomposition of chitosan's main chain and cleavage of substituent groups (Spinelli, Laranjeira, & Fávere, 2004). It was quite conspicuous that further modification might lead to lower thermal stability, with the most prominent effect by introducing both carboxymethyl and trimehtylated moieties.

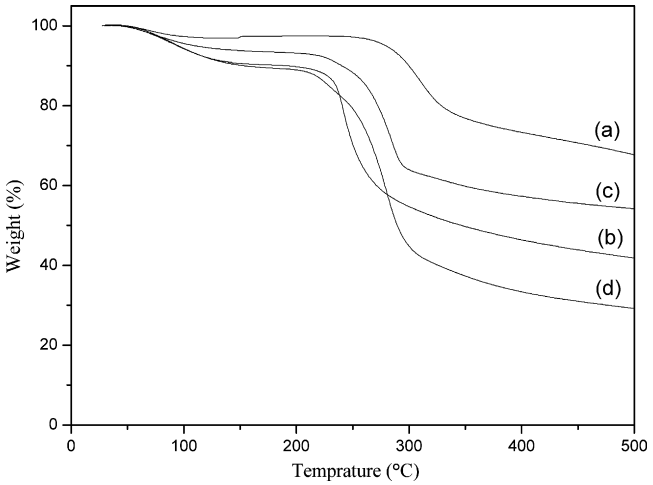

Fig. 4. TGA thermograms of (a) chitosan, (b) H-form CMC 20 h, (c) TMC 72 h, and (d) TMCMC 20 h.

3.3. Antibacterial activity

Table 2 shows MIC of chitsan and its derivatives. Fig. 5 shows the effect of pH on the antibacterial activity of chitosan and TMC 72 h. Fig. 6 shows the antibacterial activity of CMC with various degrees of substitution in both H-form and Na-form. All chitosan and its derivatives here were less active at higher pH value (Table 2 and Fig. 5), which was in good tune with previous report for quaternized chitosan (Avadi et al., 2004; Rúnarsson et al., 2007). Moreover, two species, *E. coli* representing gram-negative bacteria and *S. aureus* standing for gram-positive bacteria were used for antibacterial activity test, since the activities of chitosan itself were diverse (Zheng & Zhu, 2003). Here, all chitosan derivatives showed higher activity against *S. aureus* than *E. coli* (Table 2; Fig. 5 and Fig. 6).

Compared with chitosan, TMC displayed more effective antibacterial activity against both *E. coli* and *S. aureus* (Table 2), which was in concordance to previous report (Jia et al., 2001; Sadeghi

Table 2
Minimum inhibitory concentration of chitosan and its derivatives at pH 5.5 and pH 7.2 against *E. coli* and *S. aureus* ("—": undetectable).

Material	*E. coli*		*S. aureus*	
	pH 5.5	pH 7.2	pH 5.5	pH 7.2
Chitosan	0.1	—	0.05	—
TMC 48 h	0.0125	0.2	0.00625	0.1
TMC 72 h	0.025	0.1	0.0125	0.05
TMC 96 h	0.05	0.05	0.025	0.025
TMCMC 5 h	0.05	0.2	0.025	0.1
TMCMC 10 h	0.1	0.4	0.05	0.2
TMCMC 20 h	0.2	>0.4	0.1	0.4

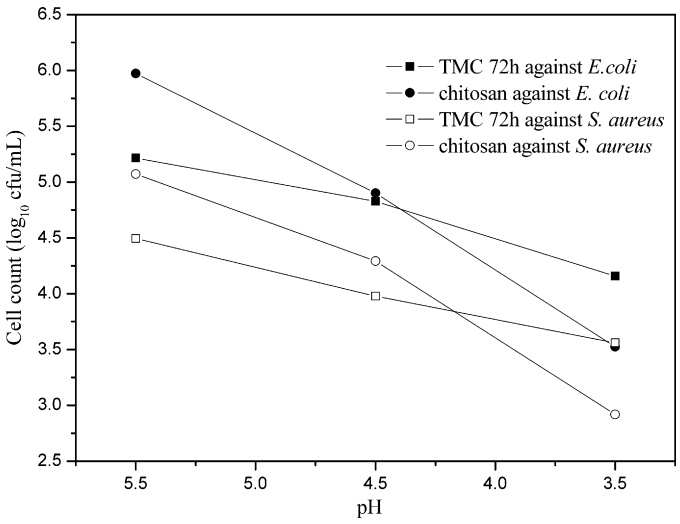

Fig. 5. Cell count treated by TMC 72 h and chitosan against *E. coli* and *S. aureus* after incubation for 1 h.

et al., 2008). Nonetheless, the antibacterial efficiency decreased as the degree of trimethylation increased at pH 5.5, and increased as the degree of trimethylation increased at pH 7.2. These results were in agreement with previous report (Rúnarsson et al., 2007). Rúnarsson and his coworkers believed that the protonated amino groups rather than trimethylated ones contributed to the antibacterial activity, and the *N*-mono- and *N*,*N*-dimethyl amino groups functioned the same as free amino groups. Actually, TMC with degree of trimethylation 94.7% still exhibited strong antibacterial activity at pH 7.2 (Table 2), proving that the trimethylated amino group contributed to the antibacterial activity. While at pH 5.5, the activity decreased as the degree of trimethylation increased, proving that the antibacterial activity of trimethylated amino groups were weaker than other quaternized amino groups. The weaker activity of trimethylated amino groups might be caused by steric hinderance effect of more methyl groups.

The antimicrobial activity of chitosan is strongly affected by pH (Avadi et al., 2004). When we investigated the effect of pH on chitosan and TMC's activity, it showed that both chitosan and TMC's activity increased as the pH value decreased (Fig. 5). TMC was more efficient than chitosan at pH 5.5, however, less efficient than chitosan at pH 3.5. Due to the shielding effect of methyl group, the repulsive force among $-N(CH_3)_3^+$ groups was weaker than that among $-NH_3^+$ groups. Compared with chitosan, the chain of TMC was more flexible and interacted more easily with cell envelope. Thus TMC was more efficient than chitosan at pH 5.5. As a salt of strong acidic and weak basic style, $-N(CH_3)_3^+Cl^-$ group of TMC could ionize as the following equations:

$$-N(CH_3)_3^+Cl^- \rightleftharpoons -N(CH_3)_3^+ + Cl^- \quad (2)$$

$$-N(CH_3)_3^+ + H_2O \rightleftharpoons -N(CH_3)_3OH + H^+ \quad (3)$$

The ionization of chitosan's $-NH_2$ group could be performed as the following equation:

$$-NH_2 + H^+ \rightleftharpoons -NH_3^+ \quad (4)$$

The lower pH could benefit the protonation of $-NH_2$ group, but repress the ionization of $-N(CH_3)_3^+Cl^-$ group. With more $-N(CH_3)_3^+Cl^-$ groups, the chain of TMC was more flexible than that at high pH. Meanwhile, because of the repulsive force among $-N(CH_3)_3^+$ groups and H^+, the chain of TMC curled more heavy than chitosan and its interaction with cell envelope reduced. Moreover, $-N(CH_3)_3^+Cl^-$ group couldnot interact with the negative charged sites on cell envelope. So TMC's antibacterial activity was weaker than chitosan's at pH 3.5.

Different from other quaternized carboxymethyl chitosan (Sun et al., 2006; Cai et al., 2009), TMCMC, with Na-form carboxymethyl group, exhibited weaker antibacterial activity than TMC against both *E. coli* and *S. aureus*. Its antibacterial activity deceased as the degree of carboxymethylation increased (Table 2). These results revealed that Na-form carboxymethyl groups had no antibacterial activity or much weaker than that of amino groups. In order to further comprehend the activity of carboxymethylation, several kinds of *O*-carboxymethyl chitosan were synthesized, either in Na-form or H-form. The antibacterial activity of them was shown in Fig. 6. To Na-form CMC, the tendency of the antibacterial efficiency versus the degree of substitution was similar as to TMCMC, and the activity was weaker than chitosan. These results were in agreement with pervious report (Liu et al., 2001). Different from Na-form TMC, the antibacterial activity of H-form CMC was stronger than chitosan, and increased as the degree of substitution increased. This might be caused by the descent of pH. According to the experimental results, it could be concluded that carboxymethyl groups didnot enhance the antibacterial activity directly.

It seems difficult to precisely compare the current results with other quaternized chitosan derivatives because many factors influencing the testing results are different, such as the original chitosan used (e.g. the source of chitosan, molecular weight and degree of deacetylation), the production synthesized (e.g. degree of substitution of each moiety, substituent site, ion exchange and molecular weight), the assay conditions (e.g. the phase of the strain, culture temperature, pH value and ion strength) and the strain of bacteria used for test. Thus, only the tendency of the activity of chitosan and its derivatives could be compared.

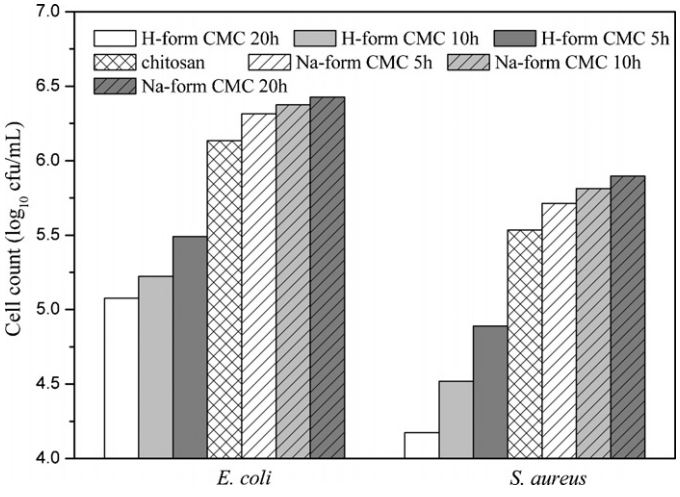

Fig. 6. Cell count treated by CMC with various degrees of substitution and chitosan against *E. coli* and *S. aureus* after incubation for 1 h at pH 5.5.

4. Conclusion

O-Methyl free TMC with various degrees of trimethylation, and TMCMC with various degrees of carboxymethylation were synthesized for different reaction time. The antibacterial activity of TMC and TMCMC coupled with CMC and chitosan were investigated and compared against *E. coli* and *S. aureus* at pH 5.5 and pH 7.2 respectively. TMC exhibited stronger activity than chitosan at both pH 5.5 and pH 7.2. However, TMC's activity decreased as the degree of substitution increased at pH 5.5, while at pH 7.2 the structure activity relationship was reversed. Both chitosan and TMC's antibacterial activity increased as the pH value decreased. However, TMC was more efficient than chitosan at pH 5.5, while at pH 3.5 was less effi-

cient than chitosan. Further carboxymethylation of TMC reduced its activity. The phenomenon was similar to the fact that Na-form carboxymethyl chitosan exhibited deceased activity than chitosan, while H-form carboxymethyl chitosan had an increased activity. Due to lack of direct evidences for antibacterial mechanism, along with the discrepancies of our results with previous reports, further investigation of quaternized and carboxymethyl chitosan are still required.

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