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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.

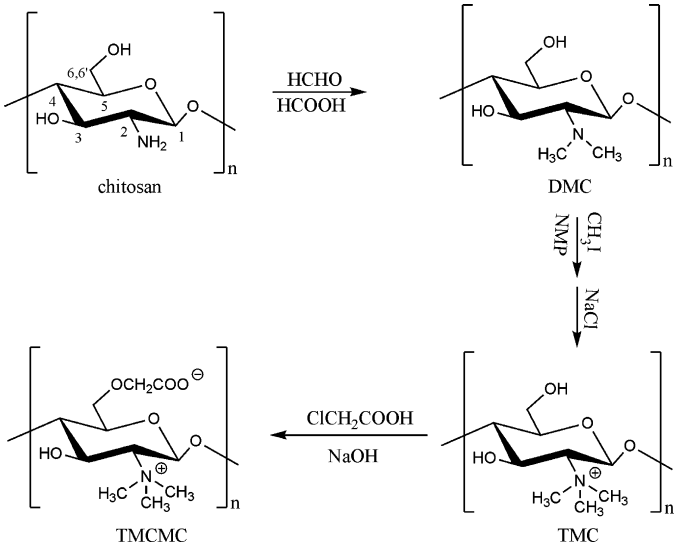

Fig. 1. Synthesis of *N*,*N*,*N*-trimethyl *O*-carboxymethyl chitosan.

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.