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Extent of shielding by counterions determines the bactericidal activity of *N*,*N*,*N*-trimethyl chitosan salts

Heveline D.M. Follmann[a,d], Alessandro F. Martins[b,d], Thatyane M. Nobre[a], Joana D. Bresolin[c], Thelma S.P. Cellet[d], Patrícia Valderrama[e], Daniel S. Correa[c], Edvani C. Muniz[d,f], Osvaldo N. Oliveira Jr.[a,*]

[a] São Carlos Institute of Physics, University of São Paulo (USP), PO Box 369, CEP 13566-590 São Carlos, São Paulo, Brazil
[b] Technological University of Paraná (UTFPR), CEP 86812-460 Apucarana, Paraná, Brazil
[c] Embrapa Instrumentation, CEP 13560-970 São Carlos, São Paulo, Brazil
[d] Polymers and Composite Materials Group (GMPC), Department of Chemistry, State University of Maringá (UEM), Av. Colombo 5790, CEP 87020-900 Maringá, Paraná, Brazil
[e] Technological Federal University of Paraná (UTFPR), Via Rosalina Maria dos Santos, CEP 87301-899 Campo Mourão, Paraná, Brazil
[f] The Graduate Program in Biotechnology Applied to Agriculture, Paranaense University (UNIPAR), CEP 87502-210 Umuarama, Paraná, Brazil

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ABSTRACT

In this study, we show that the bactericidal activity of quaternized chitosans (TMCs) with sulfate, acetate, and halide counterions against *Escherichia coli (E. coli)* and *Staphylococcus aureus (S. aureus)* correlates with the "availability" of N-quaternized groups [$-^{+}N(CH_3)_3$] in the TMCs backbones. *N*,*N*,*N*-trimethyl chitosan sulfate (TMCS) and *N*,*N*,*N*-trimethyl chitosan acetate (TMCAc) displayed the highest activities, probably due to their delocalized π system. Among TMCs with halide counterions, activity was higher for *N*,*N*,*N*-trimethyl chitosan chloride (TMCCl), whereas *N*,*N*,*N*-trimethyl chitosan iodide (TMCI) and *N*,*N*,*N*-trimethyl chitosan bromide (TMCBr) exhibited lower, similar values to each other. This is consistent with the shielding of $-^{+}N(CH_3)_3$ groups inferred from chemical shifts for halide counterions in ^{1}HNMR spectra. We also demonstrate that TMCs with distinct bactericidal activities can be classified according to their vibrational spectra using principal component analysis. Taken together, these physicochemical characterization approaches represent a predictive tool for the bactericidal activity of chitosan derivatives.

1. Introduction

The well-established bactericidal activity of chitosans and their derivatives is ascribed to their being positively charged (Kim, Choi, Chun, & Choi, 1997; Kong, Chen, Xing, & Park, 2010; Martins et al., 2014; Martins et al., 2015b; Tsai & Su, 1999). The positive charge in these cationic polyelectrolytes interact strongly with the negative charges on the surface of bacteria membranes (de Britto, Forato, & Assis, 2008; Muñoz-Bonilla & Fernández-García, 2012). Some of the chitosan derivatives are superior to parent chitosan in terms of bactericidal activity (de Britto, Celi Goy, Campana Filho, & Assis, 2011; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003; Xu, Xin, Li, Huang, & Zhou, 2010), in addition to displaying higher solubility in water under neutral and alkaline conditions as is the case of *N*,*N*,*N*-trimethyl chitosans (TMCs) (de Britto, Frederico, & Garrido Assis, 2011; Mansur, Mansur, Curti, & De Almeida, 2012; Martins et al., 2013a; Martins et al., 2014). Demonstrations of TMCs bactericidal activity are abundant, as they have been proven to be active against *Staphylococcus aureus* (Rúnarsson et al., 2010; Sajomsang, Ruktanonchai, Gonil, & Warin, 2010), *Escherichia coli* (Jia, shen, & Xu, 2001; Martins et al., 2015; Rúnarsson et al., 2010; Sajomsang et al., 2010), *Enterococcus facialis* (Rúnarsson et al., 2010), *Pseudomonasaeruginosa* (Rúnarsson et al., 2010), and *Listeriainnocua* (Belalia, Grelier, Benaissa, & Coma, 2008).

The precise molecular mechanism behind the bactericidal and antifungal activity of chitosans and derivatives is difficult to identify because such activity depends on many parameters. For instance, molecular weight (Hernández-Lauzardo et al., 2008; Sajomsang et al., 2010; Tikhonov et al., 2006), solubility in water (Je & Kim, 2006; Qin, Li, Xiao, Liu, Zhu, & Du, 2006; Sajomsang, Gonil, & Tantayanon, 2009; Xie, Xu, Wang, & Liu, 2002), positive charge density (Kong et al., 2010; Xu et al., 2010), degree of deacetylation,

* Corresponding author.
E-mail address: chu@ifsc.usp.br (O.N. Oliveira Jr.).

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and quaternization (DQ) (Follmann et al., 2012; Mellegård, Strand, Christensen, Granum, & Hardy, 2011; Sajomsang et al., 2010), pH (Follmann et al., 2012; Kong et al., 2010), type of derivatizing group (Guo et al., 2007; Sajomsang et al., 2010), position of the charged group in the polymer backbone (Holappa et al., 2006; Mohamed, Sabaa, El-Ghandour, Abdel-Aziz, & Abdel-Gawad, 2013; Rúnarsson et al., 2007; Sajomsang et al., 2010), and structure of the cationic group (Kim et al., 1997; Kong et al., 2010; Rúnarsson et al., 2010; Tan, Ma, Lin, Liu, & Tang, 2013), have all been shown to affect the bactericidal activity. This means that probing only the ionic electrostatic interactions may not be sufficient to understand bactericidal activity.

In this study, we devised experiments in which we tried to isolate most of the parameters relevant for bactericidal activity in order to test the hypothesis according to which the essential feature for the activity is the "availability" of charged groups in chitosan derivatives. Accordingly, we synthesized a series of TMCs where the counterions were varied, while all the other experimental conditions for the assays against *S. aureus* and *E. coli* were kept constant. The rationale is that the "availability" of $-^{+}N(CH_3)_3$ groups will differ in the TMCs with distinct counterions, and the differences can be captured by using standard physicochemical characterization techniques.

2. Materials and methods

2.1. Materials

Chitosan (CAS 9012-76-4) with degree of deacetylation of 85% and average molecular weight $\bar{M}_w = 8.7 \times 10^4$ g mol^{-1} was purchased from Golden-Shell Biochemical (China). Methyl iodide (CAS 74-88-4), N-methyl-2-pyrrolidone (NMP, CAS 872-50-4), formaldehyde stabilized with methanol (CAS 0050-00-0), formic acid (CAS 77-92-9), and cellulose membranes (molecular weight of cut off: 12 kDa), used for dialysis, were purchased from Sigma-Aldrich (USA). Other reagents, such as sodium hydroxide, sodium bromide, sodium chloride, sodium acetate, sodium sulfate, hydrochloric acid, ethanol, and diethyl ether were all analytical grade. The reagents were used as received without further purification.

2.2. Synthesis and characterization of TMC salts

2.2.1. Dimethylated chitosan (DMC) synthesis

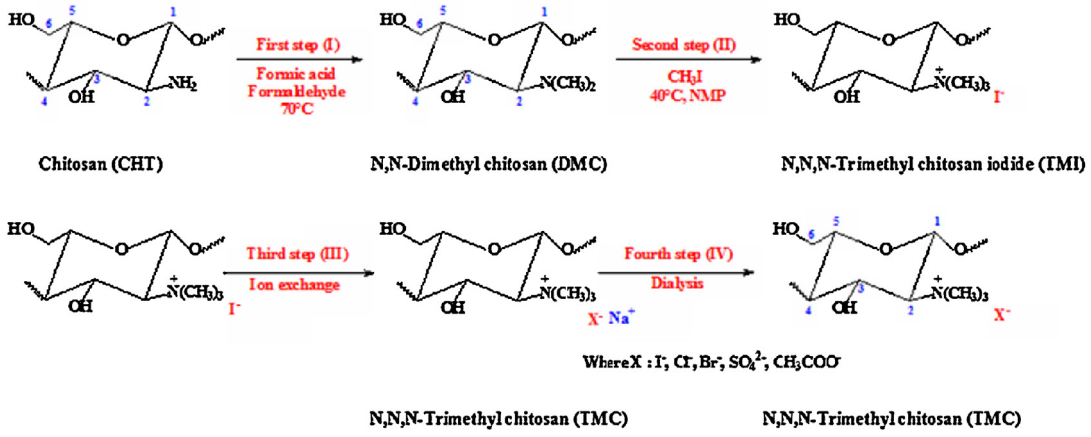
Two-step reaction pathway to synthesize TMC avoiding O-methylation is depicted in Scheme 1, and was based on the methodology by Verheul et al. (Verheul et al., 2008) and Martins et al. (Martins et al., 2013). We chose this method, among others proposed in the literature, e.g., Benediktsdóttir et al. (Benediktsdóttir et al., 2011), because it yields homogeneous TMC free of methylation, and is simple, with only two reaction steps. A mixture consisting of chitosan (10 g), formic acid (30 ml), formaldehyde (40 ml), and distilled water (180 ml) was kept in a one-neck round flask (500 ml capacity) under reflux at 70 °C and stirring for 120 h. A yellow viscous solution was obtained and made to precipitate in a NaOH solution (1.0 mol L^{-1}). The resulting gel was washed with deionized water to remove impurities and then dissolved in diluted HCl aqueous solution (pH ≈ 4.0). This solution was dialyzed against deionized water for 3 days and DMC was frozen and lyophilized.

2.2.2. Trimethylated chitosan (TMC) synthesis from DMC

TMC was synthesized by dissolving DMC (3.0 g) into distilled water (200 ml). The initial pH of solution (≈4.0) was adjusted to 11.0 by adding enough NaOH aqueous solution (1.0 mol L^{-1}). This step was performed to ensure deprotonation of the tertiary amino groups of DMC. The gel formed was washed with distilled water followed by acetone, three times, and then added to a one-neck round flask (500 ml capacity) with NMP (300 ml) and methyl iodide (9 ml). The dispersion was kept under magnetic stirring for 67 h at 40 °C. The product, *N*,*N*,*N*-trimethyl chitosan iodide (TMCI) was precipitated into 600 ml of ethanol/diethyl ether (1/1) solution. The precipitate was recovered by centrifugation and washed with diethyl ether. After drying, an amount of TMCI was dissolved in sodium chloride solution (100 ml) at 10% (w/v) to promote ion exchange (4 h, under stirring), precipitated and recovered by centrifugation to obtain *N*,*N*,*N*-trimethyl chitosan chloride (TMCCl). This procedure was performed three times. TMC bromide, TMC sulfate, and TMC acetate were produced through analogous procedures but using aqueous solutions of NaBr, Na_2SO_4, and NaAc, respectively, instead of NaCl. Each TMC salt was dialyzed against deionized water (for 7 days), frozen, and lyophilized. The TMCs were labeled as: *N*,*N*,*N*-trimethyl chitosan iodide (TMCI); *N*,*N*,*N*-trimethyl chitosan bromide (TMCBr); *N*,*N*,*N*-trimethyl chitosan chloride (TMCCl); *N*,*N*,*N*-trimethyl chitosan acetate (TMCAc); and *N*,*N*,*N*-trimethyl chitosan sulfate (TMCS).

2.2.3. Characterization by hydrogen nuclear magnetic resonance (^{1}H NMR)

TMCs and the parent chitosan were characterized by ^{1}H NMR, whose spectra are shown in Fig. S1 in the supporting information. These spectra were obtained with a Varian, Mercury Plus 300



Scheme 1. Synthetic route of DMC and TMC free of O-methyl groups.