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Enhancing antibacterial activity of chitosan surface by heterogeneous quaternization

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ABSTRACT

This research aimed to increase the antibacterial activity of the chitosan surface by introducing quaternary ammonium groups via a heterogeneous two-step process: reductive alkylation using a series of different aldehydes followed by methylation with methyl iodide. ATR-FTIR and XPS analyses, together with water contact angle and zeta potential measurements, confirmed the success of the surface quaternization. The antibacterial activity of the surface-quaternized chitosan film against *Staphylococcus aureus* and *Escherichia coli*, as model Gram-positive and Gram-negative bacteria, respectively, were superior to that of the virgin chitosan film. The apparent damaged bacterial morphology upon contact with the surface-quaternized chitosan film was verified by SEM. Thus, the introduction of additional positive charges to the chitosan surface via the versatile and yet simple process of heterogeneous quaternization can significantly improve the antibacterial activity of the chitosan surface, especially in a neutral environment.

1. Introduction

Chitosan has progressively attracted attention due to its multiple bioactivities, such as antimicrobial (Lim & Hudson, 2004; Ong, Wu, Moochhala, Tan, & Lu, 2008) and antitumor (Gorbach et al., 1994). The antibacterial activity, in particular, has been followed with great interest. Chitosan inhibits the growth of a fairly diverse range of bacteria (Choi et al., 2001; Fujimoto, Tsuchiya, Terao, Nakamura, & Yamamoto, 2006) and thus offer great benefit to a wide variety of applications, ranging from medical (Alves & Mano, 2008) to agriculture (Campaniello, Bevilacqua, Sinigaglia, & Corbo, 2008) The exact mechanism of the antimicrobial action of chitosan is still ambiguous, although six main mechanisms, none of which are mutually exclusive, have been proposed (Raafat, von Bargen, Haas, & Sahl, 2008; Rabea, Badawy, Stevens, Smagghe, & Steurbaut, 2003) as follows: (1) Interactions between the positively charged moieties on the chitosan molecules and those negatively charged ones on the microbial cell outer membranes leads to changes in the cell membrane structure and permeability inducing the leakage of proteinaceous and other intracellular constituents and so challenging the biochemical and physiological competency of the bacteria leading to loss of replicative ability and eventual death. (2) Chitosan acts as a chelating agent that selectively binds trace metals and subsequently inhibits the production of toxins and microbial growth. (3) Chitosan activates several defense processes in the host tissue, acts as a water binding agent and inhibits various enzymes. (4) Low molecular weight chitosan penetrates the cytosol of the microorganisms and, through the binding of chitosan with DNA, results in the interference with the synthesis of mRNA and proteins. (5) Chitosan on the surface of the cell can form an impermeable polymeric layer which alters the cell permeability and prevents nutrients from entering the cell. (6) Finally, since chitosan can adsorb the electronegative substances in the cell and flocculate them, it disturbs the physiological activities of the microorganism leading to their death.

Nonetheless, chitosan, shows its antibacterial activity only in acidic medium, which is ascribed to the poor solubility of chitosan above its pK_a (pH 6.5). For this reason, a number of chitosan derivatives have been developed not only to expand the use of chitosan into a broader pH range and so media but also to improve the bactericidal actions of chitosan. Amongst all the derivatives that exhibit superior antibacterial activity to native

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chitosan, quaternary ammonium-containing ones have gained the most attention. They are typically synthesized either by direct quaternization of the amino groups of chitosan using alkyl halides under alkaline conditions (Domard, Rinaudo, & Terrassin, 1986; Polnok, Borchard, Verhoef, Sarisuta, & Junginger, 2004), by reductive *N*-alkylation reaction of chitosan with aldehydes via Schiff's base intermediates followed by quaternization with methyl iodide (Muzzarelli & Tanfani, 1985; Sajomsang, Gonil, & Saesoo, 2009) or by reductive *N*-alkylation reaction of chitosan with quaternary ammonium-type aldehydes (Suzuki, Oda, Shinobu, Saimoto, & Shigemasa, 2000). Reaction of the amino groups of chitosan with glycidyltrimethylammonium chloride (GTMAC) has been introduced as an alternative for *N*-selective reaction under acidic and neutral conditions (Seong, Whang, & Ko, 2000; Sun, Du, Fan, Chen, & Yang, 2006). A recent review on the synthesis and applications of quaternized derivatives of chitosan have been available in published literatures (Mourya & Inamdar, 2009; Sajomsang, 2010).

Chitosan in its solid form (film, fiber or particles) holds promising values in many applications for which interfacial contact inhibition is sufficient, although its bactericidal action is only favorable in acidic media (pH < 6.5) when most of its amino groups hold their cationic character. As a means to permanently introduce a positive charge to solid chitosan, without having to incorporate additional cationic species or altering the processability of chitosan, heterogeneous quaternization seems to be an attractive approach. The reactions can be accomplished in the absence of tedious purification process that are certainly required if the quaternization is done homogeneously in solution. Chitosan can be fabricated into the desired solid form (film, fiber or particle) prior to the surface modification. Previously, we have demonstrated that it is conceivable to tune the surface properties of chitosan, namely hydrophilicity/hydrophobicity, and protein adsorption, by chemical modification of the chitosan surface by choosing the suitable reagents under a heterogeneous condition (Amornchai, Hoven, & Tangpasuthadol, 2004; Hoven, Tangpasuthadol, Angkitpaiboon, Vallapa, & Kiatkamjornwong, 2007; Tangpasuthadol, Pongchaisirikul, & Hoven, 2003).

This research aimed to conduct the quaternization of chitosan by a well-developed chemistry based on a two-step approach using firstly a reductive *N*-alkylation reaction of chitosan with aldehydes via the formation of Schiff's base intermediates, followed by quaternization with methyl iodide. Besides the positive charge of quaternary ammonium groups, it is envisaged that the hydrophobicity introduced from the hydrocarbon chains of the different aldehydes should help elevate the antibacterial activities, as has been formerly described by others (Badawy, 2010; Kim & Choi, 2002; Ye et al., 2007).

2. Materials and methods

2.1. Materials

Chitosan flakes (DAC of 92%, M_V = 550,000 Da) were purchased from Seafresh Chitosan (Lab) Co., Ltd. (Thailand). Chitosan films and particles were prepared according to the published procedure by Hoven et al. (2007) and Qi, Xu, Jiang, Hu, and Zou (2004), respectively. Methanol, as commercial grade, was distilled over 4A molecular sieves prior to use. Methyl iodide (CH_3I), acetaldehyde, glutaraldehyde, sodium borohydride ($NaBH_4$), sodium iodide (NaI) and were all purchased from Fluka (Switzerland), and used as received. Benzaldehyde and butyraldehyde were purchased from Merck (Germany) and Sigma Chemical Co. (USA), respectively, and used as received. *Staphylococcus aureus* (*S. aureus*) and *Escherichia coli* (*E. coli*) were purchased from the National Center for Genetic Engineering and Biotechnology (Thailand). Trypicase soy agar (TSA) and Trypicase soy broth (TSB) were purchased from PPL System Co., Ltd. (Thailand). Phosphate buffer saline (PBS) was supplied by Aldrich (USA). Ultrapure distilled water was obtained after purification using a Millipore Milli-Q system (USA).

2.2. Preparation of N-alkyl chitosan films or particles

An anhydrous methanol solution of each selected aldehyde (10 mL) at the desired concentration (0.4–1 M) was added into a flask containing chitosan films (2 cm × 2 cm) or particles (0.03 g). After stirring for a given time at ambient temperature (~28–30 °C), $NaBH_4$ (0.3 g, 0.8 mol) was added into the reaction mixture and the solution was stirred for 24 h. The films were removed from the solution, rinsed thoroughly with methanol, and dried *in vacuo*. In the case of particles, they were isolated by centrifugation at 6000 rpm. The supernatant was discarded and the particles were re-suspended in and centrifugally washed with methanol three times prior to being dried *in vacuo*.

2.3. Preparation of quaternized N-alkyl chitosan films or particles

An anhydrous methanol solution of NaI (0.2 M) was added via syringe into a flask containing *N*-alkyl chitosan films (2 cm × 2 cm) or particles (0.03 g) and NaOH (0.13 g, 0.3 mol). The total volume of the reaction mixture was 10 mL and the concentration of CH_3I was varied within a range of 0.4–2.4 M. The reaction mixture was stirred at 50 °C for the indicated time and then the films were removed from the solution, rinsed thoroughly with methanol, and dried *in vacuo*. In the case of particles, they were isolated by centrifugation, washed with methanol and dried *in vacuo* as detailed above.

2.4. Characterization of surface-quaternized chitosan films/particles

A contact angle goniometer, model 100-00, equipped with a Gilmont syringe and a 24-gauge flat-tipped needle (Ramé-Hart, Inc., USA), was used for the determination of water contact angles. The reported angle expressed as the mean ±1 standard deviation is the average of five measurements on different areas of each sample. All attenuated total reflectance-Fourier transform infrared (ATR-FTIR) spectra were collected at a resolution of 4 cm^{-1} and for 128 scans using a Nicolet Magna 750 FT-IR spectrometer (USA) equipped with a liquid-nitrogen-cooled mercury-cadmium-telluride (MCT) detector using a variable angle reflection accessory (Seagull™, Harrick Scientific, USA) with a hemispherical Ge IRE. X-ray photoelectron spectroscopy (XPS) analysis was performed using a VG ESCALAB 220i-XL instrument (UK) equipped with a monochromatic Al Kα (1486.7 eV photons) and an unmonochromated Mg Kα X-ray source (1253.6 eV photons). The zeta potential of chitosan particles was determined using a Zetasizer Nano-ZS (Malvern Instruments, UK) at 25 °C using a scattering angle of 173°. All data are displayed as the mean ± 1 standard deviation and are derived from at least three independent experiments.

2.5. Evaluation of antibacterial activity

All glasswares used for the tests were sterilized in an autoclave at 121 °C for 15 min prior to use. The quaternized *N*-alkyl chitosan films or particles were sterilized by exposing to UV radiation for 30 min prior to the tests. The quaternized *N*-alkyl chitosan films (1 cm × 1 cm) were placed one per well of a 24-well plate containing 2 mL TSB. Then 12 μL of bacterial suspension in distilled water (OD_{600} = 0.5) was pipetted into each well and the plate incubated in a shaking incubator (Model G-25, New Brunswick Scientific Co., Inc., USA) at 37 °C, 110 rpm, for 24 h. The bacterial suspension (100 μL) was then transferred from each well into a well of a 96-well plate