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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

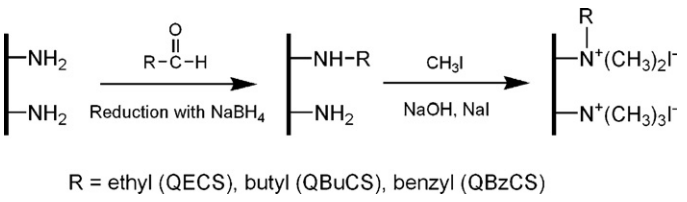
An anhydrous methanol solution of NaI (0.2 M) was added via syringe into a flask containing *N*-alkyl chitosan films (2 cm × 2 cm) or particles (0.03 g) and NaOH (0.13 g, 0.3 mol). The total volume of the reaction mixture was 10 mL and the concentration of CH_3I was varied within a range of 0.4–2.4 M. 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



Scheme 1. Surface quaternization of chitosan.

to determine the OD_{600} by UV–vis spectroscopy (Multi-Detection Microplate Reader Model All, Bio-Tek™ Instruments Inc., USA). Another 100 μL of each of the bacterial suspensions was diluted 10^{10} times and 100 μL of this diluted bacterial suspension was then spread onto TSA. After incubating at 37 °C for 24 h, the number of colonies, and thus replication competent bacteria were then counted as a measure of assumed viability. The results, after correction for the dilution factor, were expressed as the mean number of colony forming units per volume (CFU/mL). All tests of antibacterial activity were performed in triplicate per sample and upon at least three independent samples. The antibacterial ratio was calculated using the following relationship:

$$\text{antibacterial ratio (\%)} = \frac{A - B}{A} \times 100 \quad (1)$$

where A is a number of original viable bacterial cell after incubation in media alone (control) and B is a number of viable bacterial cell after incubation with the designated chitosan film.

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) version 17.0 software. Statistical comparisons were made by One-Way Analysis of Variance (ANOVA) with the Least Square Difference (LSD) tests post hoc evaluations of differences between groups. The threshold level for accepting statistical significance was set at $p < 0.05$.

2.6. Determination of bacterial morphology

Bacterial morphology on the quaternized *N*-alkyl chitosan films was examined under a scanning electron microscope (SEM, JEOL Model JSM-5800L, Japan). After incubation with the bacterial suspension ($OD_{600} = 0.5$) for 24 h, the quaternized *N*-alkyl chitosan films were removed from the bacterial suspension by sterile forceps and immersed in 3% (w/v) glutaraldehyde solution at 4 °C in order to fix the adherent bacteria on the films. After 24 h, the glutaraldehyde solution was removed and the films were washed with PBS, followed by a stepwise dehydration with 30%, 50%, 70%, 90% and 100% (v/v) ethanol in water for 10 min each. The films were then dried and sputter-coated with a thin film of gold before being characterized by SEM.

3. Results and discussion

3.1. Surface quaternization

The methods used for modifying the chitosan surface are outlined in Scheme 1. The reaction introduces quaternary ammonium groups via a heterogeneous two-step process, the reductive *N*-alkylation using selected aldehydes followed by methylation with methyl iodide to form quaternized *N*-alkyl chitosan surface. Contact angle analysis was used to monitor the extent of both reductive *N*-alkylation and quaternization as a function of reaction time and reagent concentrations. As shown in Fig. S1 (Supplementary data), the reaction with butyraldehyde and benzaldehyde, but not acetaldehyde, intrinsically introduced additional hydrophobicity to the chitosan films. Water contact angle of the virgin chitosan film is 79.8 ± 3.1°. The trend was reversed, however, in the case

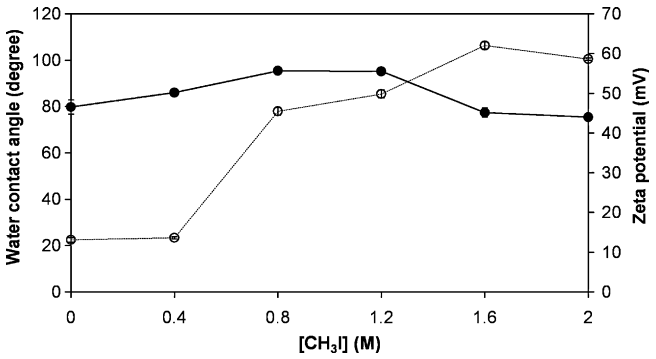


Fig. 1. Water contact angle (●) and zeta potential (○) of the quaternized *N*-benzyl chitosan film/particle prepared by methylation of the *N*-benzyl chitosan film/particle using varied [CH_3I].

of acetaldehyde. Upon using the optimized condition that yield the maximum extent of surface reductive *N*-alkylation (summarized in Table S1, Supplementary data), the water contact angle of *N*-ethyl chitosan film, *N*-butyl chitosan film, and *N*-benzyl chitosan film was 82.1 ± 0.7°, 93.8 ± 0.8°, and 93.0 ± 2.3°, respectively. A series of quaternized *N*-alkyl chitosan films were subsequently obtained by methylation of these three different *N*-alkyl chitosan films. According to Fig. S2 shown in the Supplementary data, the surface of all *N*-alkyl chitosan films essentially became more hydrophilic after quaternization. Upon using a reaction time of 12 h, the optimized methyl iodide [CH_3I] for the step of methylation of all *N*-alkyl chitosan substrates together with their lowest water contact angles obtained are displayed in Table S2 (Supplementary data).

In order to verify that the reductive *N*-alkylation followed by methylation can increase the magnitude of positive charges on the chitosan surface, a series of surface-quaternized chitosan particles prepared by conditions equivalent to those used for the films were subjected to zeta potential measurements. The results, also summarized in Table S2, reveal that, as anticipated, all surface-quaternized chitosan particles possessed higher zeta potential values than the virgin unmodified chitosan particles (zeta potential = +13.11 ± 0.6 mV). The highest zeta potential (+58.60 ± 0.3 mV) was achieved in the case of the quaternized *N*-benzyl chitosan (QBzCS) particles implying that its charge magnitude can be broadly tailored. To test this hypothesis, a series of QBzCS films/particles were prepared from *N*-benzyl chitosan films/particles using varied concentrations of CH_3I (Fig. 1), where the degree of quaternization, expressed in terms of zeta potential value, clearly increased as a function of the CH_3I concentration used. The water contact angle, on the other hand, was initially raised as the CH_3I concentration was increased up to 0.8–1.2 M, reaching a maximal value of ~95°, but the surface then became more hydrophilic as the CH_3I concentration was increased further to 1.6–2.0 M. This trend can be explained as a result of the balance between two competing effects. The methyl groups introduced from CH_3I during quaternization enhance the hydrophobicity of the *N*-benzyl chitosan film. However, the higher level of methylation leads to the greater magnitude of surface positive charge density due to the formation of quaternary ammonium groups. The surface of the modified chitosan film thus became more hydrophilic at high levels of quaternization. The lowest water contact angle obtained in the series was ~75°.

ATR-FTIR spectroscopy was also used to confirm the success of quaternization of *N*-alkyl chitosan (Fig. 2). Absorption peaks at 1650 cm^{-1} and 1590 cm^{-1} were assigned to the C=O stretching (Amide I) and N–H bending (Amide II) of the glucosamine unit, respectively. After the reaction, the peak intensity of the N–H bending of chitosan at 1590 cm^{-1} correspondingly decreased, whereas the intensity of C–H deformation peaks at 1470 cm^{-1} increased, indicating that the substitution of the alkyl groups occurred at the

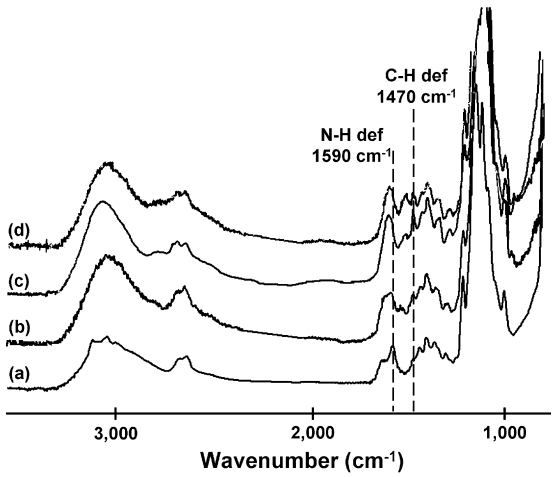


Fig. 2. ATR-FTIR spectra of the (a) chitosan, (b) QECS, (c) QBuCS, and (d) QBzCS films.

amino group of the chitosan film. The results from the ATR-FTIR analysis apparently indicated that the reaction should proceed to a depth of at least 1–2 μm (estimated depth of ATR-FTIR sensitivity).

XPS analysis further supports the success of surface quaternization on QBuCS and QBzCS films (Fig. 3), which were selected as representative samples from the series to be characterized by XPS. Besides C, O, and N, the basic elements already present in the chitosan film, there were no additional elements introduced to the chitosan surface after reductive *N*-alkylation and methylation. The only way to detect the quaternary ammonium groups after surface modification is by considering the N 1s spectrum of the surface-quaternized chitosan film in comparison with the virgin chitosan film. As seen from Fig. 3(a), the N 1s peak of the virgin chitosan film can be fitted with one peak at 400 eV. The N 1s signals of both QBuCS and QBzCS films (Fig. 3(b) and (c)), on the other hand, can be split into two peaks. One appears at the same binding energy as the chitosan film (Peak A). The other having higher binding energy emerges at 403 eV (Peak B). This latter peak can be regarded as a signal from the positively charged nitrogen atom of the quaternary

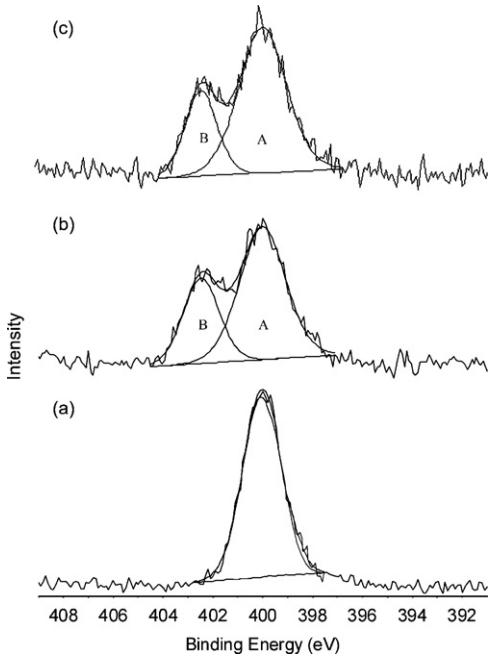
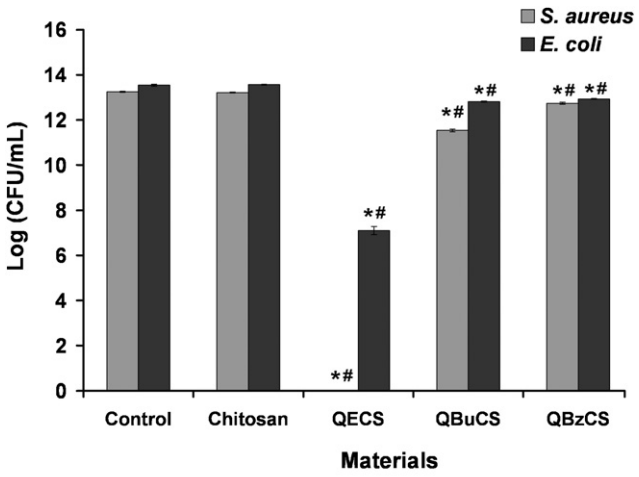


Fig. 3. XPS N 1s spectra of (a) chitosan, (b) QBuCS, and (c) QBzCS films.



% Antibacterial ratio					
S.aureus	0.00	7.80	100.00	98.04	69.27
E.coli	0.00	0.00	99.99	81.00	75.00

Fig. 4. Total replication competent (viable) cell counts of bacteria grown for 24 h in media alone (control) or in the presence of chitosan and different quaternized *N*-alkyl chitosan films, and below in the table, the corresponding antibacterial ratio. Statistical significance with $p < 0.05$ of the viable count is compared with the control (*) and the chitosan film (#).

ammonium moiety. The %DQ in the surface region could be estimated from the relative ratio of atomic composition of Peak A to Peak B. For the QBzCS series, it was found that the %DQ of the *N*-benzyl chitosan film after quaternization with 0.4 M and 1.6 M CH_3I was equal to 19.2% and 39.6%, respectively. To some extent, these values corresponded with the zeta potential values measured on the quaternized chitosan particles (see Fig. 1 for comparison).

3.2. Evaluation of antibacterial activity

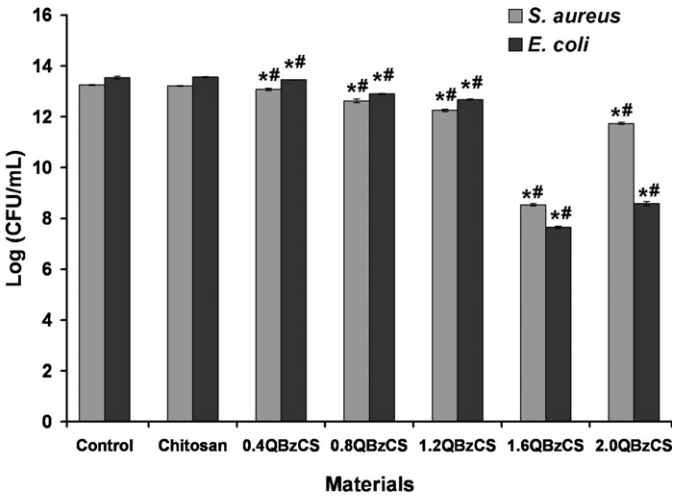
The antibacterial activity of the quaternized *N*-alkyl chitosan films was tested against the Gram-positive and Gram-negative representative bacteria, *S. aureus* and *E. coli*, respectively, in terms of the total number of replication competent (viable) cells as mean colony forming units per volume (CFU/mL). In order to determine the effect of the alkyl substituent on the antibacterial activity of the three quaternized *N*-alkyl chitosan films, QECS, QBuCS and QBzCS, having a similar zeta potential of ~30 mV were selected for the investigation. It is interesting to see from Fig. 4 that the chitosan film, at pH 7.4, showed almost no antibacterial action in comparison with the control of which the bacteria were grown in the absence of chitosan film. This is not quite unexpected, considering that the amino groups on the surface of chitosan films are in the neutral form, not positively charged, at that pH. This also implied that the chelation of the amino groups of chitosan, which has been proposed by others (Cuero, Osuji, & Washington, 1991; Kong et al., 2008) as one of the antibacterial mechanism, does not play a major role in this particular case. This outcome, in fact, suggests a potential significance for the surface quaternization in promoting antibacterial activity under a neutral environment.

Apparently, the QECS and QBuCS films exhibited a greater antibacterial activity than the QBzCS film against both bacterial species. Among all surface-quaternized chitosan films, it seems that the greater the hydrophobicity of the film was, the lower the antibacterial activity was obtained considering that all samples having the similar zeta potential. With the highest water contact angle value ($93.0 \pm 1.4^\circ$), the QBzCS film was not the best

substrate to interact with the bacteria, as previously anticipated based on the assumption that the hydrophobic character should favor the interaction with the lipid bilayers, another major component of the bacterial membrane and subsequently enhances the antibacterial activity. The QECS, on the other hand, exhibited the highest antibacterial activity despite its lowest water contact angle ($71.7 \pm 0.9^{\circ}$) in the series. This somewhat suggested that the bactericidal action of the surface-quaternized chitosan film may take place through not only the contact-inhibitory mechanism as previously expected, but also through a release-inhibitory mechanism similar to what has been observed for soluble chitosan and its quaternized derivatives. A similar speculation that the surface leaching of a soluble protonated glucosamine fraction from the chitosan film as being responsible for the antibacterial activity has also been made recently by Fernandez-Saiz, Lagaron, and Ocio (2009). The extent of weight loss which is presumably caused by surface erosion of all surface-quaternized chitosan films determined by gravimetric analysis was varied roughly in a similar range of 0.1–0.2%. Although there was no specific correlation between the magnitude of weight loss and the type of quaternized derivative, it should be more likely for the QECS to possess the greatest ability to erode out considering its lowest hydrophobicity in the series. It should be noted that there was no detectable weight loss in the case of the virgin, unmodified chitosan film.

The QECS film was detrimental to both *S. aureus* and *E. coli*, although perhaps at a greater magnitude to *S. aureus*, where it yielded a 100% and almost a 100% antibacterial ratio against *S. aureus* and *E. coli*, respectively. The QBuCS film delivered a better inhibitory effect on the Gram-positive bacteria, *S. aureus* than the Gram-negative bacteria, *E. coli*, but neither was as strong as that seen for the QECS film. It is conceivable that the absence of an outer membrane and the presence of negatively charged teichoic acid within a thick peptidoglycan layer (20–80 nm) on the surface of *S. aureus* (Talaro, 2005) should make them more attractive to the positively charged, quaternized chitosan films and easier to be damaged through the contact-inhibitory mechanism than *E. coli* (Fernandez-Saiz et al., 2009). Although *E. coli*, with its thinner double protective layer (the outer lipopolysaccharide layer embedded with a number of small channels of porins and the inner peptidoglycan layer (7–8 nm)) (Talaro, 2005) should be more structurally vulnerable to damage than *S. aureus*, which has a peptidoglycan layer that is several magnitudes thicker than that of *E. coli*, it has been recently been reported, based on AFM analysis, that the stiffness of *S. aureus* is indeed lower than that of *E. coli* (Fernandez-Saiz et al., 2009). However, that did not seem to be the case for the QBzCS films. The antibacterial activity against *E. coli* was relatively comparable to that against *S. aureus*. In fact, the higher susceptibility of *E. coli* than *S. aureus* towards chitosan has also been reported before by others (Chung et al., 2004; Devlieghere, Vermeulen, & Debevere, 2004) For comparison, the investigation was also conducted on the chitosan film that was quaternized by direct methylation with 1.2 M CH_3I for 8 h. It was found that the methylated chitosan film having a comparable zeta potential of +29.3 mV exhibited %antibacterial ratio of ~26 and 29% against *S. aureus* and *E. coli*, respectively. These significantly lower antibacterial efficiency values as opposed to those of the QECS, QBuCS, and QBzCS films helped verifying the indispensable role of the alkyl groups namely ethyl, butyl, and benzyl, respectively, introduced in the step of reductive alkylation, in enhancing the antibacterial activity of the chitosan surface via heterogeneous quaternization.

To further demonstrate the influence of the chitosan surface positive charge density on the antibacterial activity, the latter was examined against *E. coli* and *S. aureus* on a series of QBzCS films in which the charge magnitude was varied as a function of the CH_3I concentration used in the quaternization step. It should be noted that the number written in front of QBzCS displayed in the horizontal axis of Fig. 5 represents the CH_3I concentration used for preparing each QBzCS film. The viable count data shown in Fig. 5 suggested that the antibacterial activity of the surface-quaternized chitosan carrying the same hydrocarbon moiety from the step of *N*-reductive alkylation, benzyl group in this case, can be proportionally tailored as a function of the CH_3I concentration. Besides the increase in charge density, the elevated hydrophilicity introduced by quaternization may also help promote antibacterial activity of the QBzCS films, perhaps through the same mechanism previously described based on surface erosion. This may be the reason why the highly quaternized QBzCS films that were prepared from 1.6 M and 2.0 M of CH_3I and possessed relatively low water contact angles (see Fig. 1 for data) are so potent that they can almost entirely suppress the bacterial growth (~100% antibacterial ratio).



% Antibacterial ratio							
S.aureus	0.00	7.80	27.00	75.90	89.94	99.99	96.92
E.coli	0.00	0.00	17.00	77.00	86.28	99.99	99.99

Fig. 5. Total replication competent (viable) cell counts of bacteria grown for 24 h in media alone (control) or in the presence of chitosan and QBzCS films having different degree of quaternization (varied as a function of the CH_3I concentration) and below in the table, the corresponding antibacterial ratio. Statistical significance with $p < 0.05$ of the viable count is compared with the control (*) and the chitosan film (#).

In order to determine the morphological changes of bacteria upon prolonged contact with the surface-quaternized chitosan, and to be able to simultaneously correlate the extent of damage with charge magnitude of the chitosan surface, SEM analysis was conducted on the surface of the series of QBzCS films having varied charge magnitude which can be manipulated as a function of CH_3I concentration. It was our intention not to rinse the surface-quaternized film after incubation with the bacterial suspension in order to keep as many bacterial cells adhered onto the film surface as possible, so that both normal and damaged cells can be observed in case they, as is likely, vary in their adherent strength. As revealed in Fig. 6(a) and (b), the typical spherical shape and clustered formation of *S. aureus* cells (Eaton, Fernandes, Pereira, Pintado, & Malcata, 2008) was evident on the surface of chitosan and 0.4QBzCS films with no detectable signs of damage. This corresponds quite well with the fact that the CS and 0.4QBzCS film exhibited no and low antibacterial action against *S. aureus*, respectively. Although most of bacteria on the surface of the 0.8QBzCS and 1.2QBzCS films appear normal in morphology, the cells became less clustered and apparent morphological damage can be easily visualized, especially by those pointed out by arrows and magnified images shown in the inset in Fig. 6(c) and (d). Some cells were ruptured with broken

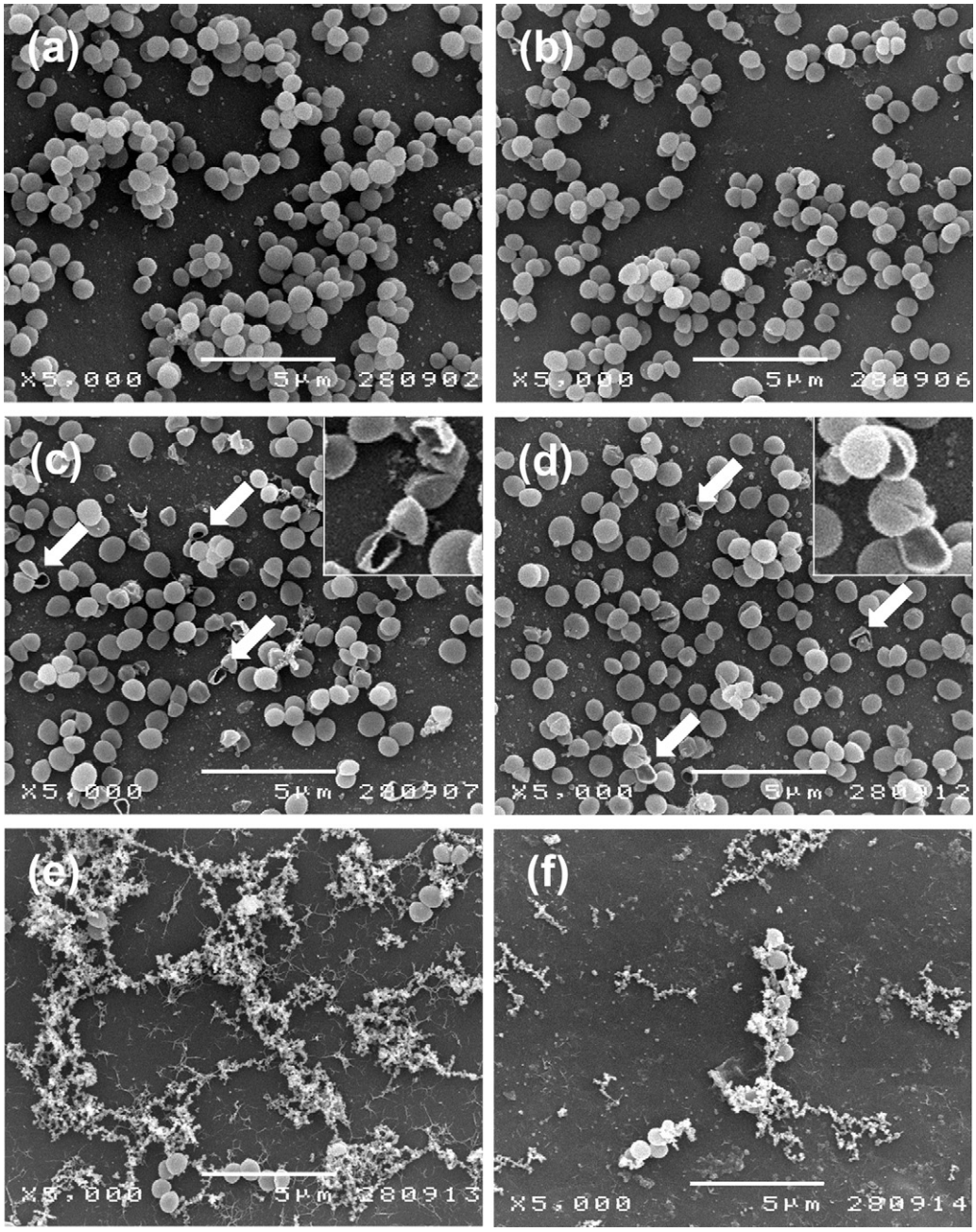


Fig. 6. SEM micrographs of (a) chitosan, (b) 0.4QBzCS, (c) 0.8QBzCS, (d) 1.2QBzCS, (e) 1.6QBzCS, and (f) 2.0QBzCS films after being incubated with the suspension of *S. aureus* (OD_{600} = 0.5) for 24 h. Micrographs shown are representative of at least 5 such fields of view per sample and 3 independent samples.

cell membranes, others were deformed, collapsed and shrunken in size. A further increase in the surface charge density caused extensive lysis of the cells and only few intact bacteria remained on the surface of the 1.6QBzCS and 2.0QBzCS films (Fig. 6(e) and (f)). It is feasible that what appear on those surfaces are fragments of disrupted cytoplasmic membrane and/or intracellular components of the bacteria.

Similar destructive features were also observed in the case of *E. coli* (Fig. 7), even though there was a higher density of *E. coli* cells in the suspension containing chitosan and 0.4QBzCS films than those containing other QBzCS films (see Fig. 5), very few of them adhered onto the surface of chitosan and 0.4QBzCS films, even despite the absence of agitation and washing prior to fixation of the samples, as can be seen from Fig. 7(a) and (b). This may imply that there are no particular attractive forces between the virgin and slightly surface-modified chitosan films under a neutral pH environment. This is entirely the opposite behavior to that observed for *S. aureus* under the same conditions. Nevertheless, the damage to *E. coli* cells became apparent with the QBzCS films having a greater extent of quaternization. As indicated by the arrows, and shown in the insets, in Fig. 7(c)–(f), *E. coli* were severely damaged and appeared in the form of deformed and collapsed rods. Fewer *E. coli* cells in the suspension, together with remnants of destroyed cell membranes and/or intracellular materials of the bacteria that were left on the surface of the 2.0QBzCS film (Fig. 7(f)), indicate that most of them were lysed and did not survive. This outcome is in excellent agreement with the antibacterial activity based on the total viable cell count data.

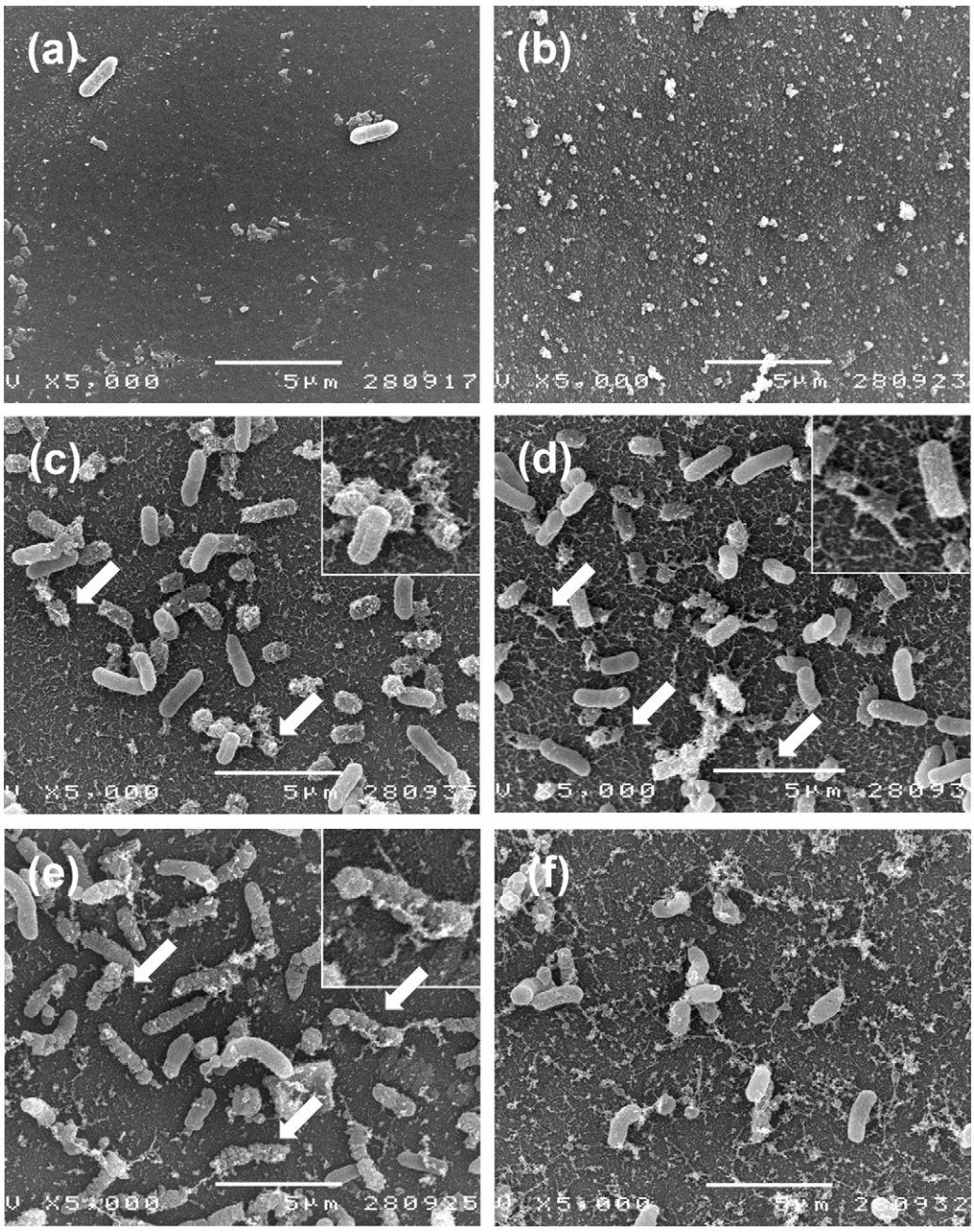


Fig. 7. SEM micrographs of the (a) chitosan, (b) 0.4QBzCS, (c) 0.8QBzCS, (d) 1.2QBzCS, (e) 1.6QBzCS, and (f) 2.0QBzCS chitosan films after being incubated with the suspension of *E. coli* (OD_{600} = 0.5) for 24 h. Micrographs shown are representative of at least 5 such fields of view per sample and 3 independent samples.

4. Conclusions

Quaternized *N*-alkyl chitosan films/particles having different alkyl substituents were successfully prepared. Conventional antibacterial test based on total viable plate counts, as well as microscopic evidences obtained from SEM analysis, support that all surface-modified chitosan samples exhibited greater antibacterial activity against *S. aureus* (Gram-positive bacteria) and *E. coli* (Gram-negative bacteria) than the virgin chitosan surface at neutral pH. The additional positive charges introduced by surface quaternization apparently made the chitosan films more antibacterial. The ability to fine tune the antibacterial activity of the chitosan surface by controlling the degree of quaternization which can readily be varied as a function of CH_3I concentration, renders the heterogeneous quaternization a powerful route for enhancing the antibacterial efficacy of chitosan. This is particularly desirable considering that the process can be done simply on the pre-fabricated solid form of chitosan without the need for a tedious purification process. These surface-quaternized chitosan films/particles should potentially be useful for many technologically important applications requiring antibacterial activity.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.carbpol.2010.08.075.

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