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Synthesis and antimicrobial activity of a water-soluble chitosan derivative with a fiber-reactive group

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Abstract—A novel fiber-reactive chitosan derivative was synthesized in two steps from a chitosan of low molecular weight and low degree of acetylation. First, a water-soluble chitosan derivative, *N*-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride (HTCC), was prepared by introducing quaternary ammonium salt groups on the amino groups of chitosan. This derivative was further modified by introducing functional (acrylamidomethyl) groups, which can form covalent bonds with cellulose under alkaline conditions, on the primary alcohol groups (C-6) of the chitosan backbone. The fiber-reactive chitosan derivative, *O*-acrylamidomethyl-HTCC (NMA–HTCC), showed complete bacterial reduction within 20 min at the concentration of 10 ppm, when contacted with *Staphylococcus aureus* and *Escherichia coli* ($1.5–2.5\times10^5$ colony forming units per milliliter [CFU/mL]).

Keywords: Water-soluble chitosan derivative; Quaternization; Fiber-reactive chitosan derivative; Acrylamidomethylation; Antimicrobial activity

1. Introduction

Chitosan, the deacetylated derivative of chitin, is the second most abundant polysaccharide found on earth next to cellulose. As a natural renewable resource, chitosan has a number of unique properties such as antimicrobial activity, nontoxicity, and biodegradability, which attract scientific and industrial interest in such fields as biotechnology, pharmaceutics, wastewater treatment, cosmetics, agriculture, food science, and textiles.[1]

The antimicrobial activity of chitosan against a variety of bacteria and fungi coming from its polycationic nature, is well known.[2–8] However, this activity is limited to acidic conditions due to its poor solubility above pH ~ 6.5, where chitosan start to lose its cationic nature.[2,5,9] Water solubility is important in applications of chitosan as an antimicrobial agent, and researchers have focused on the preparation of chitosan derivatives soluble in water over a wide pH range. Various water-soluble chitosan derivatives with enhanced antimicrobial property were reviewed in a recent paper.[10]

Market demand for antimicrobial textile products has greatly increased as consumers' standard of living has risen and they became aware of the importance of these products. Polymeric quaternary ammonium compounds have received the most attention as biocidal polymers over the years.[11] Major concerns with the antimicrobial agents used for textiles are their safety toward the human body. In this context, chitosan has received considerable attention as an antimicrobial agent for textile products.[12–15] However, the major drawback of chitosan as an antimicrobial textile finish is its lack of strong bonding with textile fibers. As the result, the antimicrobial activity of chitosan-treated fabric decreases with repeated launderings.

In this paper, we report a new water-soluble chitosan derivative (Scheme 1) with a fiber-reactive group, which can be covalently bonded to textile fibers having nucleophilic groups, especially cellulose. A water-soluble chitosan derivative, *N*-[(2-hydroxy-3-trimethylammonium)propyl]chitosan chloride (referred to here as HTCC), was prepared by reacting chitosan with glycidyltrimethylammonium chloride (GTMAC). The

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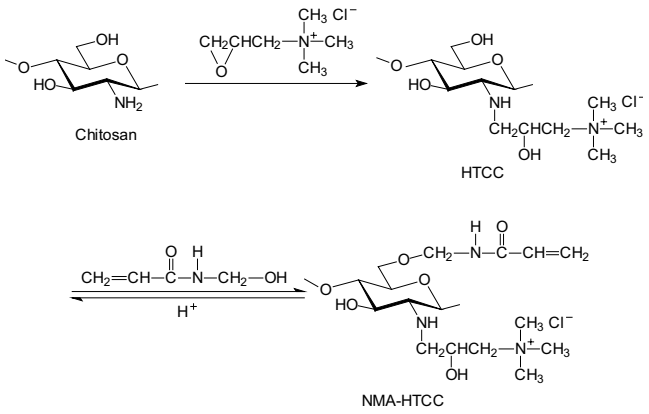


Scheme 1. Synthesis of the HTCC and NMA–HTCC.

HTCC was further modified by reacting with *N*-methylolacrylamide (NMA) to prepare a fiber-reactive chitosan derivative, *O*-acrylamidomethyl-HTCC (referred to here as NMA–HTCC). The optimal reaction conditions for introducing higher amounts of the fiber-reactive (acrylamidomethyl) groups on chitosan backbone are discussed. The antimicrobial activity of the NMA–HTCC was evaluated against *Staphylococcus aureus* and *Escherichia coli* prior to the application to textile fabrics.

2. Experimental

2.1. Materials

Chitosan (from crab shells) as a ground form with a low molecular weight was purchased from Korea Chitosan Co., Ltd. GTMAC and NMA (48 wt% aq solution) were purchased from Fluka Chemical Co. and Aldrich Chemical Co., respectively. All other reagents and solvents were purchased from either Fisher Scientific or Aldrich Chemical Co. and were used without further purification. *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25922) were obtained from American Type Culture Collection (ATCC), Manassas, VA, USA. Bacto™ nutrient broth and Difco™ nutrient agar were purchased from Becton, Dickinson and Company.

2.2. FT-IR and ^{1}H NMR spectroscopy

IR spectra were obtained using a Nicolet 510P FT-IR spectrophotometer. All samples were prepared as KBr pellets and were scanned against a blank KBr pellet background. The IR sample chamber was continually flushed with dry air. The data collection parameters employed were as follows: gain, 1; resolution, 4.0 cm^{-1}; and scans, 32.

Chitosan and its derivatives were dissolved in 2% (w/w) DCl/D_2O and D_2O, respectively, and introduced into a 5-mm NMR tube. The ^{1}H NMR spectra were obtained using a GE GN 300Ω NMR spectrometer at room temperature. As an internal reference, sodium 4,4-dimethyl-4-silapentanoate-2,2,3,3-d_4 was used. For the data collection, the number of acquisitions was 128.

2.3. Deacetylation of chitosan

Commercial chitosan (20 g) was dispersed in 200 mL of 10% (w/w) NaOH solution containing $NaBH_4$ (2 g) as an antioxidant.[16] After 5 h of stirring at 110 °C, the mixture was filtered over a glass filter and washed with distilled water until neutral to pH paper. The chitosan was further washed with MeOH and acetone and dried at 70 °C under vacuum overnight. The weight of the deacetylated chitosan was 17.6 g.

2.4. Molecular weight (MW)

The viscosity average MW ($\overline{M}_v$) chitosan was determined by the method of Wang et al.[17] using the Mark–Houwink equation. Chitosan was dissolved in aq 0.2 M AcOH–0.1 M NaOAc and the viscosity measured using a Cannon–Ubbelohde semi-micro viscometer [size 75, no. N177, viscometer constant = 0.00745 mm^2/s^2 (cSt/s)] at 30 °C.

2.5. Degree of acetylation (DA)

The DA of the chitosan was determined based on the integral ratio of proton peaks from ^{1}H NMR spectroscopy using the following equation,[18] where I_{CH_3} is the integral intensity of *N*-acetyl protons and $I_{H2\text{–}H6}$ is the sum of integral intensities of H-2, 3, 4, 5, 6, and 6′ protons.

$$\mathrm{DA} = \left(\frac{I_{CH_3}}{3}\right) \Big/ \left(\frac{I_{H2\text{–}H6}}{6}\right).$$

2.6. Synthesis of HTCC

The HTCC was prepared by a modified method of Lang et al.[19] The deacetylated chitosan (6 g, 37.0 mmol) was dispersed in distilled water (60 mL) at 85 °C. GTMAC (21.3 mL, 111 mmol) was added in three portions (7.1 mL each) at 2-h intervals. After 10 h of reaction, the clear and yellowish reaction solution was poured into cold acetone (200 mL) while stirring and kept in the refrigerator overnight. The next day, acetone was decanted and the remaining gel-like product was dissolved in MeOH (100 mL). The solution was precipitated in 4:1 acetone–ethanol (250 mL). The white product was collected by filtration and further purified by washing with hot EtOH using a Soxhlet extractor for 24 h. The final product was dried at 70 °C overnight. The weight of the final product was 10.19 g and the yield calculated was 87.8%.

2.7. Degree of quaternization (DQ)

The DQ of the HTCC was measured by titrating the amount of Cl^- ions on the HTCC with aq $AgNO_3$ solution. DQ is defined as the ratio of mol of reacted GTMAC per mol of glucosamine calculated from the original mass of chitosan used. Thoroughly dried HTCC (0.1000 g) was dissolved in deionized water (100 mL) and conductometrically titrated with 0.017 M $AgNO_3$ aq solution. Solution conductivities were monitored with an Orion Benchtop Conductivity Meter (Model 162) equipped with an Orion Conductivity Cell (Model 013030). During the titration, the temperature of the solution was kept constant (20.4–20.5 °C) using a water bath because the conductivity is a function of temperature. The value of DQ calculated was 1.00.

2.8. Synthesis of NMA–HTCC

The HTCC (1 g, 3.2 mmol) was dissolved in 48 wt% aq NMA solution (5 mL, 25.5 mmol) containing a small amount of 4-methoxyphenol (0.01 g) as a polymerization inhibitor. To the solution, different amounts of NH_4Cl (0.17–0.68 g, 3.2–12.8 mmol) were added and dissolved. The solutions were reacted at 140 °C for 8–16 min. To the reaction solution, MeOH (15 mL) was added and stirred for 10 s. The product was precipitated in acetone (100 mL) and washed thoroughly with a mixture of 1:1 acetone–ethanol and finally with ether. The white reaction product was dried at 40 °C under vacuum for 2 days. The weights of the products were 1.02–1.16 g depending on their reaction conditions.

2.9. Double-bond content

The double-bond content of the NMA–HTCC was determined by the method used by Kamel et al.[20] A known amount of NMA–HTCC (0.3 g) was dissolved in deionized water (10 mL) contained in a weighing bottle. To the solution, 3% aq 2-mercaptoethanol solution (5 mL) and 2 M NaOH (1 mL) were added and stirred in a closed weighing bottle at room temperature for 20 min to ensure the complete nucleophilic addition of mercaptoethanol to the double bond. The mixture was acidified with 1 N HCl (2.5 mL) and it was titrated against 0.1 M iodine solution, in the presence of starch indicator, until the endpoint at which the first faint blue color persisted for at least 30 s. The double-bond content on the NMA–HTCC was calculated using the following equation:

$$\text{Double bond content (mmol/g NMA–HTCC)} = \frac{(V_B - V_S) \times 0.1}{W},$$

where W is the weight of sample in grams, V_B and V_S are the amount (mL) of iodine solution used in blank and sample titrations, respectively, and 0.1 is the molarity of the iodine solution.

2.10. Antimicrobial test[21]

The antimicrobial activity of the NMA–HTCC was evaluated against *S. aureus* (ATCC 6538) and *E. coli* (ATCC 25922). Each culture was suspended in a small amount of Difco™ nutrient broth, spread on the Bacto™ nutrient agar plate, and incubated at 37 °C for 24 h. A single colony was picked off with an inoculating loop, placed in nutrient broth (5 mL), and incubated for 18 h at 100 rpm and 37 °C using an incubator shaker (New Brunswick Scientific, Edison, NJ). A final concentration of $1.5–3.0 \times 10^6$ colony forming units per milliliter (CFU/mL) was prepared by appropriately diluting each culture with a sterile buffer solution (0.3 mM phosphate buffer, pH 7.2), which was used as a diluent. These dilute culture solutions were used for the antimicrobial test. The NMA–HTCC was dissolved in the buffer solution at several different concentrations. To the 9.0 mL of NMA–HTCC solutions, which were contained in test tubes (17×100 mm) and preequilibrated at 37 °C for 1 h, 1.0 mL of each culture suspension was added. At the same time, 1.0 mL of the same culture was added to 9.0 mL of buffer solution without NMA–HTCC and this was used as a control. The initial cell concentration was enumerated by a standard plate count technique from the control solution. At various contact times at 37 °C and 150 rpm, 0.2 mL portions were taken from each control and sample solution, and diluted by mixing with 9.8 mL buffer solution. From the dilutions, 0.5 and 0.15 mL portions for the sample and control, respectively, were spread on the nutrient agar plates. The inoculated plates were incubated at 37 °C for 30 h and the surviving cells were counted. The average values of the duplicates were converted to CFU/mL in the test tube by multiplying the dilution factor.

3. Results and discussion

3.1. Deacetylation of chitosan

Commercial chitosan was further deacetylated by alkaline treatment. Figure 1 shows the difference between the commercial chitosan and the deacetylated chitosan. In the IR spectrum of the commercial chitosan, there were two absorption peaks at 1660 and 1595 cm^{-1}, which correspond to the C=O stretch of the secondary amide and the N–H bending of the primary amine, respectively. The spectrum of the deacetylated chitosan showed a decrease of the peak at 1660 cm^{-1}, indicating that the secondary amide has been further changed to primary amine by the alkaline deacetylation. The evidence of deacetylation of the commercial chitosan was

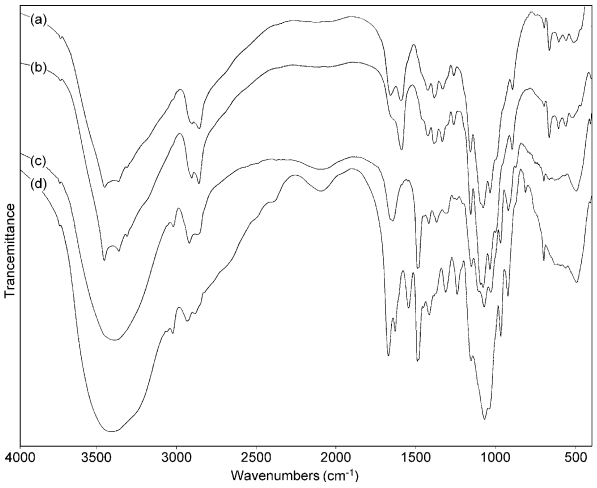


Figure 1. FT-IR spectra for (a) commercial chitosan, (b) deacetylated chitosan, (c) HTCC, and (d) NMA–HTCC.

also observed from ^{1}H NMR spectra (Fig. 2). The deacetylated chitosan shows lower intensity in the *N*-acetyl peak at 2.06 ppm than that of commercial chitosan. The properties of the commercial and deacetylated chitosan samples are listed in Table 1. As the result of the alkaline treatment, a highly deacetylated chitosan with a low MW was obtained, which is better for textile application in terms of better penetration into fiber and less stiffness of the treated fabric.

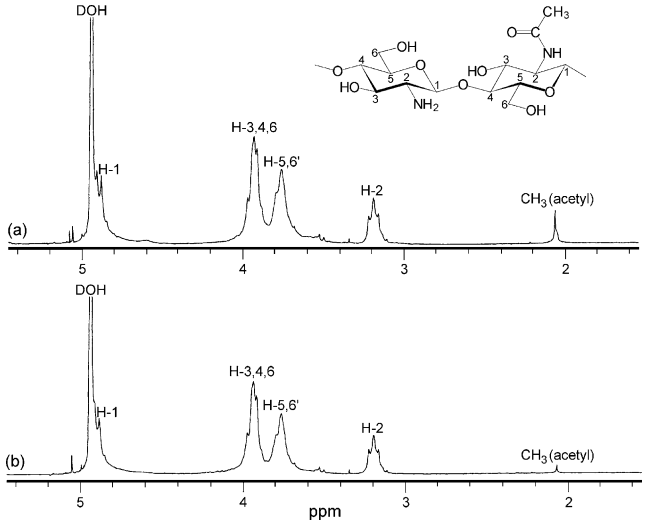


Figure 2. 300 MHz ^{1}H NMR spectra for (a) commercial and (b) deacetylated chitosan.

3.2. HTCC

Chitosan dissolves in water only in acidic conditions by the protonation of the amino groups and thus its antimicrobial activity is limited to acidic conditions.[2] Therefore, introduction of quaternary ammonium salts onto the chitosan backbone will be one of the best methods to enhance antimicrobial activity as well as the water solubility of chitosan over the entire pH range.

GTMAC was chosen as a quaternization reagent because of its ease of reaction with amino groups of chitosan and it is known that the reaction product, HTCC, shows water solubility as well as an excellent antimicrobial activity.[22,23] The HTCC was prepared by reacting chitosan with GTMAC in a neutral aq condition, in which the hydroxyl groups of chitosan are not sufficiently nucleophilic to induce ring opening of GTMAC, whereas the amino group of chitosan is nucleophilic enough to do that.[24] The IR spectrum (Fig. 1) shows evidence of the introduction of the quaternary ammonium salt group on chitosan backbone, at 1480 cm^{-1}, the C–H bending of trimethylammonium group. It should be also noted that the N–H bending (1595 cm^{-1}) of the primary amine disappeared due to the change of the primary amine to the secondary amine (aliphatic).[25] The peak at 1650 cm^{-1} was assigned for the C═O stretch of the secondary amide (2.9% of repeat units of the deacetylated chitosan) that was a shoulder of the N–H bending peak at 1590 cm^{-1} as shown in the IR spectrum of the deacetylated chitosan. In addition, the spectrum shows a broad band at around 3400 cm^{-1}, due to the increased number of hydroxyl groups. The NMR spectra of the samples are shown in Figure 3. As evidence of the reaction, methyl groups in the quaternary ammonium salt group were observed as a very strong peak at 3.2 ppm.

The DQ of the HTCC was measured by conductometric titration of Cl^- with 0.017 M aq $AgNO_3$ solution. The amount of $AgNO_3$ used at the end point equals the amount of Cl^- ions present on the HTCC. The value of DQ calculated was 1.00, which means that the amino groups of chitosan was fully substituted by quaternary ammonium salt groups.

Table 1. Properties of chitosan

Chitosan	% DA[a]	k[b]	α[b]	$[\eta]$[c] (mL/g)	$\overline{M}_v$ (g/mol)
Commercial	7.8	0.005261	0.8796	58.8	40,100
Deacetylated	2.9	0.01086	0.8296	53.3	28,100

[a]% DA was based on ^{1}H NMR.
[b]Constants for the Mark–Houwink equation.
[c]Solvent: 0.2 M AcOH–0.1 M NaOAc aq solution; temperature: 30 °C.

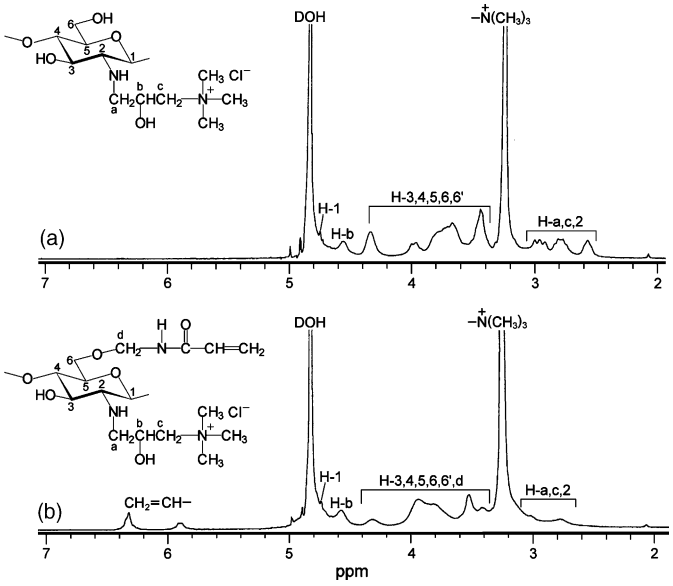


Figure 3. 300 MHz ^{1}H NMR spectra for (a) HTCC and (b) NMA–HTCC.

3.3. NMA–HTCC

NMA was chosen as a reagent to introduce a fiber-reactive group onto the HTCC because it has often been used for crosslinking of cellulose.[20,26–28] NMA (CH_2=$CHCONHCH_2OH$) has two reactive groups, which react under different conditions, a *N*-hydroxymethyl group and a double bond conjugated with a carbonyl group. By reacting NMA with HTCC under acidic conditions, NMA–HTCC is obtained as shown in Scheme 1. As an acid catalyst, ammonium chloride (NH_4Cl)[28] was used, which is a latent acid and generates HCl at an elevated temperature. The released acid can protonate secondary amine groups of HTCC, which prevent the amine groups from acting as a nucleophile. Also, the amine group of the HTCC is in a relatively bulky environment due to the quaternary ammonium salt groups. Therefore, the reaction between NMA and hydroxyl groups on C-6 position (primary alcohol) of HTCC is predominant. The NMA–HTCC has the acrylamidomethyl group as a fiber-reactive group, which has a pendant double bond that can react with the hydroxyl groups of cellulose under alkaline conditions.

The double-bond content of the NMA–HTCC, which is a measure of the extent of the acrylamidomethylation, was examined at different reaction conditions. As variable parameters, the amount of catalyst (NH_4Cl) and reaction time were selected. There were no changes in the amount of NMA (8 mol excess) and reaction temperature (140 °C). The effect of the catalyst and reaction time on the double-bond content is shown in Figure 4. The double-bond content increased as the mole ratio of catalyst increased from 1 to 3. However, further increase in the amount of catalyst showed an adverse effect. The acrylamidomethylation is a reversible reaction. It is likely that the increased amount of NH_4Cl liberated more HCl, which increased hydrolysis of the ether linkage between the acrylamidomethyl group and the HTCC. At the reaction time of 10 min, the double-bond content was maximal and considerably higher than that of 8 min. However, further increase in the reaction time decreased the double-bond content. The adverse effect at longer reaction time (12–16 min) can be attributed to the increased hydrolysis of the ether linkage between the HTCC and the acrylamidomethyl group. Thus, the optimal reaction time (10 min) is required to increase the

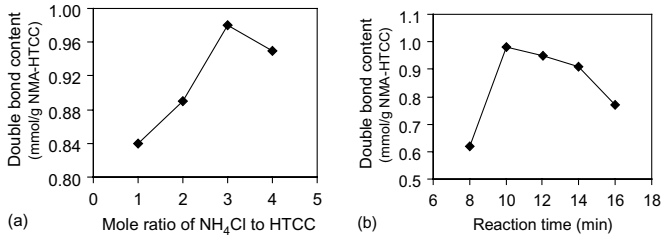
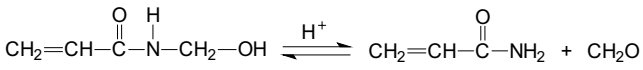


Figure 4. Effect of the amount of (a) catalyst (NH_4Cl) and (b) reaction time on the double-bond content (reaction condition: (a) NMA 8 mol excess, temperature 140 °C, time 10 min; (b) NMA 8 mol excess, NH_4Cl 3 mol excess, temperature 140 °C).



Scheme 2. Hydrolysis of NMA by HCl.

acrylamidomethylation and compensate for the hydrolysis. Another possible explanation of the decrease in double-bond content at higher concentration of catalyst and longer reaction time can be the increased hydrolysis of NMA (reactant) by HCl as shown in Scheme 2.

From the results, the optimal reaction conditions obtained for the acrylamidomethylation were as follows: MNA, 8 mol excess; NH_4Cl, 3 mol excess; reaction time, 10 min; and temperature, 140 °C. The NMA–HTCC was prepared at this reaction condition and used for the characterization.

The IR spectrum of the NMA–HTCC, shown in Figure 1, indicates the acrylamidomethylation did occur as the result of the peaks at 1670 and 1545 cm^{-1}. These peaks are most likely due to the C=O stretch and N–H bending of the secondary amide in the acrylamidomethyl group, respectively. As further evidence of the reaction, a new peak appears at 1630 cm^{-1}, which corresponds to the C=C stretch of the conjugated vinyl group. The NMR spectrum of the sample is provided in Figure 3. The acrylamidomethylation was further confirmed by the peaks at 6.3 and 5.9 ppm, which corresponds to the vinyl group.

3.4. Antimicrobial activity of NMA–HTCC

Prior to the application of the NMA–HTCC to cotton fabric, its antimicrobial activity was evaluated. Figure 5 shows log(CFU/mL) versus contact time plots for the NMA–HTCC against *S. aureus* and *E. coli*. About 1.7–2.4×10^5 CFU/mL of bacterial cells were contacted to 10, 50, 100, and 200 ppm of the NMA–HTCC dissolved in 0.3 mM phosphate buffer (pH 7.2). At the concentrations of 10, 50, and 100 ppm, all *S. aureus* cells were completely killed within 20, 40, and 60 min, respectively. Interestingly, the antimicrobial activity was inversely proportional to the concentration of the NMA–HTCC. The highest antimicrobial activity was observed at the lowest concentration (10 ppm) of the NMA–HTCC. Similarly, Sudarshan et al.[2] observed that the antibacterial activity of chitosan under an acidic condition (pH 5.8) was higher at lower concentration. It was explained by the charge interaction between the protonated chitosan and negatively charged bacterial surface. At lower concentrations, chitosan binds to the negatively charged cell surface, disturbs the cell membrane, and causes death of the cell by inducing leakage of intracellular components. Whereas, at higher concentrations, the protonated chitosan may coat the cell surface and prevent the leakage of intracellular components. In addition, the positively charged bacterial cells repel each other and prevent agglutination. It was observed that all four concentrations were effective to kill *E. coli* cells completely within 20 min. The concentration-dependant behavior, which was observed in *S. aureus*, was not observed in *E. coli* at the same concentration ranges employed.

4. Conclusion

A fiber-reactive chitosan derivative (NMA–HTCC) was prepared to overcome the drawbacks of chitosan, such as limited water solubility and antimicrobial activity, and poor laundering durability when applied on cotton fabrics. The HTCC, a water-soluble chitosan derivative with an excellent antimicrobial activity and a precursor for the NMA–HTCC, was synthesized by reacting chitosan with GTMAC in a neutral aq condition. The complete substitution of quaternary ammonium salt groups on the amino groups of chitosan was obtained when chitosan was reacted with GTMAC (3 mol excess) at 85 °C for 10 h. The preparation of the NMA–HTCC involved reaction of HTCC with NMA in the presence of an acid catalyst (NH_4Cl). Since the reaction is reversible and NMA can be hydrolyzed by the acid catalyst, the reaction time and the amount of NH_4Cl were important factors for the acrylamidomethylation. The NMA–HTCC showed complete reduction of bacteria within 20 min at the concentration of 10 ppm, when contacted with *S. aureus* and *E. coli* ($1.5–2.5\times10^5$ CFU/mL). From the test results, it has been confirmed that the NMA–HTCC has an excellent antimicrobial activity against both *S. aureus* and *E. coli* compared to chitosan,

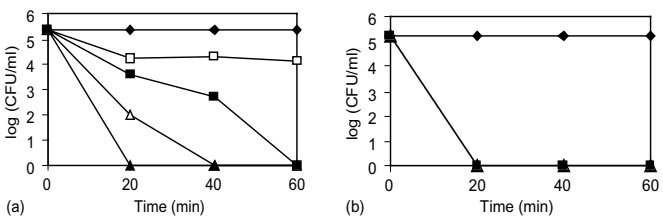


Figure 5. Antimicrobial activity of the NMA–HTCC against (a) *S. aureus* and (b) *E. coli* [◆ control (0 ppm), □ 200 ppm, ■ 100 ppm, △ 50 ppm, and ▲ 10 ppm].

which does not dissolve in pH 7.2 and does not show any antimicrobial activity under this condition.[2] The application of the NMA–HTCC to cotton fabric and its durability as an antimicrobial textile finish are under investigation in our laboratory.

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