Development of a quaternized chitosan with enhanced antibacterial efficacy

Gurpreet Kaur Khaira, Rashmi Kumariya, Manmohan Chibber and Moushumi Ghosh

ABSTRACT

The antibacterial activity of a water-soluble chitosan derivative prepared by chemical modification to quaternary ammonium compound *N*,*N*,*N*-trimethylchitosan (TC) was investigated against four selected waterborne pathogens: *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 32150. An inactivation of 4 log CFU/ml of all waterborne pathogens was noted for the quaternized chitosan as compared with chitosan over a short contact time (30 min) and low dosage (4.5 ppm) at ambient temperature. A marked increase in glucose level, protein content and lactate dehydrogenase (LDH) activity was observed concurrently in the cell supernatant to be a major bactericidal mechanism. The results suggest that the TC derivative may be a promising commercial substitute for acid-soluble chitosan for rapid and effective disinfection of water.

Key words | antibacterial activity, chitosan, disinfection, quaternized, waterborne pathogens

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ABBREVIATIONS

TC	*N*,*N*,*N*-trimethylchitosan
NMR	nuclear magnetic resonance
BHI	brain heart infusion
FE	flocculation efficiency
MIC	minimum inhibitory concentration
LDH	lactate dehydrogenase
NADH	nicotinamide adenine hydroxy dinucleotide
TAL	thin agar layer
ANOVA	analysis of variance

INTRODUCTION

Presence of microbes in drinking water caused by contamination with animal and human excreta represents the most common and widespread health risk associated with water consumption worldwide (WHO 2008). Ingestion of these microbial agents can result in various severe human diseases, including legionellosis, cholera, pneumonia, acute gastritis, bacteremia and septicemia most of which manifest as diarrhea (Ashbolt 2004). Conventionally available water treatment processes for the removal of these waterborne pathogens includes boiling, filtration, coagulation/flocculation and disinfection. Environmental considerations demand to develop strong, economically viable and eco-friendly replacements of conventional synthetic flocculants and disinfectants, based upon the renewable organic materials which are economical and degrade naturally, if ever released in the environment.

Chitosan is a copolymer of glucosamine and *N*-acetyl-glucosamine units linked by 1,4-glucosidic bonds and it is obtained through the alkaline hydrolysis of chitin (No & Meyers 1997). The high biodegradability and non toxicity to mammals have enabled chitosan to be widely used as antimicrobial agent either alone or blended with other natural polymers (Rabea *et al.* 2003; El Hadrami *et al.* 2010). The antimicrobial activity of chitosan against a variety of bacteria and fungi originates from its polycationic nature (Muzzarelli *et al.* 2001; Rabea *et al.* 2003; Xia *et al.* 2011). Unfortunately, this activity is limited to acidic conditions

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because of its poor solubility above pH 6.5, since chitosan starts to lose its cationic nature and becomes poorly soluble (Rabea *et al.* 2003). Therefore, the water solubility is an important factor in applications of chitosan as an antimicrobial agent, and subsequent efforts have focused on the preparation of derivatives soluble in water over a wide pH range (Lim & Hudson 2004). Chemical modification of chitosan and several derivatives with high solubility in water have been attempted, moreover, chitosan derivatives containing carboxyl groups such as *N*,*O*-(carboxyalkyl and -aryl) chitosans, *N*,*N*-(dicarboxyethyl) chitosan and *N*-(carboxyacyl) chitosans have been reported as water soluble compounds (Kurita *et al.* 1982; Shigemasa & Minami 1996; Hirano & Moriyasu 2004).

Whereas the bactericidal effect of concentrated chitosan against Gram-negative and Gram-positive bacteria has been reported, its action on Gram-negative bacteria seems to be less significant (Takemono *et al.* 1989; Kim & Choi 2002). Thus for practical purposes, studies with superior chitosan derivatives against a range of Gram-negative pathogens are desired as the bactericidal studies encompassing a wide range of bacterial pathogens by chitosan derivatives are rare. In the present study, we sought to evaluate the antibacterial properties of a quaternized chitosan derivative against four bacterial pathogens transmitted through contaminated water. It was envisaged that the results would be beneficial in formulating a novel approach for disinfecting water.

MATERIALS AND METHODS

Chemicals

Chitosan (85% deacetylated) was procured from Sigma-Aldrich (USA) and all other reagents were of the highest grade available commercially.

Bacterial strains and culture conditions

In total, four bacteria associated with potential to cause waterborne disease were used in this study. *Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *Escherichia coli* O157:H7 ATCC 32150 were grown in brain heart infusion (BHI) broth by incubating at 37 °C, with agitation for 6–8 h at 120 rpm.

Synthesis and characterization of *N*,*N*,*N*-trimethylchitosan derivative (TC)

Quaternized derivative of chitosan was prepared by a modified method as explained by Jia *et al.* (2001) and Belalia *et al.* (2008). Chitosan (500 mg; 10 mmole) was dissolved in 1% (v/v) aqueous acetic acid (50 ml) and formaldehyde (0.5 ml; 0.22 mmole) was added. The solution was stirred at room temperature for 30 min and sodium borohydride (41.25 mg; 1.12 mmole) was added. After 1 h of stirring, the pH of the solution was adjusted to 10 by using NaOH (1 M). White precipitates appeared that were filtered and washed to reach the pH of 7. Trapped moisture was removed by refluxing it with dry benzene through azeotropic distillation. Evaporation of benzene resulted in *N*-methylchitosan (yield 86%; 430 mg).

N-methylchitosan (400 mg; 8 mmole) was dispersed in *N*-methyl-2-pyrrolidone (12 ml) with sodium iodide (0.5 g; 3.33 mmole). The mixture was vigorously agitated at 60 °C for 1 h after which 15% w/v NaOH (2.2 ml) and methyl iodide (1 ml; 0.045 mmole) were added. The product was precipitated using acetone (36 ml), filtered and vaccum dried after the continued stirring for 6 h at 60 °C. White precipitates obtained were redissolved in deionized water and dialyzed for 24 h. The dialysate was lyophilized to obtain a white powder (yield 70%; 280 mg).

NMR analysis

^{1}H NMR spectra of chitosan and the *N*,*N*,*N*-trimethyl derivative were recorded using Bruker Avance II (400 MHz) spectrometer. The degree of quaternization (DQ) of the derivative was evaluated by titration of iodide ions (Belalia *et al.* 2008).

Determination of flocculating activity

The flocculating activity was measured according to the method described earlier (Kurane *et al.* 1986). Volumes of 10–100 µl of *N*,*N*,*N*-trimethylchitosan (TC) and chitosan were added to kaolin suspension (5 g L^{-1}). In both cases,

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40% $CaCl_2$ solution was added in order to increase the flocculation activity and reference samples were also prepared without biopolymers and the pH was adjusted to 7.0 with 1 N NaOH. The solution was thoroughly vortexed for 30 s and allowed to stand at room temperature for 5 min. Two milliliters of aliquots were withdrawn from the upper phase and absorbance was recorded at 550 nm with a spectrophotometer (U-2800, Hitachi, Japan). The activity was measured in percent as follows

$$\text{Flocculant activity } (\%) = (B - A) * 100/B$$

where A is the absorbance of the sample at 550 nm, and B is the absorbance of the reference at 550 nm.

Evaluation of antibacterial activity

The antibacterial spectrum of TC was determined against waterborne pathogens by the agar disc diffusion assay and spectrophotometric method. Cultures of the different strains (*Aeromonas hydrophila* ATCC 35654, *Yersinia enterocolitica* ATCC 9610, *Listeria monocytogenes* ATCC 19111 and *E. coli* O157:H7 ATCC 35150) were revived in BHI broth by incubating at 37 °C for 6 h. The effect of chitosan and TC on the growth inhibition of bacteria on the solid state culture was evaluated by using agar plates. Chitosan's stock solution (1% [wt/vol] in 1% acetic acid) was sterilized by autoclaving at 121 °C for 20 min and stored at 4 °C for subsequent use. The strains were grown in the respective broth at 37 °C to an absorbance of 1.0 at 600 nm and subsequently diluted in the same medium to about 10^7 CFU/ml. The discs of uniform size prepared from Whatmann filter paper were sterilized and soaked in sterile 2 ppm solution of each of the chitosan and TC. These soaked discs were then placed on the assay plates containing 100 μl of overnight grown cultures on BHI broth. The plates were then incubated at 37 °C for 12 h, after which the diameters of inhibition zones were observed. The zones of inhibition were measured with antibiotic zone scale in mm and the experiment was carried out in triplicates. *p*-value was interpreted using multivariable analysis of variance (ANOVA) analysis.

Values of the minimum inhibitory concentration (MIC) were determined by a broth microdilution assay. Briefly, serial two-fold dilutions of quaternized chitosan solutions were prepared in the appropriate culture medium in sterile 96-well round bottom polystyrene microtiter plates (Bioscreen C, Thermolabsystems, Helsinki, Finland). The solution of chitosan and quaternized chitosan (2–5 ppm) were added and their inhibitory effect was observed. Each well of the microtiter plate received 100 μl of the inoculated medium, and the plates were incubated at 37 °C for up to 24 h. The MIC was read as the least concentration of the antibacterial agents that was sufficient to completely inhibit visible bacterial growth.

Bacterial killing assay: Cultures of the indicator bacterial strains were incubated separately in the absence (control) and presence of different concentrations (0–5 ppm) of TC and chitosan, for a period of 24 h at 37 °C. The absorbance was measured at regular interval of time to record survival counts, expressed as CFU/ml. The surviving $\log_{10}$ CFU/ml was plotted against time for each of the different quaternized biopolymer and chitosan concentrations. All experiments were replicated at least three times.

Leakage of glucose, lactate dehydrogenase (LDH) and protein from treated bacterial cells

To examine the effect of water-soluble chitosan derivative on cell leakage and the viability of the indicator strain, inoculum of the test organism (1 ml) was inoculated into sterile deionized water (10 ml) with or without water-soluble chitosan derivative in a culture tube. The mixture, containing 2–5 ppm of water-soluble chitosan derivative (TC) and indicator cultures were incubated at 37 °C with shaking (120 rpm) for 12 h. During the pre-determined incubation period, aliquots were withdrawn for determination of protein and glucose contents and lactate dehydrogenase (LDH) activity. The cell suspension was centrifuged at 8,000 *g* for 15 min, and the supernatant was measured for LDH activity, protein and glucose contents. The glucose content was analyzed by a glucose assay kit (DiaSys Diagnostic Systems GmbH, Holzheim, Germany). A sample or glucose standard (0.0–3.0 mg/dl, 200 μl) was added to the reagent (1 ml) containing glucose dehydrogenase. After incubation at 25 °C for 15 min, the absorbance at 334 nm was recorded. The LDH activity was analyzed by an LDH assay kit (Clontech, USA). A 200 μl sample was added to 1 ml reagent containing

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NADH and incubated at 25 °C. LDH activity was then determined by measuring the rate of decrease of the NADH concentration which was monitored by recording the change of absorbance at 334 nm. The protein concentration was measured by absorbance at 280 nm.

Statistical analysis

The mean values and the standard deviation were from the data of triplicate trials. Mean values were compared by ANOVA with Duncan's multiple range method for comparing groups (SAS 1989). A significance level of 5% was adopted for all comparisons of triplicate trials.

RESULTS AND DISCUSSION

Synthesis of *N,N,N*-trimethylchitosan derivative (TC)

The ^{1}H-NMR spectra of the chitosan and its quaternized derivative (TC) are shown in Figures 1(a) and 1(b), respectively. The spectra (Figure 1(b)) revealed an intense signal at 3.66 corresponding to the trimethylammonium group which was found to be missing in the ^{1}H-NMR of chitosan (Figure 1(a)). According to the results of a study by Sieval *et al*. (1998), the peak at 3.6 ppm is assigned to the trimethyl amino group, the peak at 3.1 ppm is assigned to dimethyl amino groups and the peaks between 4.7 and 5.7 ppm are assigned to ^{1}H protons. For ^{1}H-NMR studies, pure chitosan was solubilized in D_2O/CF_3COOH mixture whereas TC was easily dissolved in D_2O. ^{1}H-NMR signal of the final product TC was compared with that of chitosan and the appearance of a sharp singlet at δ 3.6 ppm confirmed the quaternized methyl group (Britto & Assis 2007). The DQ of TC was determined from the titration of iodide ions, which revealed a DQ of 67%. Synthesis of TC leads to methylation of the amino groups in the C-2 position of biopolymer to form quaternary amino groups with fixed positive charges on the repeating units of the quaternized polymer chain.

Chitosan is known to possess antibacterial activity (Kendra & Hadwiser 1984; Hirano & Nagao 1989) which makes it a macromolecule of choice to explore its use in water purification. The limited solubility of chitosan in water restricts its use in water treatment. Chemical modification by quaternization resulted in improvement of physicochemical properties such as solubility in water and the antibacterial activity was significantly enhanced.

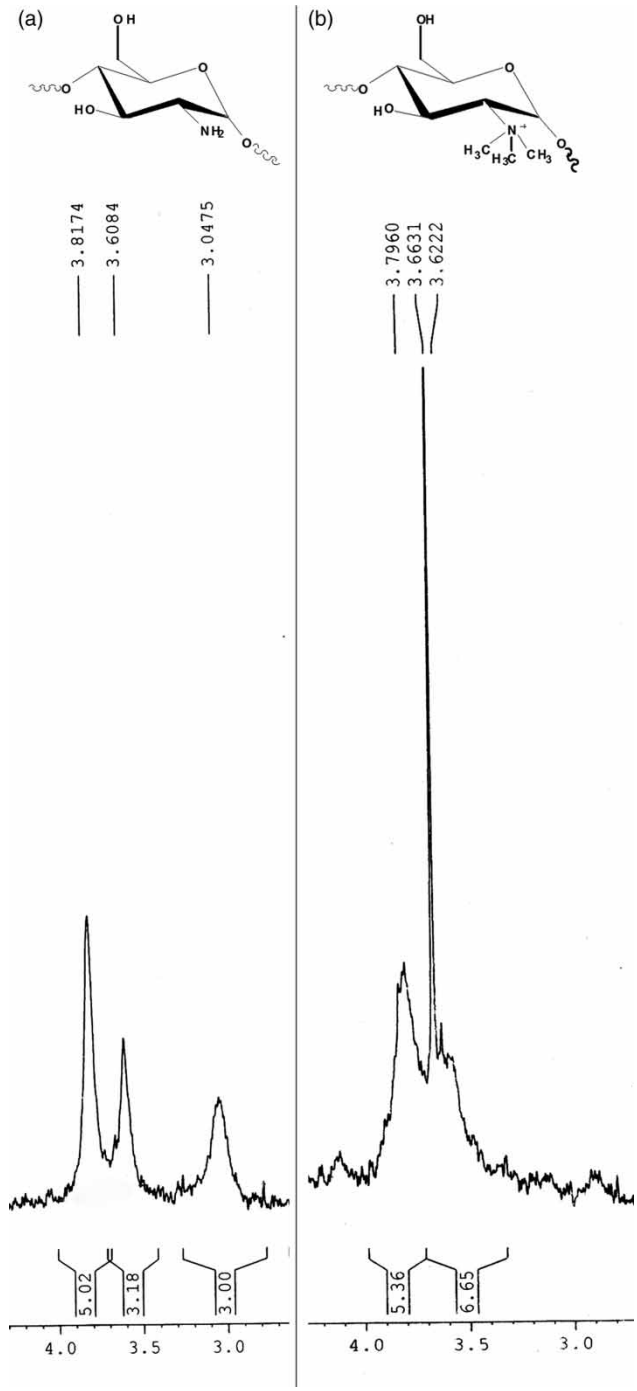

Figure 1 | (a) ^{1}H NMR spectra of the chitosan dissolved in D_2O/ CF_3COOH mixture. (b) ^{1}H NMR spectrum of the *N,N,N*-trimethylchitosan (TC) derivative dissolved in D_2O.

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N-methylchitosan was prepared by introducing a methyl group into the amine of chitosan that followed quaternization using methyl iodide which resulted in TC. The second stage of synthesis was based on direct alkylation of methyl amino group in the presence of sodium iodide and sodium hydroxide in *N*-methylpyrrolidinone as a solvent. The solvent *N*-methylpyrrolidinone, gave signals in ^{1}H NMR of the product in spite of repeated washings with acetone and water. Hence, crude quaternized product was dialyzed in deionized water for 3 days and then washed with benzene. Benzene being highly nonpolar did not dissolve the polar salt as compared with organic *N*-methylpyrrolidinone. Residual water and benzene were removed thereafter by lyophilization to obtain a pure product.

Flocculation efficiency

The flocculation efficiencies (FE) of TC at pH 4, 7 and 10 are illustrated in Figure 2. The flocculant dosage rising to the maximum FE value is considered as optimal dosage value. The results proved that TC was effective flocculating agent to kaolin suspension. The pH in a suspension determines the sign of surface charge of the particles and therefore influences FE of the flocculants. Similar trends in flocculation performance at all the measured pH was shown by the TC. Thus, TC can prove effective as a flocculant over a wide range of pH. The mechanism of the flocculation with TC may be explained by a combination of charge neutralization and polymer bridging. Electrostatic interactions are the main driving force in the flocculation of kaolin suspension, bridging also playing the major role.

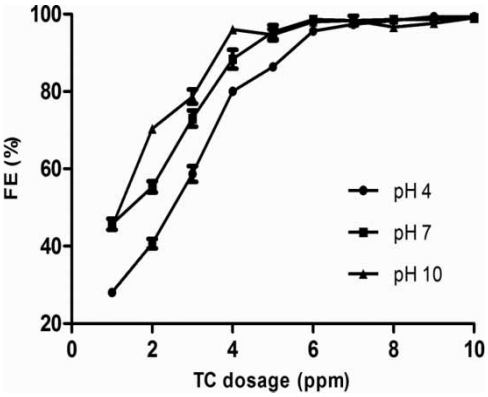

Figure 2 | Flocculation efficiencies (FE) of *N*,*N*,*N*-trimethylchitosan (TC) in kaolin suspension at different pH values.

Antibacterial activity

Results obtained by the agar disc diffusion assay of the quaternized biopolymer against the waterborne pathogens indicated that the inhibitory effect of chitosan after quaternization (in TC) was significantly ($p < 0.05$) higher than that of chitosan as evidenced by the inhibition zones (Figure 3). The order of zone of inhibition with TC were found to be *Yersinia* > *Aeromonas* > *E. coli* > *Listeria*.

Following quaternization, the chitosan became a water-soluble polyelectrolyte, with a permanent cationic charge density. The killing kinetics of chitosan and TC against the indicator bacterial strains are presented in Figure 4. The inhibitory effect of TC was higher than that of chitosan on different waterborne pathogens. These results were in agreement with the previous studies (Sajomsang *et al.* 2009) where the chitosan derivatives with the substituent having cationic charge, especially the ammonium salt, exhibited particularly high antibacterial activity. Inactivation of the indicator bacteria with the quaternized derivative could be effectively achieved with a low derivative dose of 2–5 ppm, within a contact time of 30 min at ambient temperature. Following treatment with TC, the cultures were plated on to respective selective medium and TAL (thin agar layer) plates (Wu *et al.* 2001). The TAL method resuscitates injured cells which are then identified onto their respective selective medium; the method is simple and allows enumeration of injured bacterial cells from the environment. Lack of bacterial growth on TAL plates indicated complete inactivation of the spiked pathogens (data not shown).

In order to elucidate the possible mechanism of the bactericidal action of TC, glucose, LDH and protein leakage from treated pathogens were studied. The treatment of chitosan (800 ppm) induced the leakage of glucose and LDH into extracellular media of *E. coli* cells (Tsai & Su 1999). The addition of maltose-chitosan derivative caused the cell leakage in *E. coli* O157:H7 and the glucose level increased continuously during the 12 h period (Yang *et al.* 2007). Several authors have proposed that the antimicrobial action of chitosan could be explained by a more direct disturbance

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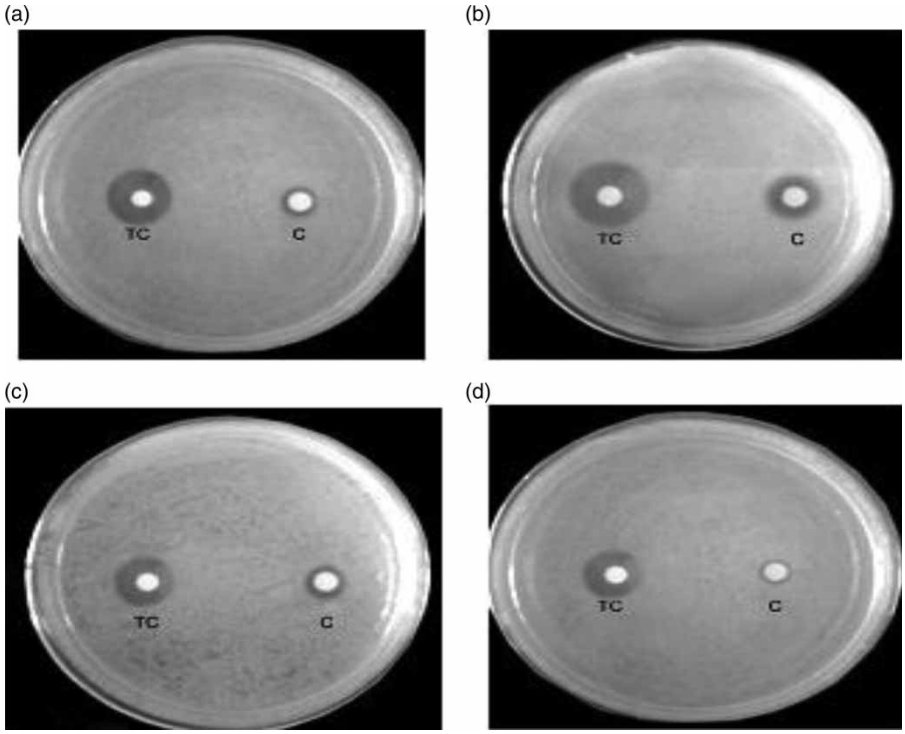

Figure 3 | Inactivation profile (zones of inhibition) of chitosan and *N,N,N*-trimethyl chitosan (TC) against (a) *Aeromonas hydrophila* ATCC 35654; (b) *Yersinia enterocolitica* ATCC 9610; (c) *Escherichia coli* O157:H7 ATCC35150; (d) *Listeria monocytogenes* ATCC 1911.

of membrane functions (Tsai & Su 1999). The reactive amino groups in chitosan could interact with a multitude of anionic groups on the surface of the cell to alter its permeability. This causes the leakage of intracellular components such as glucose, LDH and protein, resulting in a destabilized cell membrane beyond repair and subsequent cell death. For instance, Liu *et al.* (2004) showed that chitosan acetate solution increased the permeability of the outer and inner membranes of *E. coli*, and this damage was likely to be caused by the electrostatic interaction of NH_3^+ groups of chitosan acetate and phosphoryl groups of phospholipids of cell membranes. In the present study, treatment of pathogens with a concentration of 4.5 ppm of TC led to rapid leakage of proteins, glucose and LDH within a period of 3 h (Table 1), a minor increase in glucose, protein and LDH levels in the extracellular media was observed after 6 h (results not shown). No further increase in the levels of glucose, protein or LDH occurred thereafter.

The higher antibacterial activity of TC observed in the present study may be attributed to the permanent positive charges on the quaternized biopolymeric chain, as a consequence of the quaternization of the amino groups. The activity resulted from the interaction between the positively charged amino groups of the chitosan and negatively charged cell surface of Gram-negative bacteria. Quaternized chitosan acts on both Gram-positive and Gram-negative bacteria (Belalia *et al.* 2008). However, in the present study, a lower inhibitory effect was observed against Gram-positive bacterial strain in comparison to Gram-negative bacteria. Sajomsang *et al.* (2009) have reported the effect of DQ and molecular weight on bactericidal activity of trimethyl chitosan. TC could exhibit significant inhibitory effect against Gram-positive bacteria at a higher dose or concentration and at higher DQ. It has been reported that the target site of cationic disinfectants is the cytoplasmic membranes of the microbes (McDonnell & Russell 1999). The main constituents of the cytoplasmic membrane are membrane proteins and phospholipids. The phospholipids of bacteria are phosphoglycerides that have both a hydrophilic and a hydrophobic end (McDonnell & Russell 1999). Thus,

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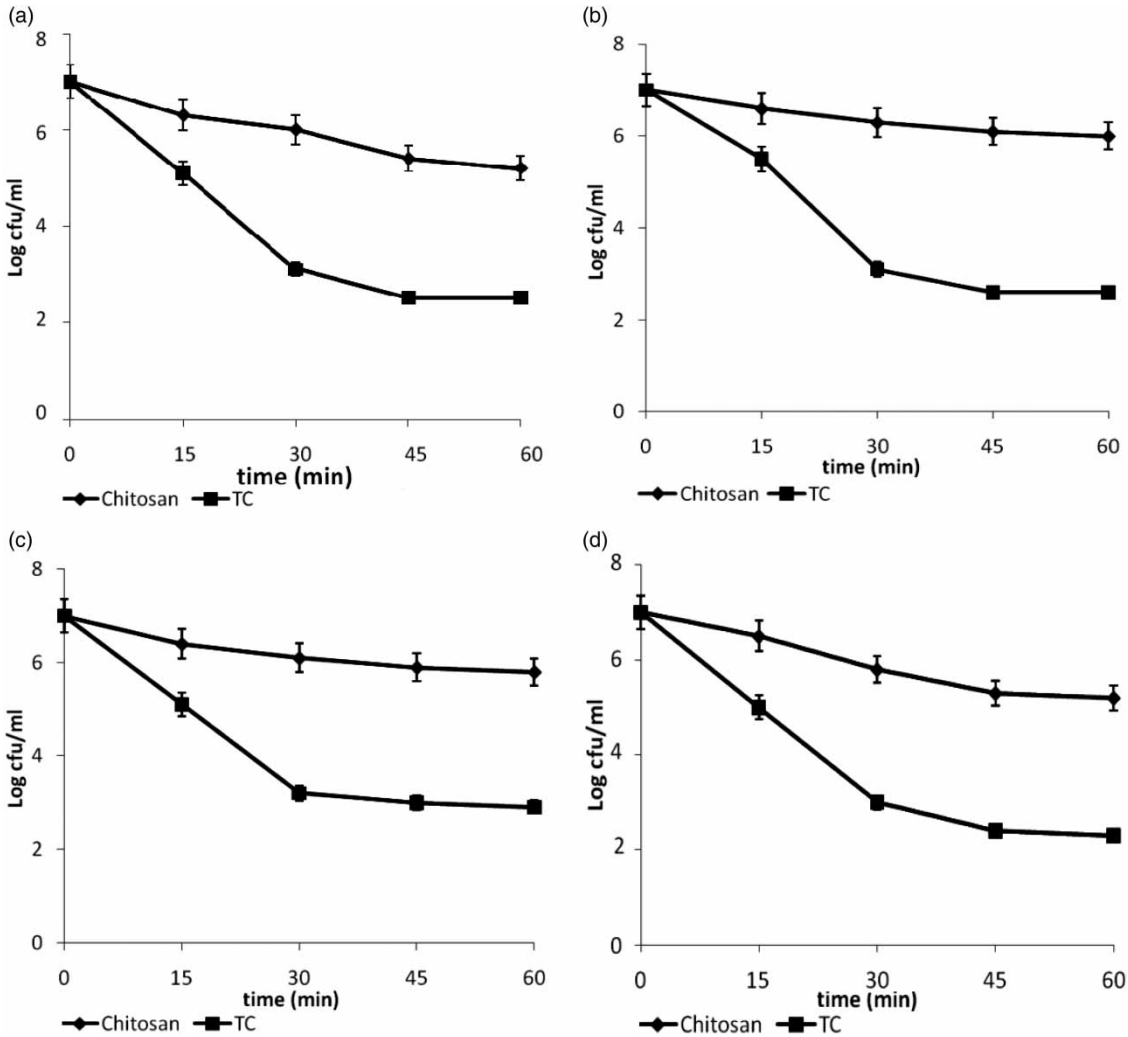

Figure 4 | Killing kinetics for chitosan (•) and *N,N,N*-trimethylchitosan (TC; ▪) against (a) *Aeromonas hydrophila* ATCC 35654 (2.5 ppm); (b) *Yersinia enterocolitica* ATCC 9610 (2 ppm); (c) *Listeria monocytogenes* ATCC 1911 (4 ppm); and (d) *Escherichia coli* O157:H7 ATCC35150 (3.5 ppm). Each value is expressed as mean ± standard deviation (SD) ($n = 3$).

Table 1 | Effect of water-soluble chitosan derivative (TC) (4.5 ppm) on glucose concentration, lactate dehydrogenase activity and protein level in the extracellular media of bacterial pathogens cultured at 37 °C for up to 3 h. Each value is expressed as mean ± standard deviation (SD) ($n = 3$)

	LDH activity (U/L)		Glucose (mg/dl)		Protein absorbance (280 nm)	
Bacterial strains	**0 h**	**3 h**	**0 h**	**3 h**	**0 h**	**3 h**
Y. enterocolitica	0	16.0 ± 0.80	0	0.49 ± 0.09	0	0.53 ± 0.02
A. hydrophila	0	15.37 ± 0.06	0	0.39 ± 0.06	0	0.50 ± 0.07
E. coli O157:H7	0	14.63 ± 0.08	0	0.35 ± 0.07	0	0.49 ± 0.09
L. monocytogenes	0	13.0 ± 0.60	0	0.33 ± 0.02	0	0.43 ± 0.03

quaternized chitosan derivatives with an alkyl group would be expected to strongly interact with cytoplasmic membranes due to the hydrophobic affinity between the introduced alkyl group and the phospholipids, leading to an enhanced bactericidal activity. Dose optimization experiments indicated a concentration of 2 ppm, sufficient to inactivate the bacterial strain of *Yersinia*; however, the dose was higher and extended for up to 4.5 ppm for

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completely inactivating *Listeria*, *Aeromonas* and *E. coli* O157:H7 over 30 min (data not shown).

Although chitosan is demonstrated to degrade easily and is not harmful to mammals, the derivate of chitosan (TC) was tested for toxicity/mutagenic activity using *Euglena gracilis* assay as described by Krizkova *et al.* (1996) where no mutagenicity was observed. Additionally toxicity studies carried out using Swiss albino mice did not indicate any toxic effect for up to a dose of 240 mg kg^{-1}. These results confirmed the safety of the chitosan derivative (unpublished results).

To the best of our knowledge, this is the first report where a low dosage of chemically modified chitosan exhibited both a high and wide inactivation spectrum against both Gram-negative and Gram-positive pathogens. The results suggest that inactivation of these bacterial pathogens using TC in water may be practically achievable. However, prior to actual application, the interactive effects of other waterborne contaminants with the chitosan derivative need to be established. The resistance to exposure of quaternary ammonium compounds to pathogens might arise (Li *et al.* 2009); however in this context it may be emphasized that the exposure time for pathogen inactivation is much less for the chitosan derivative. Therefore, resistance to TC may not arise. However, an attempt to investigate whether bacterial resistance arises from the chitosan derivative will be worthwhile in the future.

CONCLUSIONS

Chitosan was chemically modified to produce a quaternary ammonium salt using alkyl iodide. Quaternized chitosan was found to be an effective flocculant over a wide range of pH in the flocculation of kaolin suspension. TC exhibited high water solubility and enhanced bactericidal action against Gram-positive and Gram-negative pathogens as compared with chitosan. The bactericidal action resembled that of chitosan as evident from the rapid loss of intracellular glucose, LDH and proteins from treated cells. Mechanistically, an electrostatic interaction of non pH-dependent positive charge of the quaternized derivative with the negatively charged cell wall of pathogenic cells is suggested as a cause of bactericidal action. The quaternized chitosan with dual property of flocculant–disinfectant may serve as a potential alternative for water treatment.

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