Microbiocidal Activity of Chitosan-*N*-2-hydroxypropyl Trimethyl Ammonium Chloride

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ABSTRACT: Chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride (QTS) was prepared by reaction of chitosan with glycidyl trimethylammonium chloride, which was characterized by FTIR. QTS with different molecular weights (M_w 41.55 $\times$ 10^4, 9.02 $\times$ 10^4, 3.57 $\times$ 10^4, and 0.17 $\times$ 10^4) showed biocidal activity on *Staphylococcua aureus*, *Bacillus subtilis*, *Staphylococcua epidermidis*, and *Candida albicans*. QTS with high molecular weight had high biocidal activity on the gram-positive bacteria, and the biocidal effect of QTS decreased with decreasing molecular weight from 9.02 $\times$ 10^4 to 0.17 $\times$ 10^4. QTS with M_w 41.55 $\times$ 10^4 exhibits slightly lower biocidal activity on *Candida albicans* than other QTS samples. However, no remarkable biocidal activity of QTS was found on gram-negative bacteria *Escherichia coli* and *Pseudomonas aeruginosa* at the concentration up to 10 g L^{-1}. Existence of cationic surfactant, amphoteric surfactant, nonionic surfactant, Ca^{2+} and Mg^{2+} had no remarkable effect on microbiocidal activity of these QTS samples. © 2006 Wiley Periodicals, Inc. J Appl Polym Sci 103: 3851–3856, 2007

Key words: quaternized chitosan; molecular weight; microorganism; microbiocidal activity

INTRODUCTION

Chitosan is a natural nontoxic biopolymer, derived by deacetylation of chitin, a natural polysaccharide usually obtained from the exoskeletons of shellfish and insects. Chitosan is soluble in acidic solution and has found a wide variety of applications in pharmaceutics,[1] food, and cosmetics.[2] Antimicrobial activity is an important property of chitosan. The reported antimicrobial activity of chitosan was only shown in acidic medium because of its poor solubility above pH 6.5.[3] Thus, water-soluble chitosan derivatives that are soluble in both acid and basic physiologic circumstances might be good candidates for a polycationic biocide.

To increase the solubility, chitosan can be modified by the reaction with glycidyl trimethylammonium chloride. The quaternized chitosan has been reported to have a good moisture-retention capability,[4,5] and the work on its antimicrobial activity has been reported.[6]

In this article, chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride (QTS) was prepared. Six kinds of strains were tested to evaluate the microbiocidal activity of QTS as a natural disinfectant. The effect of molecular weight, concentration of QTS, pH of the medium, existence of surfactant, and existence of $Ca^{2+}$and Mg^{2+} in medium on the microbiocidal activity of QTS were also examined.

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EXPERIMENTAL

Materials and equipment

Chitosan samples (CS) were prepared in our laboratory, and their *N*-deacetylation is around 90%. Glycidyl trimethylammonium chloride (GTMAC) was prepared in our laboratory as described elsewhere.[7] Other chemicals were of analytical grade.

IR spectra taken with KBr pellets on a FTIR spectra were recorded with Nicolet380 spectrophotometer. A gel permeation chromatography (GPC) system was used for the characterization of weight–average molecular weight (M_w) of CS and QTS.[3]

Beef extract medium, consisting of 5 g beef extract, 5 g NaCl, 10 g tryptone, 15 g agar per liter, pH 7.0, was sterilized by autoclaving for 20 min at 120°C. Potato medium, consisting of 200 g potato, 20 g cane sugar, 15 g agar per liter, pH 7.0, was sterilized by autoclaving for 20 min at 120°C.

Staphylococcua aureus (*S. aureus*), *Bacillus subtilis* (*B. subtilis*), *Staphylococcua epidermidis* (*S. epidermidis*), *Escherichia coli* (*E. coli*), *Pseudomonas aeruginosa* (*P. aeruginosa*), and *Candida albicans* (*C. albicans*) were provided by Chinese Center of Type Culture Collection, Wuhan University, China. These strains were grown to the stationary phase in nutrient broth at 37°C for bacteria or at 28°C for *C. albicans*.

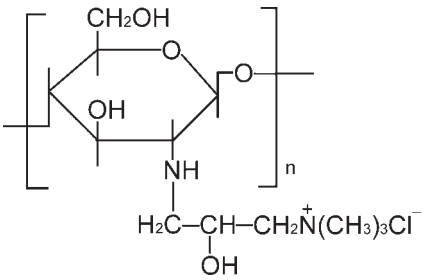

Figure 1 Structure of QTS.

Preparation of suspended microorganism solution[8]: The strains were separated into sterile saline (0.9%) at a concentration of 10^7 CFU/mL, respectively.

Synthesis of quaternized chitosan

Purified chitosan (4.0 g) was dispersed in 100 mL isopropyl alcohol, then GTMAC was added. The mixture was adjusted to pH 6 by adding aqueous HCl and stirred at given temperature for a given time.[9] The reaction product was filtered, concentrated, precipitated in acetone, and dried under vacuum at 40°C for 48 h to obtain the quaternized chitosan QTS.

Determination of the degree of substitution

The degree of substitution (DS) was determined by the method reported by Fan et al.[4]

Estimation of water solubility

The water solubility of the samples was estimated as follows. The weighed sample (0.2 g) was suspended in 10 mL distilled water at 25°C for 2 h with constant shaking. Soluble chitosan was removed by centrifugation. The precipitates were dried and weighted. The solubility of the sample in water was determined by the percent of dissolved sample.

The pH dependence of water solubility of the samples was evaluated using turbidity measurements. The sample (20 mg) was dissolved in 1% HAc (10 mL). With the stepwise addition of concentrated NaOH, the transmittance of the solution was recorded with a 722S spectrophotometer, using a quartz cell with an optical path length of 1 cm at 600 nm.

Microbiocidal activity of QTS

Effect of molecular weight of QTS

Microbiocidal activity of each sample was measured by colony count on incubated agar plates.[10] Two hundred microliters of suspended microorganism solution was added into 5 mL sterile saline solution containing 5 g L^{-1} QTS. The mixture without QTS was used as a control. Each sample was shaken and incubated at 25°C for 1 h. One hundred and fifty microliters of incubated mixtures were dispersed onto plates containing beef extract medium for bacteria or potato medium for fungi and incubated at 37°C for bacteria or 28°C for *C. albicans*. After incubation for 24–48 h, the colonies were counted to indicate the microbiocidal activity by the biocidal rate *R*. All experiments were carried out in triplicate, and average values are reported.

Effect of concentration of QTS

Two hundred microliters of suspended microorganism solution was separately added into 5 mL of sterile saline solution containing QTS of different concentration as test sample. The control was prepared using the same method except that no QTS was added. Then they were conducted according to the previous method.

Effect of pH

Tests were conducted in two sets: a test set with QTS and a control set without QTS. In the test set, 200 μL suspended microorganism solution was added into 5 mL of sterile saline solution containing 5 g L^{-1} QTS, and the mixture was adjusted to respective pH of 4–10 by HCl or NaOH. The control sets were prepared using the same method except that no QTS was added. Then they were conducted according to the previous method.

Effect of surfactant

Two hundred microliters of suspended microorganism solution was added into 5 mL of sterile saline solution containing 5 g L^{-1} QTS and surfactant 1% (w/v) as test sample. Then they were conducted according to the previous method.

TABLE I
The Preparation and Properties of QTS

CS		Preparation			QTS		
Code	M_w (10^4)	*n*(CS):*n*(GTMAC)	*T* (°C)	*t* (h)	Code	DS (%)	M_w (10^4)
CS1	50.37	1 : 4	85	14	QTS1	86.5	41.55
CS2	11.59	1 : 4	85	14	QTS2	88.2	9.02
CS3	4.36	1 : 4	85	14	QTS3	87.1	3.57
CS4	0.19	1 : 5	40	24	QTS4	86.3	0.17

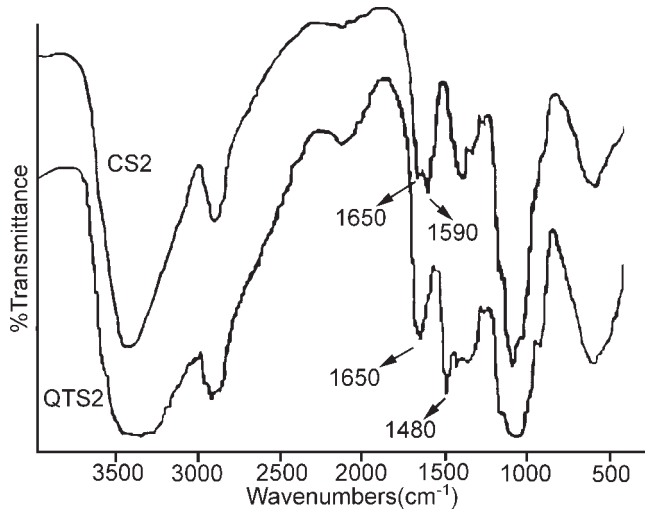

Figure 2 IR spectra of CS2 and QTS2.

Effect of Ca^{2+} and Mg^{2+} in water

Two hundred microliters of suspended microorganism solution was added into 5 mL $MgCl_2$ or $CaCl_2$ sterile saline solution containing 5 g L^{-1} QTS, respectively. Then they were conducted according to the previous method.

RESULTS AND DISCUSSION

Preparation and characterization of quaternized chitosan

The chemical modification of chitosan with GTMAC in the presence of water results in N-substitution (Fig. 1). The preparation and properties of QTS are shown in Table I.

The IR spectrum of the dried quaternized chitosan is shown in Figure 2. The absorption band at 1650 cm^{-1} in CS2 is referenced as amide I bands, and the absorption band at 1590 cm^{-1} is ascribed to N—H bending mode in the primary amine. The absorption band of $—NH_2$ in quaternized chitosan almost disappeared, suggesting that the N-alkylation in chitosan occurred. Compared with chitosan, the quaternized chitosan QTS2 produced a band at 1480 cm^{-1}, which was attributed to the methyl groups of ammonium.[11]

Water solubility of quaternized chitosan

The chitosans CS1, CS2, and CS3 were water-insoluble. After quaternization, the products QTS1, QTS2, and QTS3 became water-soluble polyelectrolyte with high charge density, so the liquor of 2% QTS was clear. CS4 with low molecular weight was water-soluble, and its quaternized product QTS4 was also soluble. Figure 3 shows that 0.2% QTS was soluble in the range of pH 2–13.

Microbiocidal activity of QTS

Effect of molecular weight of QTS

The effect of molecular weight on the microbiocidal activity of QTS is shown in Figure 4. At 5 g L^{-1}, QTS1 with M_w 41.55 $\times$ 10^4 and QTS2 with M_w 9.02 $\times$ 10^4 exhibited high bactericidal effect on *S. aureus*, *B. subtilis*, and *S. epidermidis*. The biocidal activity of QTS on gram-positive bacteria seemed to decrease with decreasing molecular weight from 9.02 $\times$ 10^4 to 0.17 $\times$ 10^4.

QTS 1 exhibits slightly lower biocidal activity on *C. albicans* than QTS2, QTS3, and QTS4.

Effect of concentration of QTS

Table II shows the microbiocidal effect of quaternized chitosan at different concentrations. At 0.63 g L^{-1} and

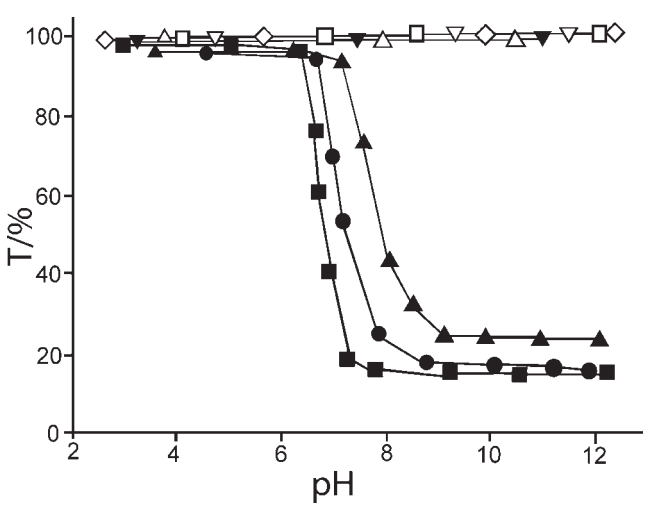

Figure 3 pH dependence of water solubility of CS and QTS. ■ CS1; ● CS2; ▲ CS3; ▼ CS4; ◇ QTS1; △ QTS2; ▽ QTS3; □ QTS4.

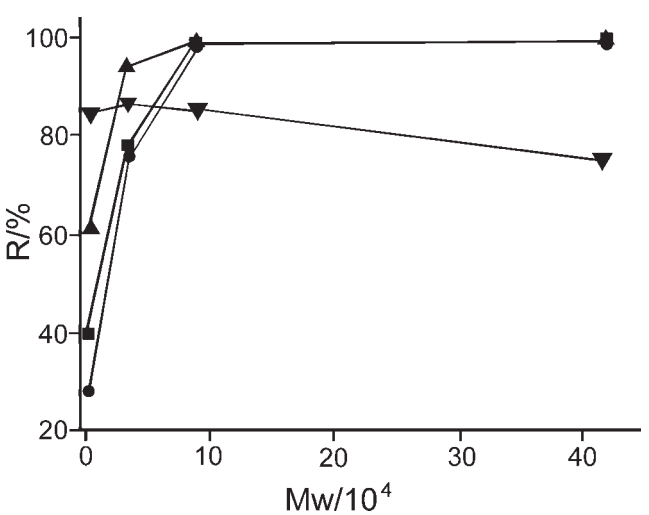

Figure 4 Effect of molecular weight of QTS on its microbiocidal activity. ■ *S. aureus*; ● *S. epidermidis*; ▲ *B.subtilis*; ▼ *C. albicans*.

TABLE II
Microbiocidal Activity (%) of QTS at Different Concentrations

Sample	Strain	Concentration (g L^{-1})					
		10.00	5.00	2.50	1.25	0.63	0.31
QTS1	*S. aureus*	99.9	99.9	99.9	99.9	99.8	77.6
	S. epidermidis	100	100	100	100	100	98.7
	B. subtilis	99.9	99.8	93.1	87.4	48.5	23.7
	C. albicans	86.8	75.2	43.3	20	0	0
	E. coli	0	0	0	0	0	0
	P. aeruginosa	0	0	0	0	0	0
QTS2	*S. aureus*	99.9	99.9	99.9	99.5	97.3	73.4
	S. epidermidis	100	100	100	100	100	96.5
	B. subtilis	99.9	99.6	87.7	74.5	31.3	0
	C. albicans	88.7	85.5	60.1	51.1	0	0
	E. coli	0	0	0	0	0	0
	P. aeruginosa	0	0	0	0	0	0
QTS4	*S. aureus*	50	40.7	19.5	0	0	0
	S. epidermidis	31.3	30.0	15	0	0	0
	B. subtilis	82.1	61.6	35.6	12.7	0	0
	C. albicans	85.5	83.8	75.2	65.4	33.9	0
	E. coli	0	0	0	0	0	0
	P. aeruginosa	0	0	0	0	0	0

above, QTS1 and QTS2 exhibited strong biocidal activity on *S. aureus* and *S. epidermidis*, but QTS4 had weaker biocidal activity on them. At 5 g L^{-1} and above, QTS1, QTS2, and QTS4 all had relatively good biocidal activity on *C. albicans*.

QTS with molecular weights from 0.17×10^4 to 41.55×10^4 exhibited no biocidal effect on *E. coli* and *P. aeruginosa* at concentrations up to 10 mg/mL.

Effect of pH

Figure 5 shows that QTS3 had high biocidal activity on *S. aureus* at different pH. Figure 6 shows that QTS3 also had high biocidal activity on *C. albicans* at different pH. The weak basic condition slightly increased the microbiocidal activity of QTS on *S. aureus* and *C. albicans*. However, Figure 7 shows that QTS3 had no biocidal activity on *E. coli* in both acidic and basic conditions. It should be mentioned that both acidic and basic media were harmful to the growth of *S. aureus* and *E. coli*, and weak acidic condition was favorable for the growth of *C. albicans*.

Jia et al. found that the antibacterial activity of quaternized chitosan, such as *N*,*N*,*N*-trimethyl chitosan, *N*,*N*-propyl-*N*,*N*-dimethyl chitosan, and *N*-furfuryl-*N*, *N*- dimethyl chitosan, in acetic acid medium is stronger than that in neutral medium.[12] Qin et al. suggested that the antimicrobial activity of chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride was stronger in weak basic conditions than in weak acidic conditions.[6]

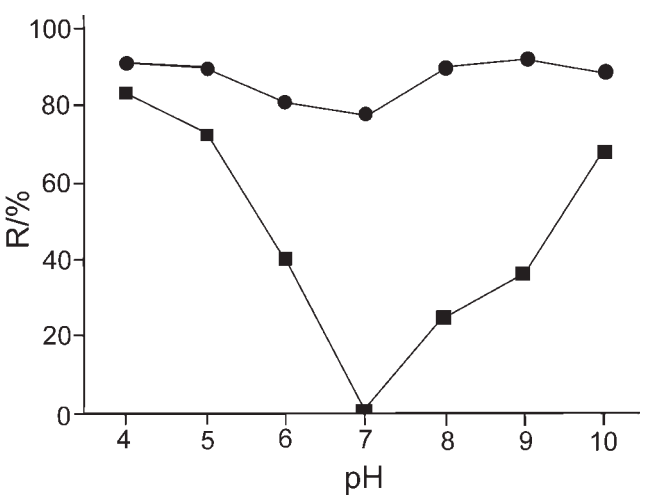

Figure 5 Effect of pH on microbiocidal activity of QTS against *S. aureus*. ● 5 g L^{-1} QTS3; ■ without QTS.

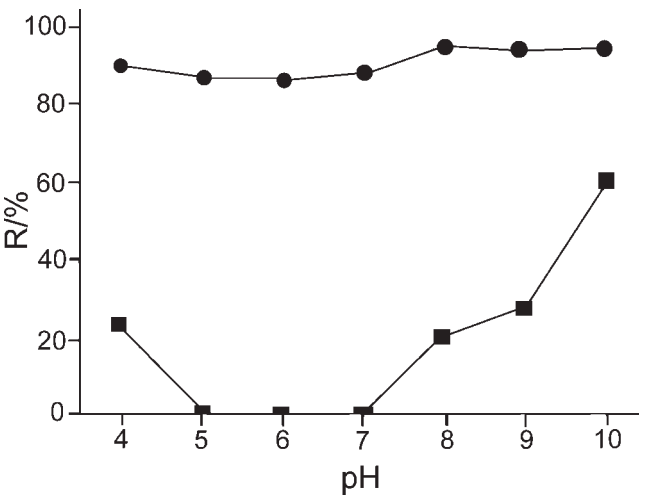

Figure 6 Effect of pH on microbiocidal activity of QTS against *C. albicans*. ● 5 g L^{-1} QTS3; ■ without QTS.

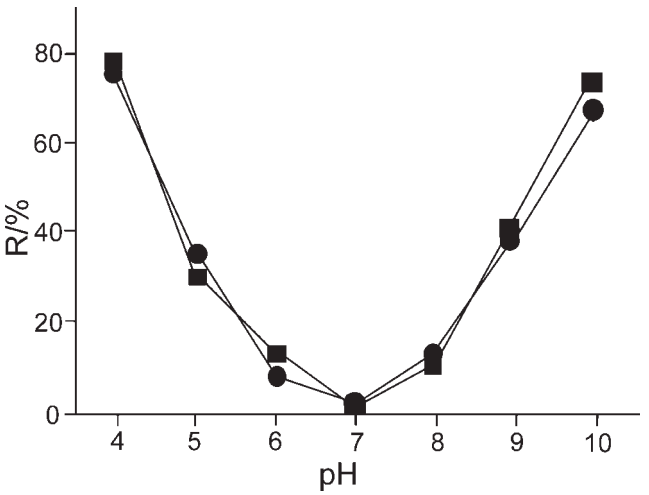

Figure 7 Effect of pH on microbiocidal activity of QTS against *E. coli*. ● 5 g L^{-1} QTS3; ■ without QTS.

Effect of surfactant

Sodium dodecyl benzene sulfonate (LAS, anionic surfactant), cetyl-trimethyl ammonium bromide (CTAB, cationic surfactant), betaine anhydrous (BA, amphotericisurfactant), and tritonX-100 (TX, nonionic surfactant) were used to test the effect of surfactant on the microbiocidal activity of QTS. The precipitate was formed when LAS reached a concentration of 1% in the solution of 5 g L^{-1} QTS3.

Table III suggested that CTAB, BA, and TX had no remarkable influence on biocidal activity of QTS3 against *S. aureus*, *E. coli*, and *C. albicans*.

Effect of Ca^{2+} and Mg^{2+} in water

There are abundant Ca^{2+} and Mg^{2+} in the natural water. Therefore, it is important to estimate the effect of Ca^{2+} and Mg^{2+} on microbiocidal activity of QTS. Table IV shows the effect of Ca^{2+} and Mg^{2+} on the biocidal activity of QTS against *S. aureus*, *E. coli*, and *C. albicans*, which revealed that Ca^{2+} and Mg^{2+} (at concentration up to 20 mmol L^{-1}) caused no remarkable change to the biocidal activity of QTS in any of the three organisms studied.

Discussion on the mode of microbiocidal activities of QTS

The antibacterial activity of chitosan has been studied. Three kinds of mechanisms were given. First, polycationic chitosan interacts with the negatively charged cell surface, which induces the metamorphosis and outlet of cell.[13,14] Second, chitosan can combine with necessary metal elements or nutrition for microorganisms and thereby inhibits their growth.[15] Third, binding of chitosan with DNA and inhibition of mRNA synthesis occurs through chitosan penetration toward the nuclei of the microorganisms and interference with the synthesis of mRNA and proteins.[16]

After quaternization, the chitosan became a water-soluble polyelectrolyte with a high charge density. Some antibacterial activity has been described with quaternized chitosan.[17–19] The target site of the cationic antibacterial agents is the negatively charged cell surface of bacteria.[20] Polycationic biocides can interact and form polyelectrolyte complexes with acidic polymers produced at the bacterial cell surface.[21] These research results suggested that microbiocidal activity of QTS might also have relationship with its molecular weight. QTS with high molecular weight are able to form polymer membrane on the cell surface of microorganisms to inhibit the exchange of materials and energy. QTS with low molecular weights are able to pass the outer membrane of cell surface of microorganisms, and absorb the cytoplasms with anion to disturb the growing of microorganisms. There may be a different optimum scope of molecular weights for microbiocidal activity of QTS on different microorganisms.

These research results also revealed that the microbiocidal activity of QTS was stronger in weak basic conditions than in weak acidic conditions, which coincided well with the regulation of general quaternary ammonium disinfectants.[22] In weak basic conditions, the microbial proteins might carry more negative charge, which led to the enhanced interactions between polycation of QTS and the cytoplasmic membrane of the bacterial cells.[6]

Effect of QTS on *E. coli* and *P. aeruginosa* was not as effective as that on *S. aureus*, *B. subtilis*, *S. epidermidis*, and *C. albicans*, which might be attributed to their different cell walls. *S. aureus*, *B. subtilis*, and *S. epidermidis* are typical gram-positive bacteria, the cell wall of which is fully composed of peptide polyglycogen. *C. albicans* is yeast, the cell wall of which is mainly made up of polysaccharide. *E. coli* and *P. aeruginosa* are typical gram-negative bacterium, the cell wall of which is made up of a thin layer of peptide polyglycogen and an outer lipopolysaccharide (LPS) layer. The outer LPS layer of *E. coli* and *P. aeruginosa* is potential barrier against foreign molecules with high molecular weight. Moreover, QTS had strong hydrophilicity but weak lipophilicity. Therefore, it was difficult for QTS to pass through the cell wall of *E. coli* and *P. aeruginosa*.

TABLE III
Effect of Surfactant on the Microbiocidal Activity (%) of QTS3

Strain	QTS3	QTS3 + CTAB	QTS3 + BA	QTS3 + TX
S. aureus	78.4	78.6	78.6	77.9
E. coli	0	0	0	0
C. albicans	87.4	87.5	87.7	87.2

TABLE IV
Effect of Ca^{2+} and Mg^{2+} on the Microbiocidal Activity (%) of 5 g L^{-1} QTS3

	Ca^{2+} (mmol L^{-1})				Mg^{2+} (mmol L^{-1})			
Strain	20	10	5	0	20	10	5	0
S. aureus	78.5	76.7	78.6	78.4	78.7	79.5	78.2	78.4
E. coli	0	0	0	0	0	0	0	0
C. albicans	87.8	86.7	85.5	87.4	86.1	87.9	87.1	87.4

CONCLUSIONS

Chitosan-*N*-2-hydroxypropyl trimethyl ammonium chloride with molecular weights 0.17×10^4 to 41.55×10^4 was water-soluble. The QTS showed microbiocidal activity against *S. aureus*, *B. subtilis*, *S. epidermidis*, and *C. albicans*. The microbiocidal activity of QTS against *E. coli* and *P. aeruginosa* was not effective. The microbiocidal activity of QTS was dependent on its molecular weight. The microbiocidal activity of QTS on these gram-positive bacteria seemed to decrease with decreasing molecular weight from 9.02×10^4 to 0.17×10^4. QTS with M_w 41.55×10^4 exhibits slightly lower microbiocidal activity on *C. albicans* than other QTS samples. The microbiocidal activity of QTS was slightly stronger in alkaline condition than in weak acidic condition. Existence of cationic surfactant, amphoteric surfactant, nonionic surfactant, and $Ca^{2+}$and Mg^{2+} in water had no remarkable effect on microbiocidal activity of QTS, but anion surfactant could not cooperate with QTS.

The quaternized chitosan with good water-solubility and moisture-retention capacity has promising applications in some advanced cosmetics and external disinfection.

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