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Synthesis of chitosan derivatives with quaternary ammonium salt and their antibacterial activity

Chun Ho Kim, Jang Won Choi, Heung Jae Chun*, Kyu Suk Choi**

Department of Industrial Chemistry, College of Engineering, Hanyang University, Seoul 133-791, Korea

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Summary

N-alkyl chitosan derivatives were prepared by introducing alkyl groups into the amine groups of chitosan via Schiff's base intermediates. Quaternization of the *N*-alkyl chitosan derivatives were carried out using methyl iodide to produce water soluble cationic polyelectrolytes, novel chitosan derivatives with quaternary ammonium salt. Their antibacterial activities against *S.aureus* were explored by the viable cell counting method in acetate buffer(pH 6.0). The antibacterial activities of the chitosan derivatives with quaternary ammonium salt increased with increase in the chain length of the alkyl substituent, and this increased activity could be ascribed to the contribution of the increased hydrophobic properties of the derivatives.

Introduction

Chitin is one of the most abundant organic materials easily obtained from natural sources, e.g., the shells of crustaceans(lobsters, shrimps, crabs, and etc.) or the broth from the industrial fungal processes(1,2). Although chitin is naturally abundant, it has a limited application because of its poor solubility and reactivity. The simple chemical modification of chitin, however, enables it soluble to the specific solvents. Chitosan, the fully or partially deacetylated chitin, is soluble to acetic acid and other organic solvents. Due to its good solubility, chitosan, as the modified biopolymer, has the wide scale of the application field(1-5). Interestingly, some antibacterial and antifungal activities have been described with chitosan and modified chitosan derivatives(6-8). In polycationic biocides, generally, it is reasonably assumed(9,10) that the charge density of the polyelectrolyte increases with increase in the molecular weight of its single coil, which leads to the enhanced adsorption of polycations onto the negatively charged cell surface. This is also favorable for the binding of the polycation to the cytoplasmic membrane of the bacterial cells. Therefore, polycationic biocides including chitosan have several advantages over other types of disinfectants; that is, they possess higher antibacterial activity, broader spectra of activity, higher killing rate and lower toxicity toward mammalian cells(9,11).

Chitosan, however, shows its antibacterial activity only in acidic area because of its poor solubility above pH6.5. Thus, water soluble chitosan derivatives soluble to both acidic and basic physiologic circumstances might be a good candidate as the polycationic biocide.

In previous article(12), it was demonstrated that water soluble chitin derivatives with some pendent amino groups and their deacetylation product have stronger antibacterial activity than chitin itself, which the activity was strongly dependent upon the molecular weight and the cationic charge of the substituent, especially ammonium salt.

* *Present address:* Research Institute of Industrial Science, Hanyang University, Seoul 133-791, Korea

** Corresponding author

In this article, we prepared the series of the water soluble chitosan derivatives with quaternary ammonium salts, in which the number of the methylene units in the alkyl spacers was varied, and examined the effect of the hydrophobic chains introduced to the amine groups of the chitosan on the antibacterial activity against *S.aureus*. The viable cell counting method was employed to determine the antibacterial activities of the prepared samples.

Experimental part

Materials

Formaldehyde, n-butyraldehyde, n-octyraldehyde, n-dodecyraldehyde, NaBH4 and *N*-Methyl-2-pyrrolidone(NMP) were purchased from Aldrich Chemical Co. Inc. CH3I and NaI were purchased from Janssen Chemical Co. Butyldimethylvinylbenzyl ammonium chloride was prepared by the procedure as described in the literature(19): Anal. Calcd for [$C_{15}H_{24}NCl \cdot 2.0H_2O$]; C, 62.16; H, 9.74; N, 4.83. Found: C, 62.70;H, 9.99; N, 4.84.

In order to obtain highly deacetylated chitosan, chitosan purchased from Tokyo Co. was treated for 1h in 47% NaOH solution at 110°C, and the alkali treatment was repeated three times by the method of Mima et al.(13). Table 1 shows the deacetylation degrees and the molecular weights(14) of chitosan and highly deacetylated chitosan, used as the starting material. The degrees of deacetylation of chitosan and highly deacetylated chitosan were calculated by the following analysis data: Anal. Calcd for [$(C_6H_{11}NO_4)_{0.77}(C_8H_{13}NO_5)_{0.23} \cdot 0.70H_2O]_n$: C,42.30; H,7.12; N,7.64. Found: C,42.07; H,6.54; N,7.64 and Calcd for [$(C_6H_{11}NO_4)_{0.98}(C_8H_{13}NO_5)_{0.02} \cdot 0.57H_2O]_n$: C,42.12; H,7.08; N,8.14. Found: C,42.06; H,7.47; N,8.16, respectively.

Characterization

Structural changes in chitosan and its derivatives due to *N*-alkylation were determined by the Nicolet 5DX FT-IR spectrophotometer. ^{13}C NMR analysis were conducted using Varian Unity 300 in D_2O (in CD_3COOD for chitosan) at concentration of 50mg/ml, with a complete proton decoupling, a spectral window of 16.5kHz, and a pulse width of 10μs.

Determination of degree of substitution

Elemental analysis was performed using a Foss Heraeus Analysentechnik GmbH. Titrations of the solution of chitosan and its derivatives(0.5g) in 0.3N HCl were carried out with 0.1N NaOH, with the aid of a Corning pH meter 220. The degree of the quaternization of the chitosan derivatives were determined by the potentiometry(15). Potentiometric titration of the halide form was carried out with the aq. silver nitrate, using a calomel electrode as the reference, and a silver electrode for the measurement.

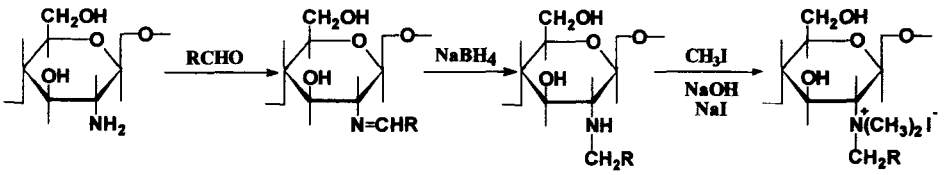
Synthesis of N-alkyl chitosan derivatives

The chitosan solution of 1% w/v was prepared by dissolving 7g of chitosan, corresponding to 42mmol of amine, into 1% aq. acetic acid. Various concentrations of the aq. aldehyde solutions, listed in Table 2, were added to the chitosan solution at room temperature. After 30min of stir-

Table 1. Properties of chitosan and highly deacetylated chitosan.

Samples	Degree of Deacetylation		[η] (cm^3/g)	Mv
	Elemental analysis	Titration		
Chitosan	0.77	0.76	1,082	1,630,000
Highly deacetylated chitosan	0.98	0.96	510	725,000

Scheme 1: Preparation route to *N*-alkyl chitosan derivatives.



ring, the pH of the solution was adjusted to 4.5. To this solution, 10% aq. solution of NaBH4 (1.5-fold excess to added aldehyde) was added, and the solution stirred for 1h. The precipitants of the *N*-alkyl chitosan derivatives were obtained by adjusting the pH of the solution to 10. These precipitants were washed with the distilled water to neutrality, and the unreacted aldehyde and the inorganic products were soxhlet-extracted with ethanol and diethyl ether for 2-7days, respectively. The resulting *N*-alkyl chitosan derivatives were filtered out and dried for 48h under vacuum at 40°C.

Synthesis of quaternized N-alkyl chitosan derivatives

Each 5g of *N*-alkyl chitosan derivatives were dispersed in 250ml of NMP during 12h at room temperature. To each dispersion, 1.4M NaOH and CH3I (2 and 15-fold excess to amine of chitosan, respectively) were added, NaI were added to adjust the concentration in the reaction medium to 0.2M. Each reaction was carried out with stirring for 12h at 50°C. The solution was collected by precipitation with acetone, which were dried to obtain the quaternized *N*-alkyl chitosan derivatives.

Antibacterial assessment

Antibacterial activities of the chitosan derivatives against *Staphylococcus aureus* (ATCC 6538P) were evaluated by using the viable cell counting method as described in the following. A loopful of each culture was spread to give the single colonies on the nutrient agar (agar 15g, bacto-peptone 10g, beef extract 3g, NaCl 3g in distilled water 1000ml; pH 7.0) and incubated at 37°C for 24h. A representative colony was picked off with a wire loop and placed in a nutrient broth (bacto-peptone 10g, beef extract 3g, NaCl 3g in distilled water 1000ml; pH 7.0), which was then incubated at 37°C overnight. By appropriately diluting with sterilized distilled water, each culture contained about 10^7cell/ml was prepared which was used for antibacterial test. Chitosan and its derivatives were dissolved in 2M sodium acetate-2M acetic acid buffer(pH 6.0). Exposure of the bacterial cells to the antibacterial agent started when the bacterial culture of 1.0 ml

Table 2. Degree of substitution of chitosan derivatives from elemental analysis.

Samples		Degree of *N*-alkylation		Degree of quaternization	
(Mol. of aldehyde/ glucosamine unit)		C/N ratio	Titration	C/N ratio	Potentiometry
N-trimethyl chitosan	(5-fold excess)	-	-	0.63	0.58
N-butyl chitosan	(5-fold excess)	0.81	0.77	0.58	0.56
N,N-dibutyl chitosan	(11-fold excess)	0.50	0.48	0.28	0.25
N-octyl chitosan	(15-fold excess)	0.90	0.86	0.24	0.20
N-dodecyl chitosan	(15-fold excess)	0.90	0.85	0.20	0.18

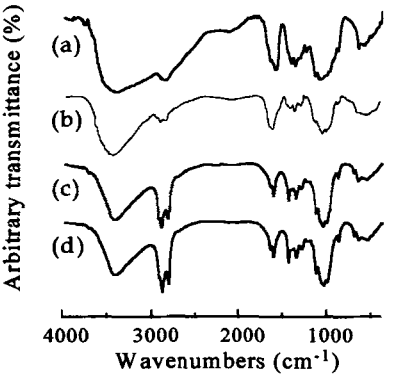


Figure 1. FT-IR spectra of (a) chitosan, (b) *N*-butyl chitosan, (c) *N*-octyl chitosan and (d) *N*-dodecyl chitosan derivatives.

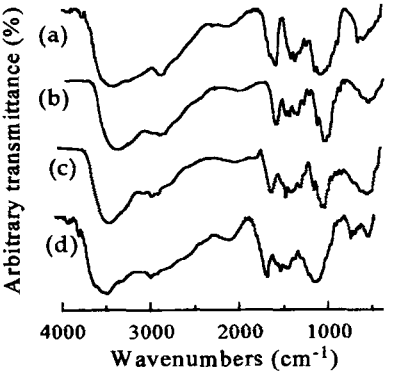


Figure 2. FT-IR spectra of (a) chitosan, (b) *N N,N*-trimethyl chitosan, (c) *N*-butyl-*N,N*-dimethyl chitosan and (d) *N*-dodecyl-*N,N*-dimethyl chitosan derivatives.

was added to the antibacterial agent solution of 9.0ml which was preequilibrated at 37°C. Concurrently, 1.0ml of the same culture was added to 9.0ml of buffer solution, and the initial cell concentration was enumerated by the spread plate method. At various exposure times, 0.2ml portions were removed and quickly spread on the nutrient agar. After inoculation, the plates were incubated at 37°C for 24h, and the colonies were counted. The counting was done in triplicate each time(12,16).

Results and discussion

Synthesis of quaternized N-alkyl chitosan derivatives

Alkyl groups were easily introduced into the amine of chitosan via Schiff's base intermediates. The resulting *N*-alkyl chitosan derivatives(Scheme 1) were quaternized using methyl iodide. Table 2 lists the degree of substitution of the *N*-alkyl and the quaternized *N*-alkyl chitosan derivatives. Figures 1 and 2 show the FT-IR spectra of chitosan and its *N*-alkyl derivatives. There

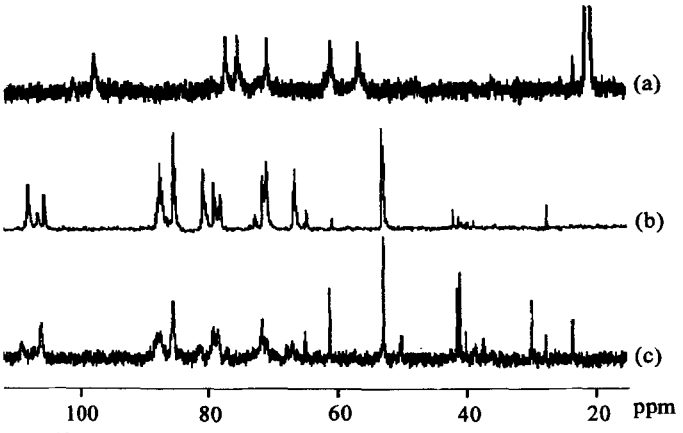


Figure 3. ^{13}C NMR spectra of (a) chitosan, (b) *N,N,N*-trimethyl chitosan and (c) *N,N*-dibutyl-*N*-methyl chitosan.

exist three characteristic peaks of chitosan at 3455, 1093 and 661cm^{-1}(17). As the modification of chitosan with alkyl chain was increased, the intensities of the peaks at 2940 and 1450cm^{-1} due to the presence of methylene groups were also increased. The intensities of two peaks are further increased with increase in the number of methylene groups. Although marked differences were not observed from the FT-IR spectra for quaternized chitosan derivative, the intensities of the peaks around 1460 and 705cm^{-1} due to the presence of methyl group were increased with the increase in the chain length.

^{13}C NMR spectra of chitosan and *N*-alkyl derivatives are shown in Figure 3. Compared with chitosan(Fig. 3(a)), *N,N,N*-trimethyl chitosan derivative(Fig. 3(b)) shows chemical shift at 53.6ppm due to the existence of the methyl groups of the quaternized ammonium salt. This is in good agreement with the reported data by Domard *et al.*(18). For *N,N*-dibutyl-*N*-methyl chitosan derivative(Fig. 3(c)), peak at 72.1ppm is assigned to C-6 carbon of chitosan, 61.6 and 50.8ppm can be attributed to the C-6 residue(C-6') of the carbohydrate unit and to the secondary carbon in α-position of the quaternized amino group of the carbohydrate unit, respectively.

Antibacterial activities of chitosan derivatives

The cationic antibacterial agents have been widely used particularly for the external disinfection. The target site of the cationic biocides is the cell envelope of bacteria(9). Polycationic biocides have been shown to possess higher activity against bacteria(10,11,16). These polymeric biocides could be powerful candidates for polymeric drugs with a high activity, which can be ascribed to their characteristic nature that carries the high local density of the active groups in the vicinity of the polymer chains(11).

Figure 4 shows plots of log(survivors) versus exposure time for the chitosan derivatives against *S.aureus* measured by the viable cell counting method. About 10^{6}cells/ml of the strain were exposed to 500, 200 and 100ppm of chitosan derivatives in acetate buffer mentioned above, respectively. According to Figure 4(a), at concentration of 500ppm, 100% of *S.aureus* was killed within 120min of contact. However, *N,N*-dibutyl-*N*-methyl chitosan(Figure 4(b)) showed the capability to kill all the bacterial cells within 120min at concentration of 100ppm, one fifth of the concentration of *N,N,N*-trimethyl chitosan. At concentration of 200 and 500ppm, all the bacterial cells were killed within 60min and 30min of contact, respectively. These results indicate that the antibacterial activity against *S.aureus* of the quaternized chitosan derivatives having different methylene spacer between the active groups is strongly affected by the space

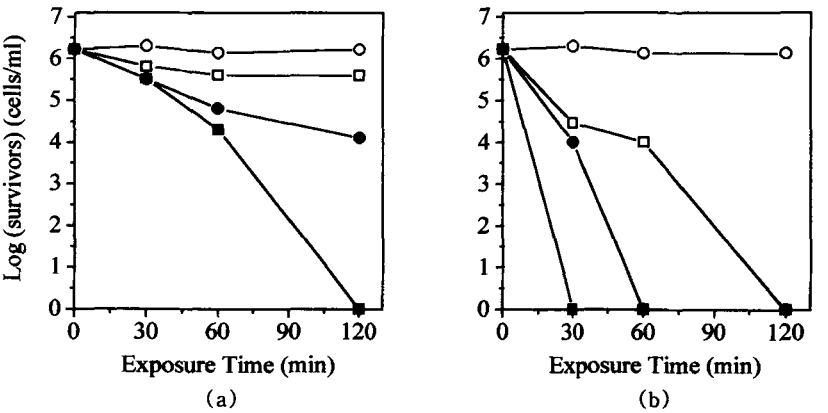


Figure 4. Plots of log(survivors) versus exposure time for (a) chitosan, (b) *N,N,N*-trimethyl chitosan and (c) *N,N*-dibutyl-*N*-methyl chitosan against *S.aureus*. Concentrations: (○) blank; (□) 100ppm; (●) 200ppm; (■) 500ppm.

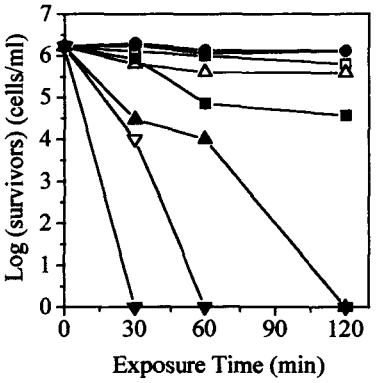


Figure 5. Plots of log(survivors) versus exposure time for chitosan and quaternized *N*-alkyl chitosan derivatives at 100 ppm concentration on acetate buffer against *S. aureus*: (○)blank; (●)butyldimethylvinylbenzyl ammonium chloride; (□)chitosan; (■)*N,N*-dibutyl chitosan; (△)*N,N,N*-trimethyl chitosan; (▲)*N,N*-dibutyl-*N*-methyl chitosan; (▽)*N*-octyl-*N,N*-dimethyl chitosan; (▼)*N*-dodecyl-*N,N*-dimethyl chitosan.

length: that is, their antibacterial activity increases with increase in the space length. The dependence of the alkyl chain length on the antibacterial activity against *S.aureus* was studied for the quaternized chitosan derivatives with a long alkyl chain. Figure 5 shows plots of log(survivors) versus exposure time for chitosan and its derivatives against *S.aureus*. About 10^6cells/ml of bacterial cells were exposed to concentration of 100ppm of antibacterial agents in acetate buffer. The antibacterial activity was found to be increased with increase in the quaternary ammonium salts moiety and with increase in the chain length of alkyl groups in the order of butyldimethylvinylbenzyl ammonium chloride<chitosan<*N,N*-dibutyl chitosan<*N,N*-dibutyl-*N*-methyl chitosan<*N*-octyl-*N,N*-dimethyl chitosan<*N*-dodecyl-*N,N*-dimethyl chitosan. This is in good agreement with previous studies on diethylaminoethyl chitin derivatives(12) which indicate that the derivatives with cationic charge of the substituent, especially ammonium salt, exhibit particularly high activity. These results clearly demonstrate that the alkyl chain length and cationic charge of the substituent strongly affects the antibacterial activity of the chitosan derivatives. Butyldimethylvinylbenzyl ammonium chloride had no activity against *S. aureus*. This is in good agreement with the reported data by Ikeda et al.(19).

It has been reported that the target site of cationic disinfectants is the cytoplasmic membranes of microbes(9,20). 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 (two long-chain fatty acid tails with a carbon number of 12-20)(21). Thus, the quaternized chitosan derivatives with a long alkyl chain could be assumed to strongly interact with the cytoplasmic membranes due to a hydrophobic affinity between the introduced alkyl chain and the phospholipids, leading to a higher antibacterial activity. Therefore, we conclude that the hydrophobic property due to the introduced long alkyl chain can be another important factor for the enhancement of the antibacterial activity against *S.aureus*.

Conclusions

1. In order to improve antibacterial activity of chitosan, several water soluble *N*-alkyl chitosan derivatives with quaternary ammonium salt were produced, and the structural changes in chitosan derivatives were confirmed with FT-IR and ^{13}C NMR.
2. Antibacterial activities was found to be increased in the order of chitosan, *N,N,N*-trimethyl chitosan, *N,N*-dibutyl-*N*-methyl chitosan, *N*-octyl-*N,N*-dimethyl chitosan and *N*-dodecyl-*N,N*-dimethyl chitosan, with increase in the chain length of the alkyl groups at pH 6.0.
3. The hydrophobicity due to the introduced long alkyl chain can be another important factor for the enhancement of the antibacterial activity against *S.aureus*.

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