Antibacterial Action of Chitosan and Carboxymethylated Chitosan

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ABSTRACT: A series of chitosan with different molecular weights obtained by γ-irradiation depolymerization and another series of deacetylated chitosan were synthesized. Several *N*,*O*-carboxymethylated chitosan and *O*-carboxymethylated chitosan were also produced. The above samples were characterized by Fourier transform infrared spectroscopy (FTIR). Their antibacterial activities against *E. coli* were explored by the optical density method. The antibacterial activity of chitosan is influenced by its molecular weight, degree of deacetylation, concentration in solution, and pH of the medium. Antibacterial activities were also found to be increased in the order of *N*,*O*-carboxymethylated chitosan, chitosan, and *O*-carboxymethylated chitosan. Fluorescence of the FITC (fluorescein isothiocyanate)-labeled chitosan oligomers at the inside of the *E. coli* cell was observed by a confocal laser scanning microscope. The antibacterial activity of chitosan oligomers seems to be caused mainly by the inhibition of the transcription from DNA. © 2000 John Wiley & Sons, Inc. J Appl Polym Sci 79: 1324–1335, 2001

Key words: chitosan; carboxymethylated chitosan; antibacterial activity; fluorescence

INTRODUCTION

As one of the most abundant natural biopolymers, and due to its unique polycationic nature, chitosan has a wide range of application fields, such as in wastewater purification,[1] coacervate formation for cell entrapment,[2] and coating of seeds for improved yield.[3]

Interestingly, some antibacterial and antifungal activities have been described with chitosan and chitosan derivatives with quaternary ammonium.[4–7] Chitosan inhibits the growth of a wide variety of bacteria and fungi (cf. Table I). Moreover, chitosan has several advantages over other types of disinfectants, that is, it possesses a higher antibacterial activity, a broader spectra of activity, a higher killing rate, and lower toxicity toward mammalian cells.[8,9]

Several mechanisms were proposed for the antimicrobial activity by chitosan.[10] In one mechanism, the polycationic nature of chitosan interferes with the negatively charged residues of macromolecules at the surface.[11] Chitosan interacts with the membrane of the cell to alter cell permeability.[12] For example, fermentation in bakers' yeast is inhibited by certain cations, which act at the yeast cell surface to prevent the entry of glucose.[13] UV-absorption studies indicated that chitosan caused considerable leakage of proteinaceous material from *Pythium oaroecandrum* at pH 5.8.[14]

The other mechanism involves the binding of chitosan with DNA to inhibit RNA synthesis.[15] It has been proposed that when chitosan is liberated from the cell wall of fungal pathogens by plant host hydrolytic enzymes chitosan penetrates the nuclei of fungus and interferes with RNA and

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Table I Antimicrobial Activities of Chitosan

Bacteria	MIC[a] (ppm)
Agrobacterium tumefaciens	100
Bacillus cereus	1000
Corinebacterium michiganence	10
Erwinia sp.	500
Erwinia carotovora subsp.	200
Escherichia coli	20
Klebsiella pneumoniae	700
Micrococcus luteus	20
Pseudomonas fluorescens	500
Staphylococcus aureus	20
Xanthomonas campestris	500
Fungi	
Botrytis cinerea	10
Fusarium oxysporum	100
Drechstera sorokiana	10
Micronectriella nivalis	10
Piricularia oryzae	5000
Rhizoctonia solani	1000
Trichophyton equinum	2500

[a] MIC: minimum growth inhibitory concentration.

protein synthesis. The organism may be impaired by both its own chitosan and host phytoalexin induced by the liberated chitosan.[16]

Chitosan, however, shows its antibacterial activity only in an acidic range because of its poor solubility above pH 6.5. Thus, water-soluble chitosan derivatives soluble to both acidic and basic physiologic circumstances may be good candidates for the polycationic biocide.

In this article, we prepared a series of chitosan that has various molecular weights (MW) and degree of deacetylation (DDA) and two types of carboxymethylated chitosan. Also, we examined whether their antibacterial activity against *Escherichia coli* (*E. coli*) depended on the MW, DDA, chitosan concentration (C), and pH of the medium. We observed fluorescence of the FITC (fluorescein isothiocyanate)-labeled chitosan oligomers at the inside of the *E. coli* cell by a confocal laser scanning microscope.

EXPERIMENTAL

Materials

The first type of chitosan was provided by Qingdao Medicine Institute (Qingdao, Shangdong Province, China). One of this type had 1.08×10^6 M_ν calculated by the Mark–Houwink equation.[17] $[\eta] = KmM^a$, where $Km = 1.81 \times 10^{-3}$, $a = 0.93$, and the DDA was 85%. The other of this type had 2.74×10^5 M_ν and a 74% DDA.

The second type of chitosan was obtained from the Wuxi University of Light Industry (Wuxi, Jiangsu Province, China). One of this type had a weight-average molecular weight (M_w) of 8000, a molecular weight distribution (M_w/M_n) of 1.80, and a 75% DDA, and the other of this type had a M_w of 5000, an M_w/M_n of 2.10, and 73% DDA.

The *E. coli* was from the School of Biology Science, NanKai University (Tianjin, China), and was stored at 4°C until it was used. Biochemical reagents of beef extract, peptone, and agar powder were purchased from the Tianjin Dongfang Biomaterial Co. (Tianjin, China).

Monoclonal anti-FITC (mouse IgG1 isotype) was Sigma Immuno Chemicals's product No. F-5636 (Sigma Company, USA). All other reagents were analytical grade.

Characterization

IR spectra of all the samples were recorded with a Nicolet 170SX IR spectrophotometer. After being dried completely at 50°C under a vacuum, the sample could be used for analysis.

γ-Irradiation Depolymerization of Chitosan

The γ-irradiation of samples was carried out in a γ cell (Co-60 source, Gamma 400 A, Tianjin Atomic Physical Research Institute, Tianjin, China). Chitosan samples in glass vials were irradiated with doses of up to 100 kGy under anoxia conditions. All irradiated vials were placed in a desiccator for at least 1 week prior to sample characterization. A series of chitosans with different MW was obtained (cf. Table II).

Deacetylation of Chitosan

To obtain different deacetylated chitosan (cf. Table III), chitosan ($M_\nu = 2.74 \times 10^5$) was refluxed for 10–55 min at 95°C with a 40% (w/v) NaOH solution under nitrogen gas conditions. The samples were washed with distilled water, refluxed again under similar conditions, and washed with distilled water until neutral.[18] The DDA of the chitosan was determined by the FTIR baseline method as explained by Roberts and Domsey,[19] using the relationship % DDA = $(1 - A_{1655}/A_{3340} \times 1/1.33) \times 100$, where A is the logarithmic ratio of the absorption and transmittance at the given wavenumber.

Table II Chitosan with Different MW for Antibacterial Activity Analysis

Samples	Irradiation Dose (kGy)	M_ν ($\times 10^4$)	DDA (%)	M_w	M_w/M_n
Chitosan	0	108	85	—	—
Irradiated chitosan	15	65	85	—	—
Irradiated chitosan	50	9.16	86	—	—
Irradiated chitosan	100	5.11	88	—	—
Chitosan	—	27.4	74	—	—
Chitosan oligomer	—	—	75	8000	1.8
Chitosan oligomer	—	—	73	5000	2.1

Preparation of *N,O*-Carboxymethylated Chitosan (*N,O*-CM-Chitosan)

Chitosan ($M_\nu = 2.74 \times 10^5$, 20 g) is suspended in isopropanol (200 mL) under agitating, and sodium hydroxide (50.4 mL, 10*M*) is added in six equal portions over a period of 20 min. The alkaline slurry is stirred for an additional 45 min, and the solid monochloroacetic acid (24 g) is added in five equal portions at 5-min intervals. The reaction mixture is heated at 60°C for 3 h. Then, cold distilled water (17 mL) is incorporated into the mixture and its pH is adjusted to 7.0 with glacial acetic acid. The reaction mixture was filtered and the solid product was washed with a 70% methanol/water mixture (300 mL) and then with anhydrous methanol. The resultant *N*,*O*-CM-chitosan is dried in an oven at 60°C under a vacuum.[20] The degree of carboxymethylation (DCA) is determined by pH titration.[21]

Preparation of *O*-Carboxymethylated Chitosan (*O*-CM-Chitosan)

Chitosan $M_\nu = 2.74 \times 10^5$, 10 g) suspended in a 100 mL NaOH solution (42%) is reacted with sodium monochloroacetate obtained by adding monochloroacetic acid (11.7 g) at 0–30°C for 5–24 h. The pH is adjusted to 7 with HCl and the solution is dialyzed for 3 days against deionized water.[22] The DCA is determined by pH titration.[21]

Table III Properties of Chitosan and Deacetylated Chitosan

Samples	Reflux Time (min)	DDA (%)	M_ν ($\times 10^4$)
Chitosan	0	74	27.4
Deacetylated chitosan	10	75.6	26.1
Deacetylated chitosan	25	82.7	20.3
Deacetylated chitosan	40	89.9	16.9
Deacetylated chitosan	55	96	12.7

Antibacterial Assessment

Antibacterial activities of the series of chitosan and its derivatives against *E. coli* were evaluated by using the optical density method as described. A loopful of each culture was spread to give the single colonies on the nutrient agar (agar 15 g, peptone 10 g, beef extract 3 g, NaCl 3 g in distilled water 1000 mL; pH 7.0) and incubated at 37°C for 24 h. A representative colony was picked off with a wire loop and placed in a nutrient broth (peptone 10 g, beef extract 3 g, NaCl 3 g in distilled water 1000 mL; pH 7.0), which was then incubated at 37°C overnight. Then, a culture where *E. coli* grew in a logarithmic growth phase was prepared for an antibacterial test. Two percent of them was inoculated to the medium containing different chitosans and their derivatives, which were dissolved in 2*M* acetic acid, under shaken cultivation at 37°C for 24 h. During incubation, the turbidity of the medium was measured at 610 nm (by a 756MC UV-vis spectrophotometer, Shanghai, China) every 2 h.

Fluorescence Observation on Antibacterial Activity of FITC-labeled Chitosan Oligomer

Preparation of FITC-labeled Chitosan Oligomer

Chitosan, 0.2 g ($M_w = 8000$), was dissolved in 20 mL of a H_2O:C_2H_5OH = 1:1 mixed solution at pH 9.0 and 0.04% of FITC was reacted with the chitosan oligomer at an ice-cold temperature under stirring overnight. An FITC–chitosan oligomer was precipitated with ethanol and air-dried after extensive rinsing with ethanol.

Fluorescence Observation

Five percent of the culture medium adapting *E. coli* was inoculated in the medium containing

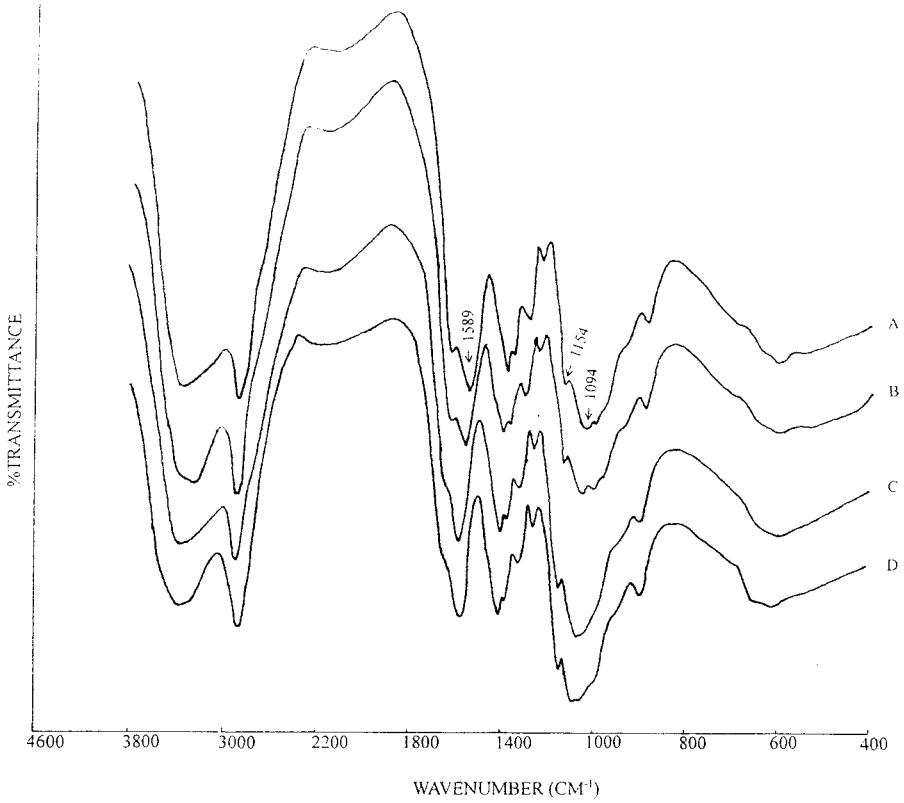


Figure 1 IR spectra of the original chitosan: (A) $M_\nu = 108 \times 10^4$), and the irradiated chitosans: (M_ν = (B) 65×10^4; (C) 9.16×10^4; (D) 5.11×10^4).

0.01% of the FITC–chitosan oligomer under a shake culture at 37°C for 24 h in the dark. *E. coli* cultured with the FITC–chitosan oligomer was collected by centrifugation (3000 rpm for 15 min) and rinsed extensively with physiological saline. The aggregated organisms were dispersed by ultrasonication in the physiological saline and loaded on the fluorescence free slide glass followed by air-drying. Then, a coverglass was set after treatment with 50% aqueous glycerol containing sodium azide and localization of the fluorescence was observed by a Confocal laser scanning microscope (Model: TSC-NT 165123, US).[23]

RESULTS AND DISCUSSION

IR Spectra Analyses

Figures 1 and 2 show the FTIR spectra of chitosan with different viscosity-average molecular weights (M_ν) and DDA. The main characteristic peaks of chitosan are at 3455 (O—H stretch), 2867 (C—H stretch), 1589 (N—H bend), 1154 (bridge O stretch), and 1094 cm^{-1} (C—O stretch).[24] From the spectra (cf. Fig. 1), there is no qualitative difference in peak locations between the irradiated chitosan and the started chitosan (cf. Table II), especially in the amino characteristic peak at about 1589 cm^{-1}. For different deacetylated chitosan (cf. Table III), the higher the DDA of chitosan is, the weaker the acetyl group absorption (ca. 1566 and 1658 cm^{-1}) is and the stronger amino peak is (cf. Fig. 2).[25]

For *N*,*O*-CM-chitosan (cf. Table IV), its spectrum is different from the spectrum of chitosan (cf. Fig. 3). The bands at 1597–1610 and 1414 cm^{-1} correspond to the carboxy group and —CH_2COOH group, respectively.[26] Compared with the peak of chitosan, the peak of *N*,*O*-CM-chitosan at 1589 cm^{-1} (N—H bend) decreases, whereas those at the peak, 1066–1109 cm^{-1} (C—O stretch), increase. These bands become more evident in that carboxymethyl has substituted the amino and hydroxyl of chitosan. Figure 3 also shows the IR spectra of *O*-CM-chitosan (cf. Table IV), and the 1730 (—COOH), 1080–1154 (—C—O—), and 1624 and 1516 cm^{-1} (—NH_3^+) bands appear.[22] Therefore, that the carboxymethyl simply substituted the hydroxyl of chitosan is evident.

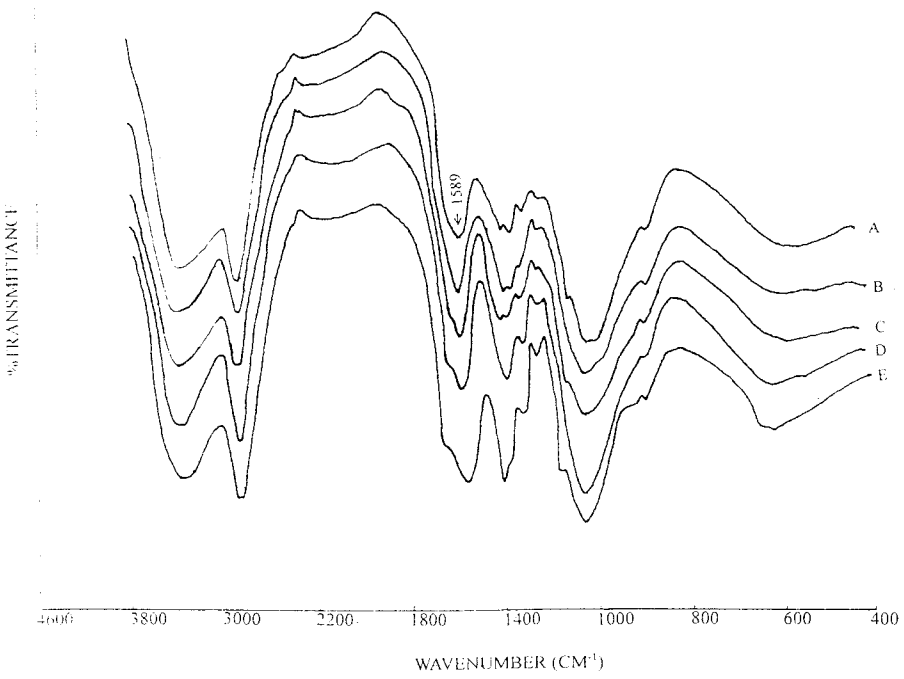


Figure 2 IR spectra of different deacetylated chitosans: DDA% = (A) 74; (B) 75.6; (C) 82.7; (D) 89.9; (E) 96.

Antibacterial Activities of Chitosan and CM-Chitosan

Chitosan, a cationic antibacterial agent, has been widely used, particularly for external disinfection, and the target site of the cationic biocides is the cell envelope of bacteria. For Sudarshan et al., the mechanism of antibacterial activities that the amino group of chitosan is bound to surface components of the bacteria and then inhibits their growth was developed.[10] They thought that at lower concentration chitosan may have bound to the negatively charged bacterial surface to disturb the cell membrane and cause cell death due to leakage of intracellular components; at high concentration, chitosan may have additionally coated the bacterial surface to prevent leakage of intracellular components as well as to impede mass transfer across the cell barrier.

MW-dependent Antibacterial Activity

Figures 4 and 5 show the optical density versus the culture time for the chitosan with different MW against *E. coli.* Seven types of chitosan with MW ranging from 5000 to 1.08×10^6 were studied for their antibacterial activities. The smaller the optical density (OD) of the medium is, the higher is the antibacterial activity of the correspondent chitosan. According to Figure 4, compared to the OD of the medium without chitosan, the OD of that with chitosan was much lower, and it de-

Table IV DCA of CM-Chitosan

	Sequence					
	A	B	C	D	E	F
	Sample					
	N,*O*-CM-chitosan	*N*,*O*-CM-chitosan	*N*,*O*-CM-chitosan	*N*,*O*-CM-chitosan	*O*–CM-chitosan	*O*-CM-chitosan
DCA (%)	97.9	91.0	69.6	47.7	41.9	73.1

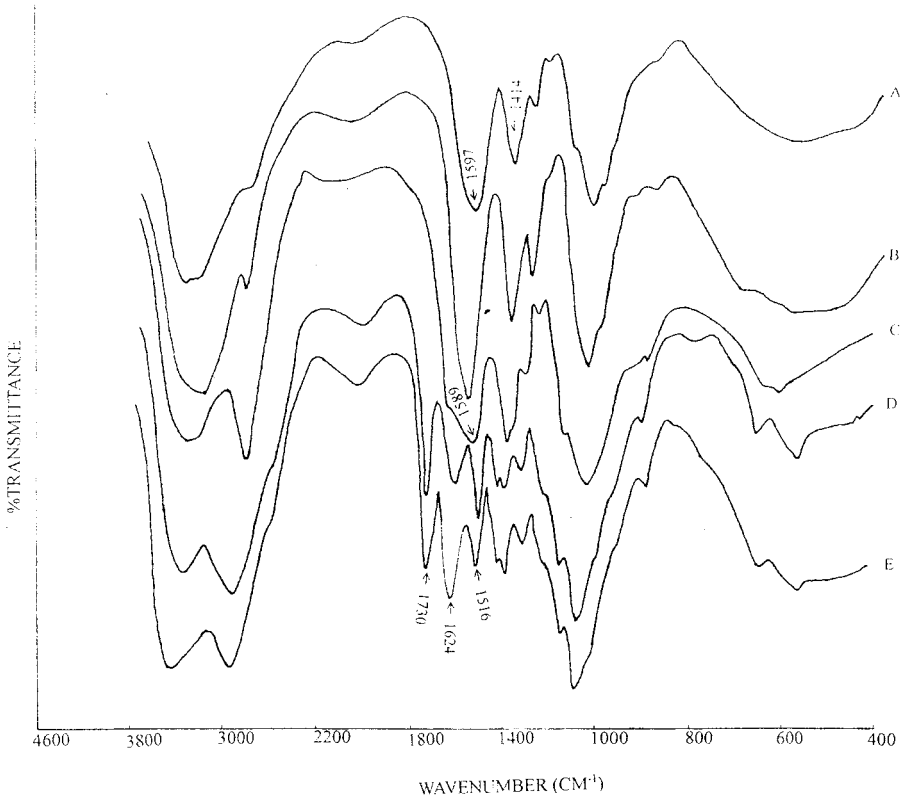


Figure 3 IR spectra of *N*,*O*-CM-chitosans: DCA % = (A) 47.7; (B) 97.9; chitosan: (C) $M_v = 27.4 \times 10^4$; and *O*-CM-chitosan: DCA% = (D) 41.9; (E) 73.1.

creased gradually, with the MW varying from 5000 to 9.16×10^4. This is evidence that chitosan has good antibacterial activity, and it was increased with the MW varying from 5000 to 9.16×10^4. However, from Figure 5, the OD of the medium with the chitosan MW varying from 9.16×10^4 to 1.08×10^6 was increased gradually. This also meant that the antibacterial activity of chitosan decreased while its MW increased.

Sudarshan et al. argued that the growth inhibitory activity of chitosan markedly increased with lengthening of the polymer,[10] while Groboillot et al. held that the crosslinked chitosan did not inhibit bacterial growth, suggesting that only soluble chitosan is inhibitory.[27] They both think that water-soluble chitosan affects bacterial transport mechanisms through the cell walls by binding membrane macromolecules.

Summing up the result, it was seen that the antibacterial activity of chitosan, which is a polycationic compound due to a large amount of $—NH_3^+$ in the solution, may be depend on the concentration of the $—NH_2$ of the polymer. Also, when the MW is under 9.16×10^4, the antibacterial activity of chitosan increases with increasing of the $—NH_2$ content (in other words, with increasing of the MW). When the MW exceeds about 9.16×10^4, amino groups of chitosan may be too many, which promotes a fictitious crosslinked structure through their strong intramolecular hydrogen bondings and then they are no longer available to attach to bacteria surfaces. So, the antibacterial activity of chitosan decreased with increasing of its MW when it was above 9.16×10^4.

Antibacterial Activity Regulated by DDA

Figure 6 shows the OD versus the culture time for the chitosan with different DDA against *E. coli*. The OD of the medium with five types of different DDA chitosan was observed. The curves demonstrate that the OD decreased gradually with the percent of the DDA of chitosan, heightening from 74 to 96. So, the antibacterial activity was increased respectively.

The results evidence that the heightening of DDA causes increase of the $—NH_2$ concentration and then increases the $—NH_3^+$ numbers. Thus, more $—NH_3^+$ positive charges may have bound to

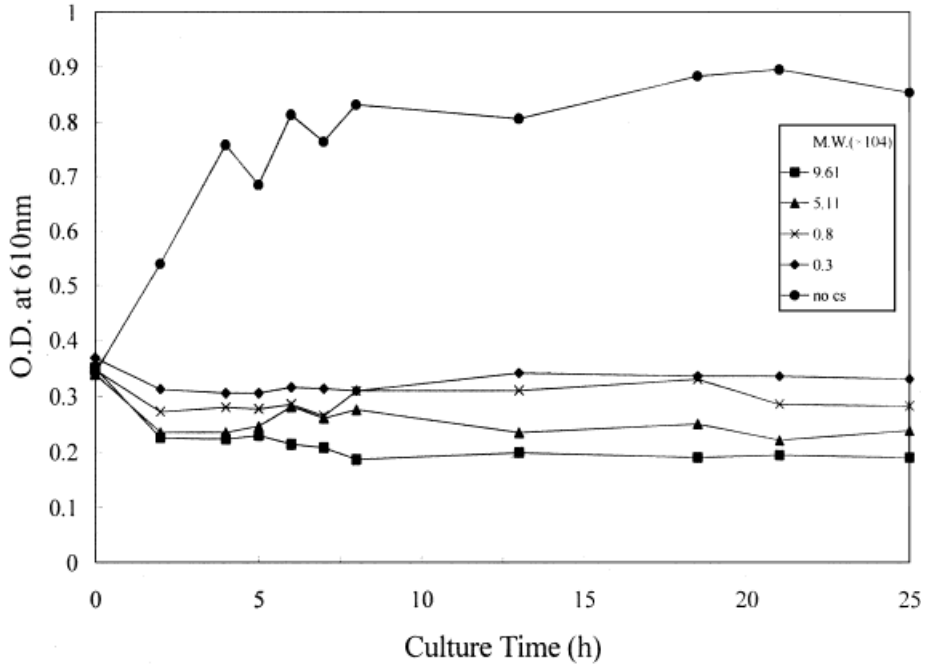


Figure 4 OD versus culture time for the chitosan with MW ranging from 5000 to 9.61 $\times 10^4$ against *E. coli* ("no cs" means no chitosan in the medium).

the negatively charged bacterial surface to cause agglutination. The study of Morimoto and Shigemasa about the inhibition of bacterial growth by deacetylated chitins with various DDA (from 66 to 91%) also show that the antibacterial activity was increased with increase of the DDA.[28] This conclusion is in accordance with our study result that the antibacterial activity of chitosan strongly depended on the DDA.

Dependence of Antibacterial Activity on Concentration (C) of Chitosan in Solution

Figures 7 and 8 show the OD versus the culture time for two kinds of chitosan with different con-

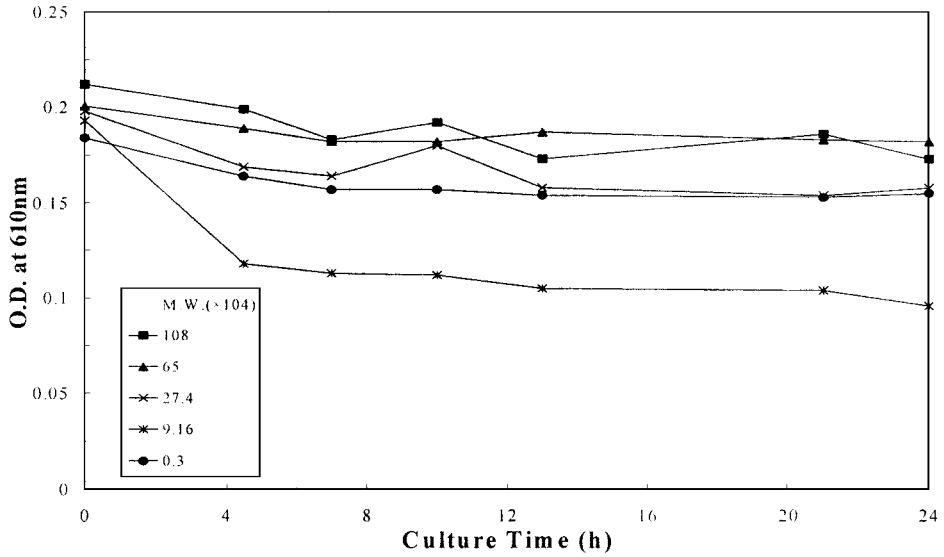


Figure 5 OD versus culture time for the chitosan with MW ranging from 5000 to 1.08 $\times 10^6$ against *E. coli.*

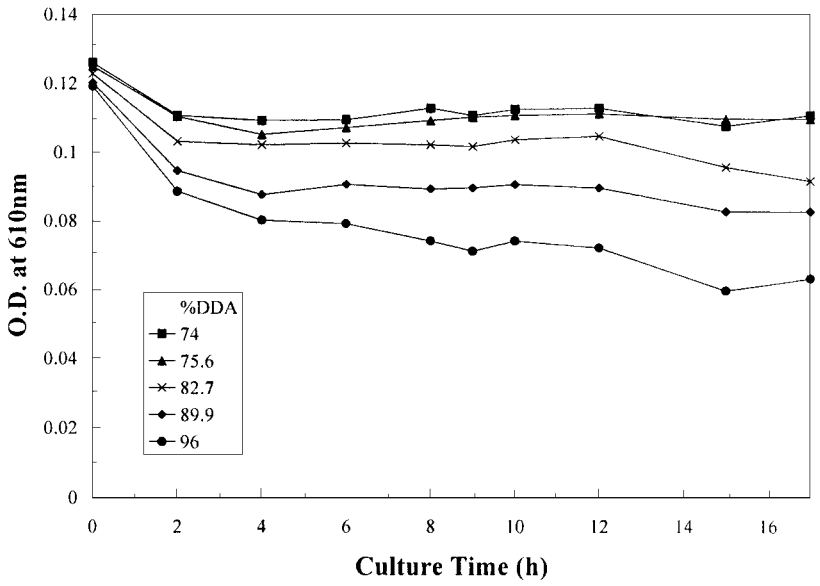


Figure 6 OD versus culture time for the chitosan with % DDA ranging from 74 to 96 against *E. coli.*

centrations against *E. coli.* While some chitosan was added to the medium, its OD was obviously lower than that of the medium without chitosan. Moreover, with increase of the concentration of chitosan in the medium, which varied from 0.01% (w/v) to 0.1% (chitosan $M_v = 5.14 \times 10^4$) and from 0.01 to 0.5% (chitosan $M_w = 5000$), their OD values decreased, respectively. In other words, the antibacterial activity of chitosan in the medium would increase if the concentration of chitosan had been increased. As the concentration of chitosan in the medium also indicates the $—NH_3^+$ concentration, the above result evidenced that the inhibitory effect of bacteria depended on the amount of $—NH_3^+$ and was strengthened with the $—NH_3^+$ concentration in the experimental range. However, we can not check the antibacterial activity of chitosan with a higher concentration because of the poorly solubility of chitosan.

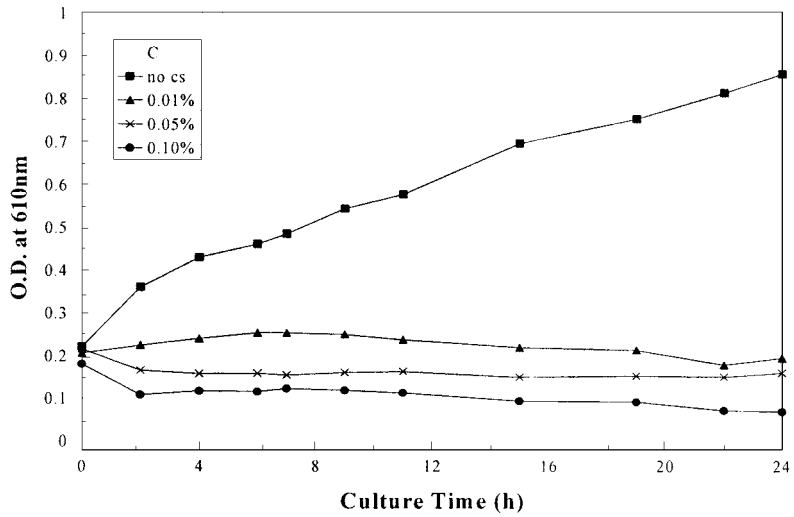


Figure 7 OD versus culture time for the chitosan ($M_v = 5.11 \times 10^4$) whose C in the medium is 0.01, 0.05, and 0.10% against *E. coli* ("no cs" means no chitosan in the medium).

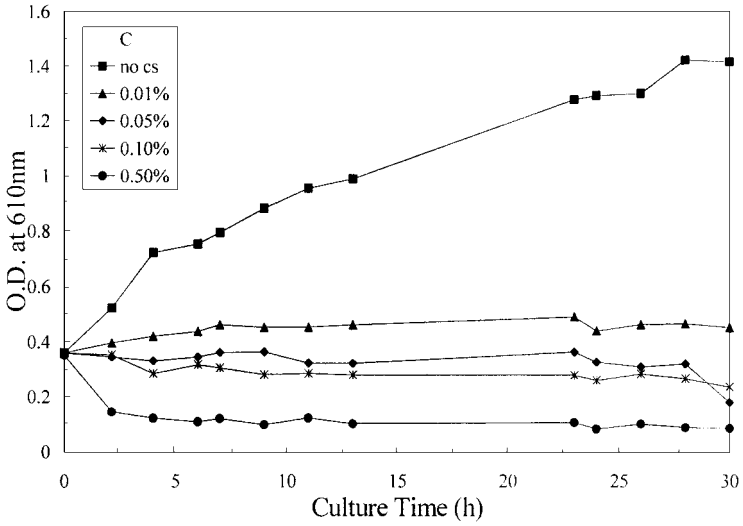


Figure 8 OD versus culture time for the chitosan (MW = 5000) whose *C* in the medium is 0.01, 0.05, 0.10, and 0.50% against *E. coli* ("no cs" means no chitosan in the medium).

Effects of pH on Antibacterial Activity

Figure 9 exhibits the OD versus the culture time for the chitosan dissolved in different pH's of media against *E. coli.* In the same culture conditions, when the pH of the media was lower than 6.3, their OD values were larger than that of the medium with pH 6.3. So, when the medium's $pH < 6.3$, the corresponding antibacterial activity of chitosan was lower than that of chitosan in medium with pH 6.3 and decreased gradually with pH varying from 6.3 to 4.0, then increased again with pH to 3.0. When a solution's pH > 6.3, their ODs were larger than that of the medium with pH 6.3, and the antibacterial activity of chitosan in it was clearly decreased compared with that of chitosan in medium with pH 6.3. No antibacterial activity was observed in chitosan in media with pH 7.0 or above, which may be due to chitosan's poor solubility in this condition.

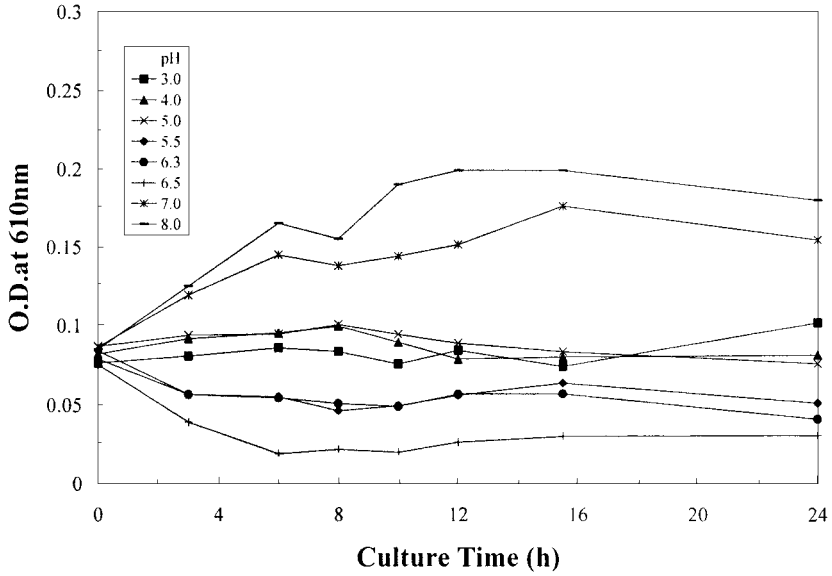


Figure 9 OD versus culture time for the chitosan ($M_v = 5.11 \times 10^4$) solved in the medium whose pH ranges from 3 to 8 against *E. coli.*

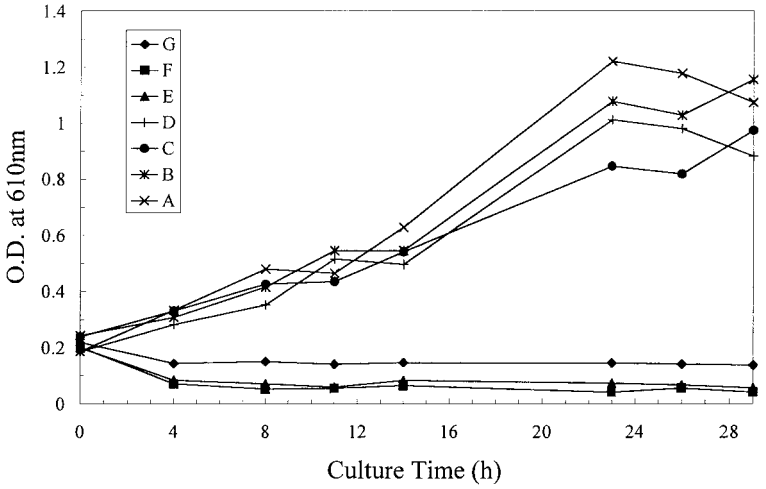


Figure 10 OD versus culture time for (A–D) *N*,*O*-CM-chitosan (cf. Table IV), (E,F) *O*-CM-chitosan (cf. Table IV), and (G) chitosan $M_\nu = 27.4 \times 10^4$) against *E. coli.*

Chitosan is a polyelectrolyte, and its pK_a is approximately 6.3.[10] In this condition, the —NH_2 of chitosan was significantly charged, existed as —NH_3^+, and showed a good antibacterial activity. With pH > 6.3, the amount of —NH_3^+ decreased; moreover, the solubility of chitosan declined, so the antibacterial activity of chitosan diminished. With pH < 6.3, the amount of —NH_3^+ was not varied, but the number of H^+ was increased. The two cationics competed in binding to the negatively charged bacterial surface, but only the polycationic can cause agglutination. So, the antibacterial activity of chitosan in media with this pH decreased.

Antibacterial Activity of CM-Chitosan

Figure 10 shows the OD versus the culture time for the chitosan, four kinds of *N*,*O*-CM-chitosan, and two kinds of *O*-CM-chitosan against *E. coli.* The results indicated that the OD of *N*,*O*-CM-chitosan whose DCA varied from 36.6 to 97.7% was much larger than that of chitosan. The antibacterial activity of these *N*,*O*-CM-chitosan was hardly observed compared with its original chitosan. However, the ODs of *O*-CM-chitosan (% DCA = 41.9 and 73.1) were lower than that of chitosan, and the antibacterial activities were slightly enhanced.

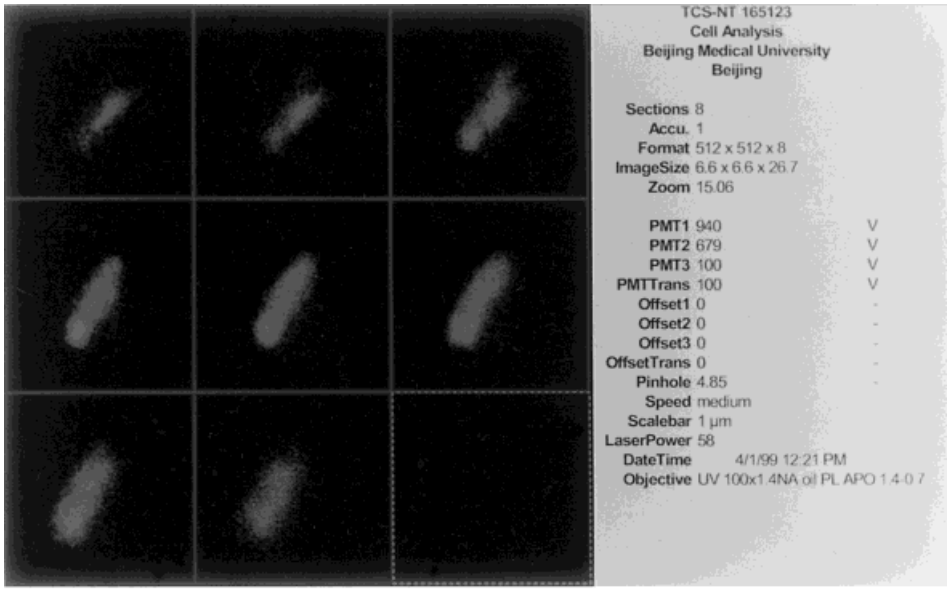
N,*O*-CM-chitosan is a product where —NH_2 groups and —OH groups were substituted by —CH_2COOH groups. So, compared with chitosan, its —NH_2 content was lower, then its antibacterial activity decreased, and no antibacterial activity appeared. *O*-CM-chitosan is the substitution of chitosan with —CH_2COOH only to —OH; its number of —NH_2 is not changed. Moreover, its —COOH group may have reacted with the NH_2 group intra- or intermolecularly and charged these NH_2 groups. So, in the same condition, the number of —NH_3^+ groups of *O*-CM-chitosan is more than that of chitosan. Therefore, the antibacterial activity of *O*-CM-chitosan was increased.

Fluorescence Observation on FITC–labeled Chitosan Oligomer

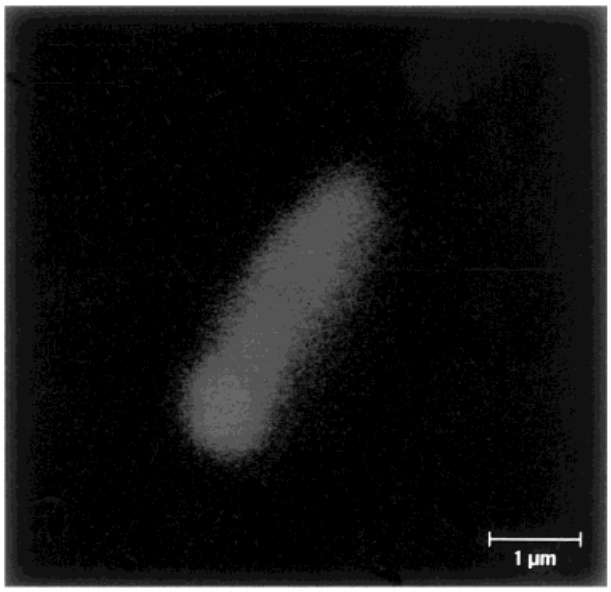
The fluorescence micrographs of *E. coli*-accumulated FITC-labeled chitosan oligomers (MW = 8000 and 5000) are displayed in Figures 11 and 12. In these micrographs, the fluorescence (bright) areas are FITC-labeled chitosan oligomers, and the elliptic area containing the bright ones is the *E. coli* cell. In Figure 11(a), the nine small pictures, left to right, then up and down, expressed a series of cross-sections of *E. coli* containing FITC-labeled chitosan oligomers from the outside to the inside to the outside. It evidenced that the FITC-labeled chitosan oligomers were observed at the inside of the cell. Thus, permeated chitosan oligomers [MW = 8000 and 5000; cf. Figs. 11(b) and 12] were suggested to block the transcription from DNA to inhibit the growth of bacteria. As this fluorescence study corresponds closely to the results of antibacterial activity by chitosan oligomers, the antibacterial activity of chitosan oligomers seems to be caused mainly by the inhibition of the transcription from DNA.[23]

CONCLUSIONS

To analyze the antibacterial activities of chitosan, one series of chitosans with MW ranging from 5.11×10^4 to 1.08×10^6 was obtained by γ-irra-



(a)



(b)

Figure 11 Fluorescence micrographs of FITC-labeled chitosan oligomer (MW = 8000) accumulated in the *E. coli* cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

diation depolymerization, and another series of different deacetylated chitosans whose DDA varied from 74 to 89.9% was synthesized. Several kinds of *N*,*O*-CM-chitosans and *O*-CM-chitosans were also produced to analyze the antibacterial activities of chitosan derivatives.

The study evidenced that the water-soluble chitosan has a good antibacterial activity against *E. coli.* The antibacterial activity was increased with the MW varying from 5000 to 9.16×10^4 and decreased with MW varying from 9.16×10^4 to 1.08×10^6. When the DDA or concentration in a solution of chitosan increased, the antibacterial activities of chitosan also increased. While the pH of the medium was lower than 6.3, its antibacterial activity of chitosan was lower than that of chitosan in pH 6.3. When the solution was pH > 6.3, its antibacterial activity of chitosan was clearly decreased compared with that of chitosan in pH 6.3. Also, no antibacterial activity was ob-

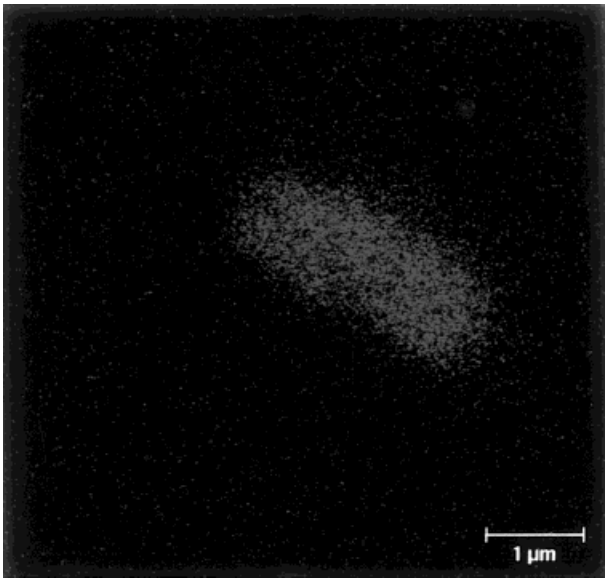


Figure 12 Fluorescence micrograph of FITC-labeled chitosan oligomer (MW = 3000) accumulated in the *E. coli* cell. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

served at pH 7.0 or above because of chitosan's poor solubility in this condition. Antibacterial activities were found to increase in the order of *N*,*O*-CM-chitosan, chitosan, and *O*-CM-chitosan.

Summing up these results, an identical conclusion can be drawn: The variety of the above effect factors on the antibacterial activity of chitosan means the varying numbers of NH_2 groups of chitosan. The antibacterial activity of chitosan depended on the concentration of —NH_2 of the polymer and increased with the addition of the —NH_2 content. In the solution, chitosan is a polycationic compound due to a large number of —NH_3^+, so the antibacterial activities of chitosan and carboxymethylated derivatives also depend on the effective number of —NH_3^+ groups. Also, a mechanism of antibacterial activities of water-soluble chitosan was that chitosan may have bound to the negatively charged bacterial surface to disturb the cell membrane and cause cell death.

The FITC-labeled chitosan oligomers (MW = 8000 and 5000) were observed at the inside of the *E. coli* cell, and they show good antibacterial activities. Thus, another mechanism of the antibacterial activity of chitosan oligomers was that the permeated chitosan oligomer may have blocked the transcription from DNA and interfered with the RNA and protein systhesis.

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