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Chitosan Derivatives Killed Bacteria by Disrupting the Outer and Inner Membrane

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Six kinds of water-soluble chitosan were prepared by grafting aminofunctionality onto chitosan at the C-6 position, and their antimicrobial activities were investigated against three Gram-negative and three Gram-positive bacteria. Among the derivatives, dimethylaminoethyl-chitosan (DMAEC) has the highest potential to suppress the growth of bacteria. To elucidate detailed antimicrobial modes of action against bacteria, cell integrity, outer membrane (OM), and inner membrane (IM) permeabilization assays were investigated. When treated with DMAEC, the release of 260 nm absorbing materials quickly increased for both *Escherichia coli* and *Staphylococcus aureus*, but the absorbance value was different due to the difference in cell structures. In OM and IM permeabilization assays, DMAEC rapidly increased 1-*N*-phenylnaphthylamine uptake and the release of cytoplasmic β-galactosidase via an increase in the permeability of OM and IM. Moreover, DMAEC90 prepared from 90% deacetylated chitosan had more activity than DMAEC50 prepared from 50% deacetylated chitosan, and these results revealed that the antimicrobial action of water-soluble chitosans was dependent on the degree of deacetylation and the substituted group.

KEYWORDS: Water-soluble chitosan; antimicrobial action; membrane permeabilization

INTRODUCTION

Chitosan, which is a copolymer consisting of β-(1→4)-2-acetamido-D-glucose and β-(1→4)-2-amino-D-glucose units, is derived from chitin by deacetylation in the presence of alkali. It exhibits a wide variety of biological activities such as antitumor activities (*1*), immunostimulating effects (*2*), antimicrobial effects (*3*), free radical scavenging activities (*4*), and cholesterol-reducing effects (*5*). Because of the biological functions, it has attracted much attention; however, the applications of chitosan are limited because of its insolubility. Unlike chitosan, its hydrolyzed products and chitosan oligosaccharides (COS) are readily soluble in water due to their shorter chain lengths and free amino groups in D-glucosamine units (*6*). However, recent studies on the antimicrobial activity of chitosan and COS have revealed that chitosan is more effective in inhibiting the growth of bacteria than COS (*7*−*9*).

Several studies have described the antimicrobial activities of water-soluble chitosan derivatives such as quaternary ammonium chitosan (*10*, *11*), hydroxylpropyl chitosan (*12*), N-carboxybutyl chitosan (*13*), carboxymethylated chitosan (*14*), and sulfated chitosan (*15*). In addition, branched chitin and chitosan with N-acetyl-D-glucosamine and D-glucosamine at the C-6 position exhibited antimicrobial activities (*16*). The actual mechanism of antimicrobial activity of chitosan and its derivatives is not yet fully understood but has been suggested to involve cell lysis, breakdown of the cytoplasmic membrane barrier, and the chelation of trace metal cations by the chitosan could be necessary for the microorganism's growth (*17*−*19*). In the killing of Gram-negative bacteria, a cationic chitosan must interact with both bacterial cell envelope membranes.

Therefore, the aim of this study was to investigate the antimicrobial activity and to elucidate an antimicrobial mechanism of water-soluble chitosan derivatives with different degrees of deacetylation.

MATERIALS AND METHODS

Materials. Chitin (average molecular mass, 140−310 kDa,) prepared from crab shells was donated by Kitto Life Co. (Seoul, Korea). Partially deacetylated chitosans were prepared by *N*-deacetylation with 40% (w/w) sodium hydroxide solution at 100 °C for various times according to our previous method (*4*) and were designated as chitosan90 (90% deacetylated chitosan) and chitosan50 (50% deacetylated chitosan). For the preparation of chitosan derivatives, 2-chloroethylamino hydrochloride was purchased from Fluka, and 2-(dimethylamino)ethylchloride hydrochloride and 2-(diethylamino)ethylchloride hydrochloride were obtained from Sigma Chemical Co. (St. Louis, MO). The microorganisms tested for antibacterial activity were obtained from KCTC (Korean Collection of Type Cultures). *O*-Nitrophenyl-β-D-galactoside (ONPG) and 1-*N*-phenyl-naphthylamine (NPN) were obtained from Sigma Chemical Co.. All other reagents were of the highest grade available commercially.

Synthesis of Water-Soluble Chitosan Derivatives. Water-soluble chitosans were prepared according to our previous method (*20*). Water-soluble aminoethyl-chitosan (AEC), dimetylaminoethyl-chitosan (DMAEC), and diethylaminoethyl-chitosan (DEAEC) were designated as AEC90, DMAEC90, and DEAEC90 when prepared from 90% deacetylated chitosan, and AEC50, DMAEC50, and DEAEC50 were prepared from

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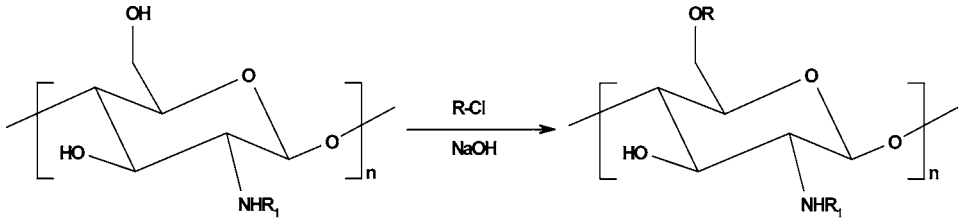
Scheme 1. Synthesis Pathway and Chemical Structures of Water-Soluble Chitosans



Chitosan (C)

AEC : R=$(CH_2)_2NH_2$; R_1=H, $COCH_3$

DMAEC : R=$(CH_2)_2N(CH_3)_2$; R_1=H, $COCH_3$

DEAEC : R=$(CH_2)_2N(CH_2CH_3)_2$; R_1=H, $COCH_3$

50% deacetylated chitosan. Fourier transform infrared spectra showed the new peak at 2965 cm^{-1} due to C−H stretching of substituted groups. The ^1H NMR spectra of DEAE-chitosan in D_2O showed a peak at 1.30 ppm for the methyl, a peak at 3.28 ppm for the methylene protons of the DEAE group, and peaks between 1.5 and 1.6 ppm for the methyl protons of the protonated DEAE groups. In the same manner, the peak observed between 2.9 and 3.0 ppm for methyl and methylene protons of the DMAE group and the peak at 2.9 ppm for methylene protons of the AE group were also observed (*20*). AEC50, DMAEC50, and DEAEC50 were characterized in the same manner.

Assay for Antimicrobial Activity. The antimicrobial activity of water-soluble chitosans was investigated against three Gram-negative (*Escherichia coli*, *Salmonella typhimurium*, and *Pseudomonas aeruginosa*) and three Gram-positive (*Enterococcus faecalis*, *Listeria monocytogenes*, and *Staphylococcus aureus*) bacteria purchased from KCTC. The minimum inhibitory concentration (MIC) was tested by 2-fold serial broth dilution as follows. Bacteria cultures (10^6−10^7 colonies/mL) grown in 5 mL of Mueller−Hinton broth (Difco, MI), which contained 1 mL of derivatives with various concentrations in 50 mM acetate buffer (pH 5.5), were incubated at 37 °C for 18 h. The MIC was defined as the lowest concentration of derivatives at which the cell growth was not visible with the naked eye or microscopy.

Cell Integrity. The bacterial cell membrane integrity was examined by determination of the release of material absorbing at 260 nm (*21*). Bacterial cultures grown were harvested by centrifugation at 11000*g* for 10 min, washed, and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to an absorbance of 0.7 at 420 nm. A 1.5 mL portion of derivatives solution was mixed with 1.5 mL of bacterial cell suspension, and the release over time of materials absorbing at 260 nm was monitered with a UV spectrometer (Varian, Cary 1C, Austrailia).

Outer Membrane (OM) Permeabilization Assay. The OM permeabilization activity of water-soluble chitosans was determined by the NPN assay described by Ibrahim et al. (*22*). *E. coli* cultures were harvested by centrifugation at 11000*g* for 10 min, washed, and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to obtain an A_{420} of 1.0. To a 1 mL volume of bacteria in a quartz cuvette, 20 μL of 1 mM NPN was added. The background fluorescence was record using a Hitachi F-3000 Fluorescence spectrophotometer, with 1 cm path length cuvettes. Excitation and emission wavelengths were set at 350 and 429 nm, respectively. Aliquots of derivative (various concentrations) were added. An increase in fluorescence due to partitioning of NPN into the OM was recorded as a function of time until no further increase in intensity. Control tests were performed to verify that the enhanced fluorescence was due to NPN uptake by bacteria.

Inner Membrane (IM) Permeabilization Assay. IM permeabilization was determined by measuring the release of cytoplasmic β-galactosidase activity from *E. coli* into the culture medium using ONPG as the substrate (*22*). Logarithmic-phase bacteria grown in nutrient broth containing 2% lactose were harvested, washed, and resuspended in 0.5% NaCl solution. The final cell suspension was adjusted to obtain an A_{420} of 1.2. The bacterial suspension (200 μL) was pipetted into the wells of a standard microtiter plate, and 10 μL of ONPG (30 mM) was added to each well. The production of *o*-nitrophenol over time was determined by monitoring the increase in A_{420} using a spectrophotometer.

Table 1. MIC Values of Chitosan Derivatives against Gram-Negative Bacteria

	MIC value (μg/mL)		
derivatives	*E. coli*	*S. typhimurium*	*P. aeruginosa*
AEC50	125	125	62.5
DMAEC50	62.5	62.5	31.25
DEAEC50	500	500	62.5
chitosan50	1250	625	1250
AEC90	125	125	62.5
DMAEC90	62.5	62.5	15.6
DEAEC90	250	>500	125
chitosan90	625	625	625

RESULTS AND DISCUSSION

Antimicrobial Activity of Water-Soluble Chitosans. The antimicrobial activity of chitosan has been evaluated against several fungi, bacteria, and phytopathogens, and its activity was influenced by a number of factors such as the type of chitosan, molecular mass, and some of its other physicochemical properties (*23*, *24*). According to the literature (*7*−*9*), chitosan is more effective in inhibiting the growth of bacteria than COS, and the major factor of activity is dependent on the molecular mass and degree of deacetylation. Recently, to improve the solubility of chitosan in water, several chitosan derivatives were developed via quaternary ammonium salt and were investigated for their antimicrobial activity (*10*, *11*). In the present study, we synthesized water-soluble chitosan derivatives by grafting aminofunctionality onto chitosan at the C-6 position (**Scheme 1**) because biological activities of chitosan may be affected by their free amino groups at the C-2 position. Antimicrobial activities of the derivatives were evaluated using MIC values against Gram-negative and Gram-positive bacteria (**Tables 1** and **2**). All derivatives inhibited in the growth of most Gram-negative and Gram-positive bacteria in low concentrations. Among the derivatives, DMAEC had the highest potential to suppress the growth of bacteria. All chitosan derivatives were more active against *P. aeruginosa* than the other bacteria tested and showed lower MIC values than those of native chitosan. In our previous study, chitosan with a high degree of deacetylation revealed a higher antimicrobial activity than chitosan with a low degree of deacetylation (*3*). This result suggested that a free amino group plays a pivotal role in the antimicrobial activity. In this study, we introduced an aminofunctionality onto chitosan at the C-6 position by aminoalkylation. This might be the first reason for improving the antimicrobial activity of chitosan derivatives as compared to native chitosan. Further-

Table 2. MIC Values of Chitosan Derivatives Against Gram-Positive Bacteria

	MIC value (μg/mL)		
derivatives	*S. aureus*	*L. monocytogenes*	*E. faecalis*
AEC50	125	125	62.5
DMAEC50	125	62.5	62.5
DEAEC50	250	250	125
chitosan50	625	625	1250
AEC90	125	62.6	125
DMAEC90	125	62.5	62.5
DEAEC90	250	250	125
chitosan90	625	625	625

more, chitosan derivatives might interact with cellular components of bacteria via hydrophobic interactions due to the hydrophobic moiety of the substituted group.

Jeon et al. (*8*) prepared 89% deacetylated chitosan, and the MIC values of chitosan against Gram-negative and Gram-positive bacteria were less than 0.06%. Seo et al. (*25*) tested the effect of chitosan on the growth of 11 different bacteria and found that the MIC values of chitosan ranged from 10 to 1000 ppm. Among the organisms tested, the growth of *E. coli*, *P. fluorescens*, *B. cereus*, and *S. sureus* was inhibited by chitosan concentrations of 20, 500, 500, and 1000 (that is, 0.002, 0.05, 0.05, and 0.1%, respectively), respectively. Uchida et al. (*24*) reported that the MIC values of chitosan for *E. coli* and *S. aureus* were 0.025 and 0.5%, respectively. Recently, several chitosan derivatives with water-soluble properties have been developed and investigated for their antimicrobial activity against several bacteria (*12*, *26*, *27*). According to the literature, the MIC values of chitosan derivatives were significantly decreased against all tested bacteria than those of native chitosan. In comparison with the above-published results, water-soluble chitosans prepared in this study were more effective in inhibiting the growth of bacteria. Furthermore, we previously reported (*28*) that all derivatives prepared in this study were less toxic against human normal cell lines such as MRC-5 and ECV304. These results suggest that water-soluble chitosan derivatives will be useful as food additives or for developing antimicrobial packing materials.

Cell Integrity. The cytoplasmic cell membrane undoubtedly is the target for many antimicrobial agents. When antimicrobial agents interact with bacterial membranes, they cause fundamental changes in bacterial membrane and function (*29*). When bacterial membranes become compromised by interaction with antimicrobial agents, first, low molecular mass species such as K^+ and PO_{43}^- tend to leach out, followed by DNA, RNA, and other materials. These intracellular components are easily detected by UV at 260 nm as an indication of membrane damage (*21*).

DMAEC, which has a superior antimicrobial activity as compared to other derivatives, was used for detailed study of antimicrobial action. The release of intracellular components from *E. coli* and *S. aureus* suspensions treated with DMAEC is shown in **Figure 1**. The absorbance at 260 nm was increased with time dependent upon the addition of DMAEC, and the release of intracellular components with treated DMAEC90 was higher than that of DMAEC50 despite similar MIC values. Up to 60 min, there was a dramatic increase; thereafter, the absorbance was almost unchanged. This result indicated that DMAEC90 more quickly disrupts cell membranes than DMAEC50. The absorbance of *S. aureus* suspensions was higher than that of *E. coli* suspensions because *S. aureus* does not have the OM to prevent the influx of foreign molecules (*21*).

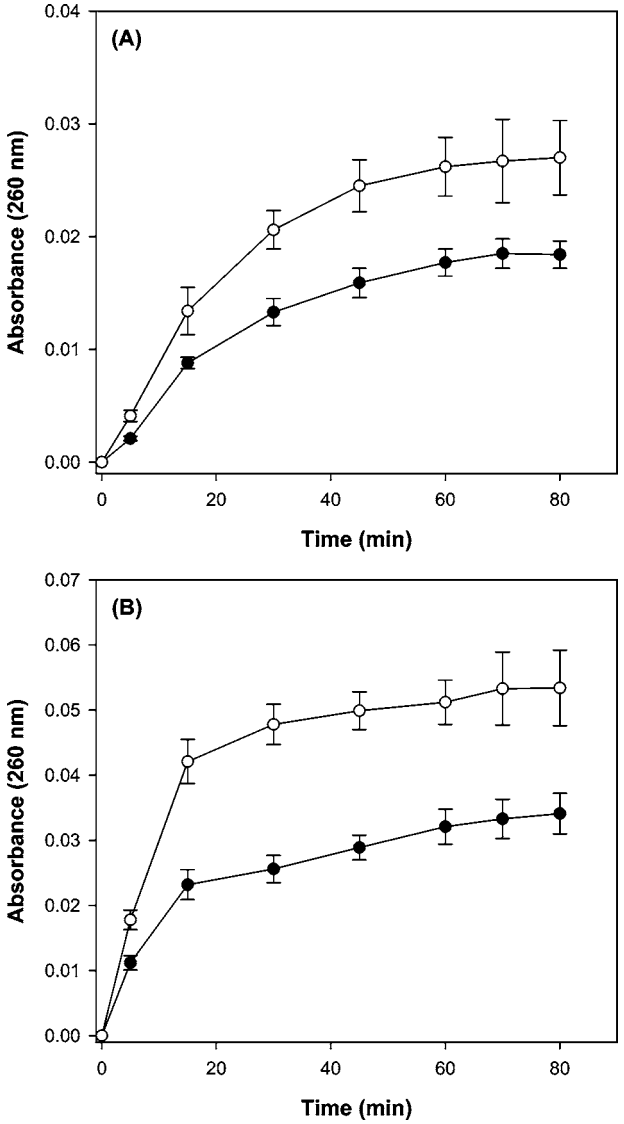


Figure 1. Release of intracellular components of *E. coli* (**A**) and *S. aureus* suspensions (**B**) treated with 73.5 μg/mL of DMAEC. DMAEC90, ○; DMAEC50, ●. The values represent means ± SD ($n = 3$).

OM Permeabilization of *E. coli*. The OM in Gram-negative bacteria plays a role as a drug barrier. NPN, which is a hydrophobic probe, is normally excluded from the OM. When the OM was damaged and functionally invalid, NPN partitioned into the perturbed OM, exhibiting increased fluorescence. On the basis of this principle, the OM permeabilization of *E. coli* by DMAEC was evaluated using the hydrophobic NPN fluorescent probe. As shown in **Figure 2**, the addition of DMAEC to *E. coli* suspensions in the presence of NPN caused a dose-dependent increase in fluorescence, which indicates that *E. coli* cell membranes were damaged by DMAEC. Moreover, DMAEC90 showed higher fluorescence values than DMAEC50. This result also strengthens the fact that DMAEC90 quickly disrupts cell membranes of bacteria more so than DMAEC50.

The OM of Gram-negative bacteria consist of lipopolysaccharides (LPSs) and proteins, and these are maintained together by electrostatic interactions with divalent cations that are required to stabilize the OM. Polycationic molecules could bind to the negatively charged O-specific oligosaccharide units of *E. coli* LPSs, thus disrupting the integrity of the OM and resulting in loss of the barrier function or blocking of the nutrient flow with concomitant bacterial death due to depletion of the nutrients. In the present study, DMAEC could bind to LPSs by

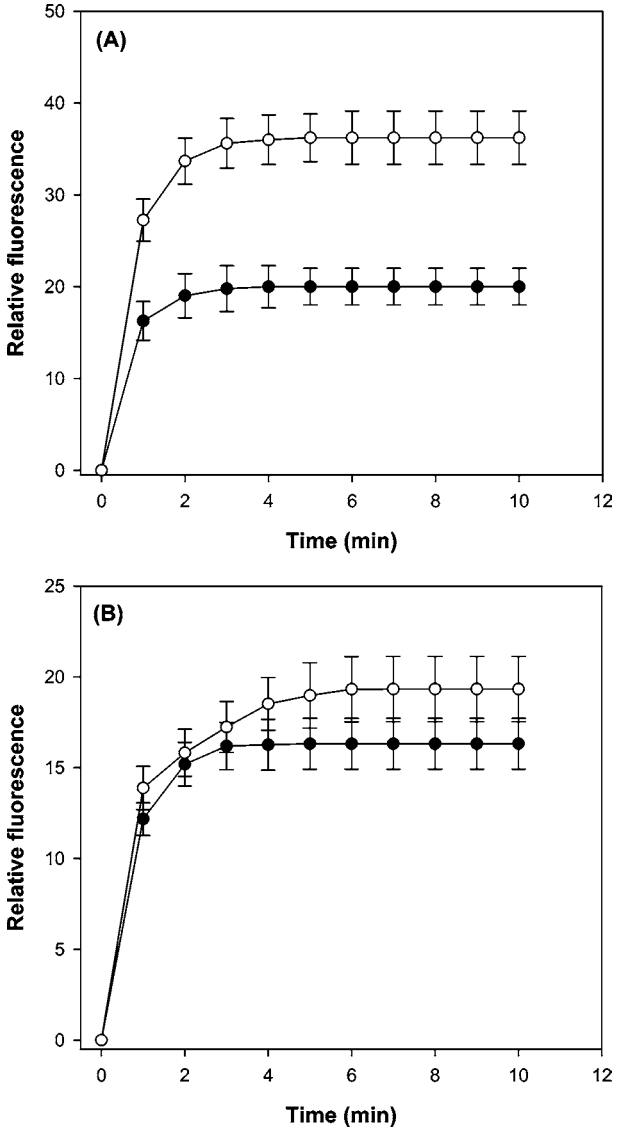


Figure 2. Uptake of NPN by *E. coli* suspensions with addition of DMAEC90 (**A**) and DMAEC50 (**B**) with 147 (○) and 73.5 μg/mL (●). The values represent means ± SD ($n = 3$).

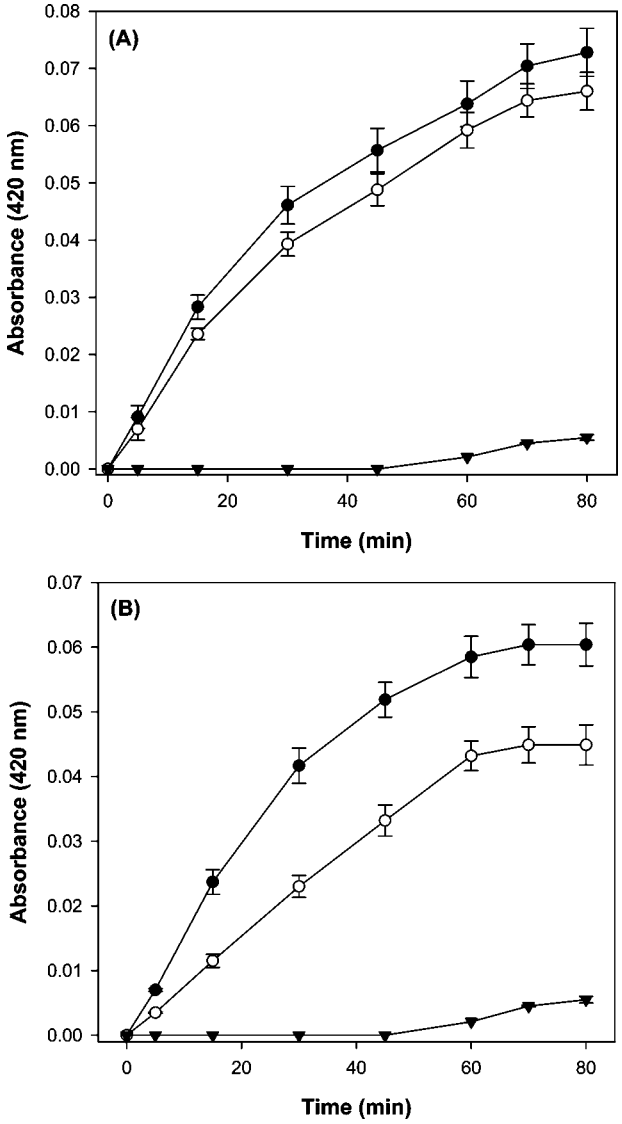


Figure 3. Release of cytoplasmic β-galactosidase activity of *E. coli* cells treated with DMAEC90 (**A**) and DMAEC50 (**B**). Control, ▼; 73.5 g/mL, □; and 147 μg/mL, ●. The values represent means ± SD ($n = 3$).

electrostatic interactions because they carried a strong positive surface charge due to the primary amines.

IM Permeabilization of *E. coli*. IM permeabilization of *E. coli* was evaluated as a function of cytoplasmic β-galactosidase release. Destabilization of the OM is necessary to gain access to the IM. In the OM permeabilization assay, DMAEC effectively interacted with the highly negatively charged O-specific oligosaccharide units of the OM consisting of the anionic LPS. Therefore, DMAEC could interact with IM components because the IM of Gram-negative bacteria is composed of anionic lipids like phosphatidyl glycerol and cardiolipin, which favor the association of cationic molecules. As shown in **Figure 3**, the release of cytoplasmic β-galactosidase was increased dose and time dependently when treated with DMAEC in *E. coli* suspensions. In control suspensions, a lag of about 40 min was followed by a relatively slow release of cytoplasmic β-galactosidase up to 80 min. This means that DMAEC has IM permeabilization activity. In comparison, the absorbance values of suspensions treated with DMAEC90 were higher than those of DMAEC50; this result also supported the fact that DMAEC90 is more active against *E. coli* than that of DMAEC50. It also agrees with the result of the OM permeabilization assay.

Conclusion. In this study, we prepared water-soluble chitosan derivatives with different degrees of deacetylation by grafting aminofunctionality onto chitosan at the C-6 position. All derivatives showed a higher antimicrobial activity than native chitosan and killed bacteria by disrupting the OMs and IMs of bacteria. These results suggested that water-soluble chitosan derivatives may be good candidates as antimicrobial agents.

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