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Antibacterial activity of aminoderivatized chitosans against methicillin-resistant *Staphylococcus aureus* (MRSA)

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ABSTRACT

This work describes the anti-MRSA activity of aminoderivatized chitosans. Two kinds of aminoethyl-chitosans (AEC), AEC90 and AEC50, having degrees of deacetylation of 90% and 50%, respectively, exhibited the strongest anti-MRSA activities by presenting MICs of 16–64 μg/mL against two standard strains and twelve clinical isolates. The bactericidal activity, thermal and pH stability, and cell membrane integrity effects of AEC90 and AEC50 are also discussed.

1. Introduction

The resistance to a number of antibiotics is of growing concern in the medical field, and has been well recognized as a global nosocomial problem in recent years. Since its discovery in early 1961, methicillin-resistant *Staphylococcus aureus* (MRSA) has been a bacterium responsible for serious infections due to its resistant to a large group of antibiotics, and has now emerged as a predominant and serious pathogenic bacterium, leading to high morbidity and mortality.[1,2] MRSA is resistant not only to β-lactams, but also to aminoglycosides, fluoroquinolones, chloramphenicol, and macrolides, because many strains of MRSA possess a multi-drug resistant genotype. Over the last decade, glycopeptide antibiotics such as vancomycin and teicoplanin have been considered the only effective antibiotics against MRSA, and are widely used to treat MRSA infections.[3] However, *S. aureus* that is resistant to glycopeptide antibiotics has now also emerged.[3–5] In view of these problems, there is an urgent need for new anti-MRSA compounds.

Chitosan, a polycationic polymer comprised of mainly glucosamine units, is known for its biocompatibility, biodegradability, and less toxic nature. Chitosans are regarded as physiologically bioactive materials since they possess antibacterial, hypocholesterolemic, antitumor, immuno-stimulating, and antioxidant characteristics.[6–10] In spite of their unique biological aspects, the water-insoluble property of chitosans is a major limiting factor for industrial applications. To this end, studies on the chemistry of chitosans, for basic data and applications, have recently increased to improve not only their water-soluble characteristic but also their biological activity. In recent years, we developed aminoderivatized water-soluble chitosans with different degrees of deacetylation, which exhibited versatile biological activities such as antioxidant, antihypertensive, enzyme inhibition, and antimicrobial characteristics.[11–14] To date, however, there is no information regarding the anti-MRSA activity of chitosan derivatives. Therefore, as part of our ongoing investigation on the biological activities of chitosan derivatives, we evaluated the antibacterial activity of aminoderivatized chitosans against MRSA.

2. Results and discussion

2.1. Antibacterial activities of aminoderivatized chitosans against MRSA

To investigate the anti-MRSA activities of the aminoderivatized chitosans, we employed a growth inhibition assay using the paper disk method. All paper disks containing aminoderivatized chitosan at 100 μg/mL made clear zones with different levels of inhibition, in which AEC90 and AEC50 showed the highest growth inhibition activities (data not shown). It is well known that antibacterial activity of chitosans was dependent on their molecular weight and degree of deacetylation. Generally, chitosans with high molecular weight and degree of deacetylation exhibited potent antibacterial activity. In particular, degree of deacetylation was major

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factor affecting antibacterial activity, and chitosan with high amounts of free amino groups showed more potent than chitosan with low degree of deacetylation.[14] In this study, we employed aminoalkyl group at C-6 position, thereby AEC90 and AEC50 exhibited more potent antibacterial activity than DMAECs and DEAECs due to their newly introduced free amino groups. Therefore, we further examined anti-MRSA activity using AEC90 and AEC50.

In order to quantitatively evaluate antibacterial activity against methicillin-susceptible *S. aureus* (MSSA) and against the MRSA strains, the MIC values of AEC90 and AEC50 were determined by a twofold serial dilution method. Among the 15 strains tested in this study, two standard MRSA and 12 clinical isolate strains were *mec*A gene positive, and one standard MSSA strain was found to be *mec*A gene negative (Table 1); this gene is responsible for the specific penicillin-binding proteins (PBP2a or PBP2́) that can reduce affinity to β-lactam antibiotics. All *mec*A gene positive strains were highly resistant to β-lactam antibiotics, including ampicillin, penicillin, and oxacillin, and their MIC values were equal to and/or greater than 64 μg/mL. However, the MIC values of these β-lactam antibiotics against MSSA were less than 1 μg/mL, respectively. The MIC values of AEC50 were in the range of 16–32 μg/mL, which was less than those of the β-lactam antibiotics against the MRSA strains. With the exception of two clinical isolates, AEC90 also showed dominant effects with MIC values in the range of 16–32 μg/mL, and exhibited higher antibacterial activities than the β-lactam antibiotics (Table 1).

It is well known that β-lactam antibiotics covalently bind with penicillin-binding proteins (PBP2), and also inactivate several enzymes, including transpeptidase and carboxypeptidase, that are responsible for catalyzing the final transpeptidation step of bacterial cell wall biosynthesis.[15,16] However, MRSA expresses PBP2a from the *mec*A gene, and PBP2a takes over the biosynthetic functions of normal PBP2 in the presence of β-lactam antibiotics, as PBP2a reduces the binding affinity to these antibiotics.[17,18] The resulting antibacterial activities of the β-lactam antibiotics used in this study (ampicillin, penicillin, and oxacillin) agree to this phenomenon (Table 1). The β-lactam antibiotics only possessed antibacterial activity against MSSA, which cannot produce PBP2a encoded by the *mec*A gene, and did not display antibacterial activity against MRSA, which can produce PBP2a. These results indicate that the antibacterial activity of the β-lactam antibiotics was greatly dependent on PBP2a. However, AEC90 and AEC50 exhibited antibacterial activities against not only MSSA, but also MRSA and the clinical isolates. These results suggest that the antibacterial activities of AEC90 and AEC50 were not specific toward MRSA, and more clearly the activities were not related to PBP2a. Our previous report also revealed that aminoderivatized chitosans exhibited good antibacterial activities against food pathogens and spoilage microorganisms.[14] These results further confirm that aminoderivatized chitosans present antibacterial activities against a broad spectrum of bacteria.

2.2. Bactericidal activity of AEC90 and AEC50

Considering their antibacterial activities, we investigated the bactericidal activities of AEC90 and AEC50 by time-kill experiment using an MRSA strain (KCCM 40510). The time-kill curves of AEC90 and AEC50 at the MIC, two times the MIC, four times the MIC, and at eight times the MIC are shown in Figure 1. The results are expressed as the log value of the number of surviving bacteria in the anti-MRSA assay at various time intervals. The initial antibacterial-free control count was 4×10^5 CFU/mL, which increased to almost 10^{10} CFU/mL after 24 h. At the MIC concentration, both AEC90 and AEC50 suppressed bacterial growth, indicating a bacteriostatic effect. In the treatments of two times the MIC, four times the MIC, and eight times the MIC, MRSA survival was dramatically decreased with increasing time and treatment concentration. In particular, at four and eight times the MIC, MRSA were completely killed after incubation for 24 h.

2.3. Thermal and pH stability of AEC90 and AEC50

Thermal stability is an important physical parameter in the design and selection of novel drug candidates. For this experiment, AEC90 and AEC50 were incubated at various temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 1 h or were autoclaved at 121 °C for 15 min, and then their anti-MRSA activities were estimated turbidimetrically at 640 nm. As shown in Figure 2, the relative anti-MRSA activities of AEC90 and AEC50 were different at high incubation temperatures. The anti-MRSA activity of AEC90 did not change at any of the test temperatures; however, the anti-MRSA ability of AEC50 was decreased at over 90 °C. In particular, the anti-MRSA activity of AEC50 was decreased around 10% after autoclaving at 121 °C, indicating that AEC50 does not have good thermal stability at high temperatures.

Generally, chitosans bears a positive charge at pH levels below 6.5, and many of their observed biological properties are supposedly due to free amino groups at the C-2 position. Therefore, we

Table 1
Minimum inhibitory concentrations (MIC) of AEC50, AEC90, and β-lactams against methicillin-susceptible *Staphylococcus aureus* (MSSA) and methicillin-resistant *S. aureus* (MRSA)

Strain	Source or reference	*mec*A [b]	MIC (μg /mL)				
			AEC50	AEC90	Ampicillin	Penicillin	Oxacillin
MSSA (KCTC 1927)	Standard strain	−	16	16	<1	<1	<1
MRSA (KCCM 40510)	Standard strain	+	32	32	512	512	128
MRSA (KCCM 40511)	Standard strain	+	32	32	512	256	128
MRSA D-3	Clinical isolate[a]	+	32	64	128	128	512
MRSA D-4	Clinical isolate	+	32	32	128	256	128
MRSA D-5	Clinical isolate	+	32	32	256	256	128
MRSA D-6	Clinical isolate	+	32	32	256	128	256
MRSA D-8	Clinical isolate	+	32	64	256	128	512
MRSA D-10	Clinical isolate	+	32	32	128	128	128
MRSA D-12	Clinical isolate	+	16	16	128	128	64
MRSA D-13	Clinical isolate	+	32	32	128	256	256
MRSA D-14	Clinical isolate	+	32	32	128	64	128
MRSA D-17	Clinical isolate	+	32	32	128	128	128
MRSA D-18	Clinical isolate	+	32	32	128	128	512
MRSA D-19	Clinical isolate	+	16	16	128	128	64

[a] MRSA strains were isolated at Dong-A University Medical Hospital.
[b] +, *mec*A positive; −, *mec*A negative.

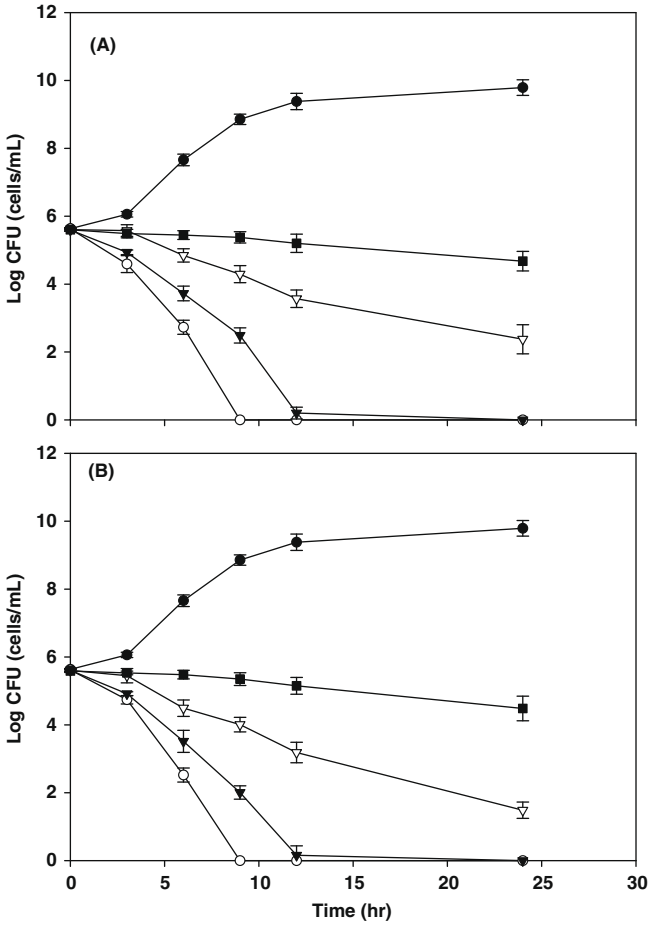


Figure 1. Comparative bactericidal activities of AEC50 (A) and AEC90 (B) against MRSA KCCM 40510. ●, Growth control; ■, MIC; ▽, two times the MIC; ▼, four times the MIC; ○, eight times the MIC. All assays were done in triplicate.

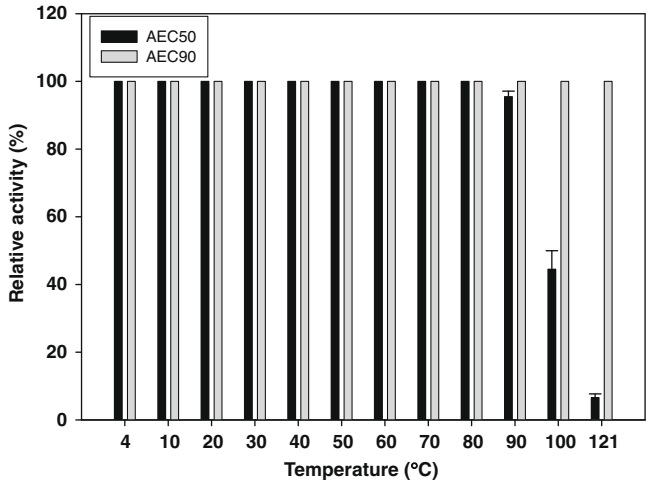


Figure 2. The thermal stability of AEC50 and AEC90. AEC50 and AEC90 were incubated at the indicated temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 1 h or at 121 °C for 15 min. All assays were done in triplicate.

also assessed the pH stabilities of AEC90 and AEC50 with respect to anti-MRSA activity. Here, AEC90 and AEC50 were incubated for 1 h in buffer solutions with different pH conditions in order to evaluate the effects of pH on their anti-MRSA activities. As shown in Figure 3, AEC90 and AEC50 maintained their anti-MRSA activities in spite of different pH conditions, indicating they were stable in the tested pH conditions.

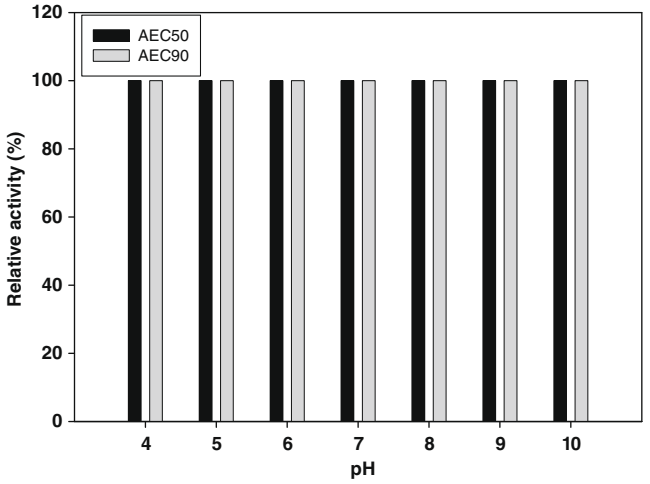


Figure 3. The pH stability of AEC50 and AEC90. AEC50 and AEC90 were suspended in 0.1 M citrate phosphate buffer at a pH range of 4–7 and in 0.1 M Tris–HCl buffer at a pH range of 8–10. They were then kept in each buffer for 1 h. All assays were done in triplicate.

2.4. Cell membrane integrity

Many antibacterial agents have been developed to target the cytoplasmic cell membrane. Bacterial cell membrane and function are frequently changed by antibacterial agents by means of interacting with the bacterial membrane.[19] If the membrane becomes compromised, low molecular weight species such as K^+ and PO_4^{3-} tend to leach out first, followed by nucleotides such as DNA, RNA, and other materials. The release of these intracellular components is determined by a strong UV absorption at 260 nm, and indicates membrane damage.[20]

The release of intracellular components upon the addition of AEC90 and AEC50 to the MRSA suspensions is shown in Figure 4. When the MRSA suspensions were treated with AEC90 and AEC50, the absorbance of the suspensions at 260 nm dramatically increased up to 40 min. Thereafter, the absorbance was almost unchanged. In addition, the release rate of the intracellular components was dose-dependent. The modes of action of antibacterial agents are related primarily to cell wall structure and to the arrangement of the outer membrane. MRSA is a Gram-positive bacterium, which does not have an outer membrane to prevent the influx of foreign molecules. Several reports have been published regarding the antimicrobial activity of chitosan, which is related to membrane permeability.[21] Liu et al.[21] studied the integrity of cell membranes using chitosan against *Escherichia coli* and *S. aureus*. The results showed that A_{260} increased rapidly at first, and then at a decreasing rate up to 120 min, and the A_{260} value was greater in the suspension treated with 0.5% as compared to 0.25% chitosan. Our previous study also revealed that A_{260} increased with increasing chitosan derivative concentration, and the A_{260} value of the *S. aureus* suspension was greater than that of the *E. coli* suspension. Considering this, the outer membrane plays a key role in protection against antibacterial agents. As shown in Figure 4, AEC90 and AEC50 both induced a fast release of A_{260} absorbing materials from MRSA, within about 40 min, suggesting that AEC90 and AEC50 killed MRSA quickly.

3. Conclusion

In the present study, water-soluble aminoderivatized chitosans were prepared and their anti-MRSA activity was evaluated. Among the chitosan derivatives, AEC90 and AEC50 exhibited the strongest anti-MRSA activities, and their MIC values ranged from 16 to

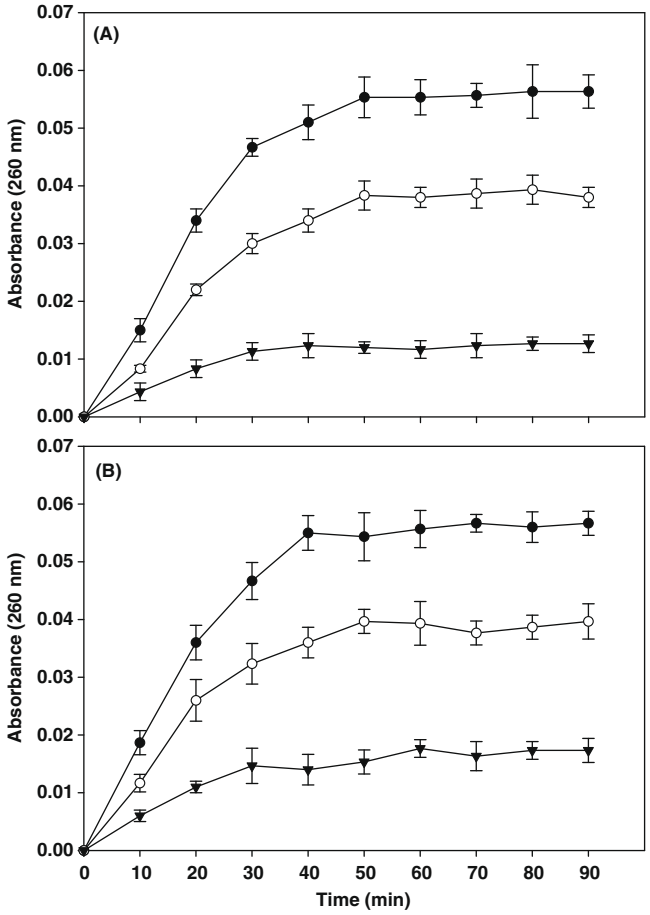


Figure 4. Cell membrane integrity effects of AEC50 (A) and AEC90 (B) against MRSA. The release of cell materials absorbing at 260 nm from MRSA suspensions induced by AEC50 and AEC90. ●, four times the MIC; ○, two times the MIC; ▼, MIC.

64 μg/mL against MRSA and clinical isolates. These values were lower than those of β-lactam antibiotics, including penicillin, ampicillin and oxacillin. Furthermore, AEC90 and AEC50 showed good thermal and pH stabilities, and killed MRSA by disrupting the cell membrane. These results suggest that aminoderivatized chitosans may be good candidates as anti-MRSA agents.

4. Materials and methods

4.1. Bacterial strains and medium

Two standard MRSA strains (KCCM 40510 and KCCM 40511) were purchased from the Korea Culture Center of Microorganisms (KCCM; Seoul, Korea), and twelve clinical MRSA isolates were kindly provided by the Donga-A University Hospital (Busan, Korea). The single methicillin-susceptible *S. aureus* (MSSA) tested in this study was purchased from the Korean Collection for Type Cultures (KCTC; Daejeon, Korea). All strains were grown aerobically at 37 °C in Mueller Hinton broth (MHB; Difco, USA), and were subsequently used in experiments to measure antibacterial activity.

4.2. Preparation of aminoderivatized chitosan derivatives

The chitosans, with their degrees of deacetylation (90% and 50%) determined by FT-IR and a titration method in our previous report, were prepared from crab chitin by N-deacetylation under akali conditions.[22] The average molecular weights of the chitosans were 3.1×10^5 as determined by viscosimetry.[23] The aminoderivatized chitosans were prepared according to our previous method (Scheme 1).[13] Aqueous 3.0 M (15 mL) aminoalkyl hydrochloride was added to the chitosan (0.30 g) with stirring at 65 ℃. NaOH of 3.0 M (15 mL) was added to the reaction mixture dropwise, and continuously stirred for 18 h. Subsequently, the reaction mixture was acidified with HCl (0.1 M and 0.0001 M), and dialyzed against water for 2 days. The product was freeze-dried to give the aminoderivatized chitosans (AEC90: 0.412 g, DMAEC90: 0.393 g, DEAEC90: 0.489 g, AEC50: 0.401 g, DMAEC50: 0.376 g, and DEAEC50: 0.476 g), which were found to dissolve well in water and at all pH ranges. The water-soluble chitosan derivatives prepared from 90% deacetylated chitosan were designated as aminoethyl-chitosan (AEC90), dimetylaminoethyl-chitosan (DMAEC90), and diethylaminoethyl-chitosan (DEAEC90), and those prepared from 50% deacetylated chitosan were designated as AEC50, DMAEC50, and DEAEC50. The FT-IR spectra showed a new peak at 2965 cm^{-1} due to C–H stretching of the substituted groups. The ^1H NMR spectra of DEAEC90 in D_2O showed the following peaks: a peak at 1.30 ppm for the methyl unit, at 3.28 ppm for the methylene protons of the DEAE group, and between 1.5 and 1.6 ppm for the methyl protons of the protonated DEAE groups. In the same manner, peaks were observed between 2.9 and 3.0 ppm for the methyl and methylene protons of the DMAE group, and a peak at 2.9 ppm for the methylene protons of the AE group.[13] AEC50, DMAEC50, and DEAEC50 were characterized in the same manner.

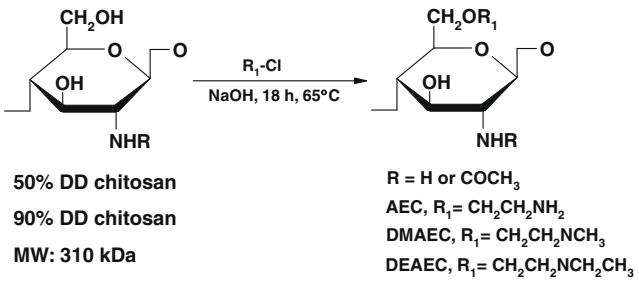
4.3. Measurement of anti-MRSA activity

The anti-MRSA activity of the derivatives was estimated by a growth inhibition assay. The MRSA were spread on Mueller Hinton agar plates and paper disks containing the chitosan derivatives were then placed on the plates. After 24 h at 37 °C, the paper disk containing the chitosan derivatives (100 μg/mL), which made a clear zone indicating MRSA growth inhibition, was selected.

The minimum inhibitory concentrations (MICs) of the chitosan derivatives and β-lactams, including ampicillin, penicillin, and oxacillin, against MRSA were determined by the twofold serial dilution method as described by the National Committee for Clinical Laboratory Standards.[24] The MIC was defined as the lowest concentration that demonstrated no visible growth after incubation at 37 °C for 24 h.

4.4. Bactericidal activity of chitosan derivatives

Here, the chitosan derivatives were added to MH broth inoculated with an MRSA strain (KCCM 40510), which was adjusted to an estimated cell density of 4×10^5 CFU/mL, followed by culture at 37 °C for 24 h. The final concentrations of the chitosan derivatives consisted of the MIC, two times the MIC, four times the MIC, and eight times the MIC. Samples were taken at several time intervals. The anti-MRSA activity was evaluated by determining the viable cell counts of the MRSA strain after 24 h of incubation.



Scheme 1. Structure of aminoderivatized chitosans

4.5. Thermal and pH stability of the chitosan derivatives

To investigate their thermostability, the chitosan derivatives were incubated at several temperatures (4, 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100 °C) for 1 h, or at 121 °C for 15 min. For pH stability, the derivatives were suspended in 0.1 M citrate phosphate buffer at a range of pH 4–7, and in 0.1 M Tris–HCl buffer at pH 8 to 10; they were kept in each buffer for 1 h. After treatment, anti-MRSA activity was estimated turbidimetrically at 640 nm.

4.6. Cell membrane integrity

Bacterial cell membrane integrity was examined by determining the release of cell materials absorbing at 260 nm, as described by Chen and Cooper.[20] The seed cultures of MRSA (KCCM 40510) in MHB were harvested by centrifugation at 11,000 *g* for 10 min, washed twice, and resuspended in 0.9% NaCl solution. The absorbance was adjusted to 0.7 at 420 nm. The MRSA suspensions were exposed to the chitosan derivatives at the MIC concentration, two times the MIC concentration, and at four times the MIC concentration. Then, the release over time of materials absorbing at 260 nm was measured with a UV spectrometer at 10-min intervals.

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