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Quaternized chitosans bind onto preexisting biofilms and eradicate pre-attached microorganisms

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Abstract

Quaternized chitosans, N,N,N-trimethylchitosans (TMC) with different degree of quaternization were synthesized by reacting methyl iodide with chitosan. The reaction was confirmed by FT-IR and ¹H-NMR characterization. Antimicrobial assay showed that the prepared TMC had potent biocidal effects against planktonic Gram-positive bacteria *Staphylococcus epidermidis*, Gram-negative bacteria *Escherichia coli*, and yeast *Candida albicans*. Bacterial and fungal biofilms were formed on poly(methyl methacrylate) (PMMA) films and then treated with TMC aqueous solutions. Zeta potential measurement suggested that TMC bonded onto the preexisting biofilms. Biofilm-binding kinetics was evaluated in UV studies using phenyl group-labeled TMC as model compounds, which revealed that quaternized chitosans bonded onto the preexisting biofilms rapidly. Colony-forming unit (CFU) determination and SEM, confocal laser scanning microscopy (CLSM) and fluorescence microscopy studies demonstrated that the bonded TMC had powerful biocidal activities to eradicate the pre-attached bacterial and fungal cells in the preexisting biofilms. The biocompatibility of the TMC samples with rat skin fibroblast cells was evaluated in the MTT assay.

Keywords

Quaternized chitosan; Biofilm-binding; Biocides; Biocompatible

1 Introduction

Microbial biofilm formation on medical devices has been a continuing problem, often leading to serious morbidity and mortality, excess length of hospital stay, and extra costs.¹⁻⁵ Biofilms are likely initiated by just a few microorganisms that come into contact with and

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adhere to a clean surface.⁶⁻¹⁰ Once adhered to the surface, microorganisms multiply and accumulate in multilayered cell clusters. Many factors, such as polysaccharide intercellular adhesin, extracellular accumulation-associated protein, physiological status changes, and intercellular signaling, can affect accumulation and biofilm formation.¹¹⁻¹⁴ Microorganisms living in a biofilm are up to 1,000 times more resistant to antimicrobial agents than their planktonic counterparts.^{1,15-17} This high resistance is likely due to the combined actions of many factors, including delayed penetration of the antimicrobial agents into the biofilm matrix, the presence of neutralizing enzymes, and the expression of resistant phenotypes.^{1,5,17} Although biofilms are firmly attached to the surface, microorganisms may dislodge from the macro-colony and drift into the environment, causing more serious problems.

Because biofilms are complex, multilayered structures,^{1,5,7} effective approaches to disinfect preexisting biofilms should include antimicrobial agents that can bind onto biofilms and eradicate pre-attached microorganisms, which are still lacking in general practice. Here, we report that quaternized chitosans are such *biofilm-binding biocides*. Chitosan is a natural cationic polymer produced by deacetylation of chitin, an abundant natural polysaccharide that exists in the exoskeleton of crustaceans and the cell walls of fungi. Chitosan is hypoallergenic, biocompatible, antimicrobial, and biodegradable, making it an attractive biopolymer for biomedical applications.¹⁸⁻²¹ Since chitosan has limited water solubility under neutral and basic conditions, a range of water-soluble chitosan derivatives, including quaternized chitosans, have been prepared.²²⁻³⁶ It has been found that quaternized chitosans are antimicrobial, biocompatible, and bioadhesive, which can be potentially used in tissue engineering, permeation enhancement, drug delivery, gene delivery, and wound care. Although the activity of quaternized chitosans in killing free-swimming microorganisms and preventing the formation of biofilms on clean surfaces has been studied previously,²²⁻³¹ the binding of quaternized chitosan onto preexisting biofilms and its effects on pre-attached microorganisms have not been reported. In this study, we found that quaternized chitosans are capable of binding onto preexisting bacterial and fungal biofilms and kill the pre-attached microbes. These findings suggest that it is feasible to use quaternized chitosans to treat preexisting biofilms and kill the pre-attached bacterial and fungal cells so as to reduce the risk of biofilm-related complications, shedding new lights on managing biofilms and controlling infections in a wide range of biomedical applications.

2 Results and Discussion

2.1 Preparation of Quaternized Chitosans, N,N,N-trimethylchitosan (TMC)

In the current study, the quaternized chitosans, TMC, were synthesized by reacting methyl iodide with chitosan.³² The reaction was confirmed by FT-IR studies, as shown in Figure 1a. The bands at 3400 cm^{-1} , 1654 cm^{-1} , 1473 cm^{-1} , and $1150\text{-}890\text{ cm}^{-1}$ were in correspondence with the hydroxyl and amino groups, carbonyl groups, N-CH_3 groups, and the β -1,4-linked GlcN/GlcNAc saccharide structure in TMC, respectively, in good agreement with the published data.²⁷ In $^1\text{H-NMR}$ study (Figure 1b), the signal at 3.10 ppm was related to the protons on the quaternized ammonium group $[\text{N}(\text{CH}_3)_3]$, and the signal at 5.10 ppm corresponded to the H-1 protons of the acetylglucosamine units. Thus, degree of

quaternization (DQ) could be calculated according to the previously reported method.^{26,32,33,37}

The DQ of TMC prepared with 1:1, 2:1, and 3:1 of methyl iodide to chitosan (the molar ratio of methyl iodide to the amino groups on chitosan) was 28%, 47% and 56%, respectively. The samples were labeled as TMC28, TMC47 and TMC56 for the following studies. Their intrinsic viscosities and viscosity average molecular weights (Table 1) were tested following published procedures.²⁸ All the three samples were soluble in water in the pH range of 1.0-10.0.

TMC with higher DQ (>56%) could be synthesized by further increasing the amount of methyl iodide in the quaternization reactions. However, these TMC samples had much lower solubility in water. Our ¹H-NMR studies showed distinct signals at 3.47 ppm and 3.55 ppm (spectrum not shown), in addition to the characteristic signals of TMC, suggesting the presence of O-methylated chitosan with limited water solubility.³² Since the purpose of the current study was to test whether water soluble quaternized chitosan could bind onto preexisting biofilms and kill pre-attached microorganisms, those samples with decreased water solubility were not investigated in the following studies.

2.2 Biocidal Activities of TMC against Planktonic Bacterial and Fungal Cells

In biocidal tests, *Staphylococcus epidermidis* (*S. epidermidis*), *Escherichia coli* (*E. coli*), and *Candida albicans* (*C. albicans*) were selected to represent Gram-positive bacteria, Gram-negative bacteria, and yeast, respectively. These microorganisms are known for their involvement in various biofilm-related infections.¹⁻⁵ All the three TMC samples, TMC28, TMC47 and TMC56, demonstrated biocidal activities against the test microorganisms (Table 2). Interestingly, higher DQ led to lower minimal bactericidal concentration (MBC) for *S. epidermidis*; lower DQ resulted in lower MBC for *E. coli*; and in the case of *C. albicans*, however, increasing DQ from 28% to 56% had little effect on the value of MBC. These findings showed the different biocidal efficiencies of TMC against different microorganisms.

Although the biocidal mechanisms of quaternized chitosans are not completely clear, it is generally accepted that electrostatic interactions between the polycationic structure of TMC and the anionic components on microorganisms play a critical role in antibacterial activity.^{23,38} Higher DQ could lead to stronger interaction with the bacterial cell surfaces to disturb cell wall functions, and this could at least partially explain the findings in the testing of the Gram-positive bacteria *S. epidermidis* (Table 2). In the case of the Gram-negative bacteria *E. coli*, the MBC values were much higher than those of *S. epidermidis* (Table 2). This could be caused by the barrier functions of the outer membrane in Gram-negative bacteria. On the other hand, in addition to electrostatic interactions, the free amino groups on TMC could form complexes with Mg²⁺ and Ca²⁺ cations on the outer membrane, which could comprise membrane integrity, a prerequisite for antimicrobial actions against Gram-negative bacteria.^{23,39,40} Since TMC samples with lower DQ have more remaining free amino groups to react with Mg²⁺ and Ca²⁺ cations, it was not surprising that TMC28 had a lower MBC value than TMC47 and TMC56 against *E. coli*. As for *C. albicans*, the three TMC samples showed the same, low MBC value of 0.25 mg/mL. It was believed that the

antifungal activities of TMC could also be originated from the electrostatic interactions between the cationic groups on the polymer and the anionic residues on fungal cell surfaces.^{23,41-44} The absorption of TMC chains onto the fungal cell surface could block the transport of nutrients into the cells, leading to death.⁴⁵

2.3 Binding of TMC onto Preexisting Bacterial and Fungal Biofilms

In this study, poly(methyl methacrylate) (PMMA) was selected as a typical example of biomedical materials because of its multiple dental and medical applications including complete denture bases, bone cements, screws for bone fixation, fillers for bone cavities and skull defects, and vertebrae stabilization in osteoporotic patients, in which biofilm formation is of great concerns.⁴⁶ We found that *S. epidermidis*, *E. coli* and *C. albicans* readily formed biofilms on the un-treated PMMA. As shown in Figure 2, in zeta potential studies, pure PMMA film had a zeta potential of -0.08 ± 0.15 mV, suggesting a neutral/weakly anionic surface. After the formation of *S. epidermidis*, *E. coli* or *C. albicans* biofilms on PMMA surfaces, the zeta-potential values were -31.31 ± 1.81 mV, -42.32 ± 1.57 mV, and -5.32 ± 1.01 mV, respectively. This significant change in zeta potentials was believed to be caused by the anionic components on the microbial cells and/or the extracellular matrixes (ECMs) of the formed biofilms.³⁸⁻⁴⁷ After TMC treatment, the biofilm-containing films were transformed into highly positively charged surfaces with zeta potentials at 17.71 ± 1.81 mV, 12.62 ± 1.62 mV, and 20.90 ± 0.99 mV for *S. epidermidis*, *E. coli* and *C. albicans* biofilms, respectively, suggesting that TMC bonded onto the preexisting biofilms and covered the biofilm surfaces.

TMC in aqueous solutions cannot be detected by conventional spectroscopic methods, making it difficult to follow the biofilm-binding process. To solve this problem, we synthesized phenyl group labeled TMC (Ph-TMC) as a model compound of TMC. Ph-TMC has UV absorbing property to allow for biofilm-binding kinetic studies. As illustrated in Figure 3, TMC28 did not show any UV absorption. However, after labeling with phenyl groups, the new Ph-TMC showed UV absorption centered at 235.2 nm. Thus, the binding amounts of the quaternized chitosans onto preexisting microbial biofilms could be calculated using UV absorptions (original Ph-TMC concentration minus Ph-TMC concentration in the media after different periods of binding).

It was found that when Ph-TMC was added to the control PMAA films, no Ph-TMC binding onto the PMMA surfaces was observed. On the other hand, binding of Ph-TMC was detected on the biofilm-containing PMMA films after 1 h incubation in the range of tens of micrograms per square centime ($\mu\text{g}/\text{cm}^2$, Figure 4). Further increase in treatment time had no significant effects on Ph-TMC binding amounts ($p > 0.05$).

To our best knowledge, these zeta potential and UV (using Ph-TMC as a model compound) results are the first to demonstrate that the quaternized chitosans bind onto preexisting bacterial and fungal biofilms. This biofilm-binding activity could be due to the electrostatic interactions between the positively charged TMC polymer chains and the negatively charged microbial biofilms.

2.4 Effects of Bonded TMC against Pre-attached Bacterial and Fungal Cells

Binding of antimicrobial agents onto preexisting biofilms could increase local antimicrobial concentration within the biofilms and extend residence time, both of which could lead to potent biocidal effects against the pre-attached microorganisms. To evaluate these effects, TMC28 was used for the following study since this sample had the highest biocompatibility with model mammalian cells (see Section 2.5). The effects of TMC28 concentration in the solution on biocidal effects against the pre-attached microbes in the preexisting biofilms were shown in Figure 5. As low as 0.5 mg/mL of TMC28 in the solution significantly decreased the level of recoverable adherent bacteria, *i.e.*, *S. epidermidis* and *E. coli* after 24 h of treatment, even though the MBC of TMC28 against free-floating *E. coli* was 3.0 mg/mL. Further increase in TMC28 concentration in the solution did not markedly enhance biocidal activities against the pre-attached bacteria. This could be explained by the binding of TMC28 onto the bacterial biofilms: in such a system, biocidal activities were determined by the local concentration of TMC28 within the biofilm. When binding saturation was achieved, further increase in TMC28 concentration in the solution would not increase its local concentration in the biofilm. A similar trend was observed in the evaluation of pre-attached *C. albicans*, except that higher concentrations (2.0 mg/mL) of TMC28 were required to significantly reduce the level of recoverable colony-forming units (CFUs). These results further demonstrated that *Candida* cells living in biofilms are more resistant to antifungal agents than their free-swimming counterparts, *e.g.*, the MBC of TMC28 for *C. albicans* was 0.25 mg/mL (Table 2).

TMC28 provided fast antibacterial activities against pre-attached *S. epidermidis* and *E. coli* in the preexisting biofilms. In the control biofilms (without TMC28 treatment), *S. epidermidis* was recovered at a level of $(8.32 \pm 2.48) \times 10^6$ CFU/cm². After 30 min of TMC28 treatment, the recoverable bacterial level was significantly reduced to $(6.42 \pm 0.78) \times 10^5$ CFU/cm² (92.3% of reduction, Figure 6 A). When the contact time was increased to 2 h, the reduction level was increased to 99.8%. A similar bactericidal trend was observed in the testing of *E. coli* (Figure 6, B), even though the MBC of TMC28 against free-floating *E. coli* was 3.0 mg/mL (Table 2). Again, this could be explained by the binding of TMC28 from the solution to the biofilm (Figures 2-4), which increased the local TMC28 concentration, leading to potent antibacterial effects against the pre-attached *E. coli*.

As discussed above, the antibacterial activities of the TMC could be originated from electrostatic interactions between TMC and the microorganisms and/or competition for divalent cations to disturb cell wall functions. Apparently, these two effects occurred rapidly, leading to fast antibacterial activities of TMC28 against the pre-attached *S. epidermidis* and *E. coli*.

However, the antifungal effects of TMC28 against pre-attached *C. albicans* were much slower than antibacterial activities (Figure 6, C). In the control, $(5.21 \pm 2.65) \times 10^4$ CFU/cm² of adherent *C. albicans* were recovered. No significant differences were detected in the level of recoverable adherent *C. albicans* after 4 h of contact. After 8 h of contact, the recoverable level was significantly decreased to $(2.26 \pm 0.24) \times 10^4$ CFU/cm² ($p < 0.05$). When the contact time was extended to 48 h, the reduction level was increased to 99.3%. The slow

antifungal reaction against pre-attached *C. albicans* suggested that *C. albicans* biofilms had different characteristics from the bacterial biofilms and/or the pharmacology effects of TMC were different between the fungi and the bacteria. TMC would take a longer time to reach equilibrium with *Candida* cell wall and it had lower efficacy on killing pre-attached *C. albicans* in biofilms.

The biocidal effects of TMC against pre-attached bacterial and fungal cells were further confirmed with SEM and CLSM/fluorescence microscopy studies. In SEM evaluations, before TMC28 treatment, a large amount of *S. epidermidis* (Figure 7, A), *E. coli* (Figure 7, C), and *C. albicans* (Figure 7, E) adhered and aggregated on the surface of PMMA films, forming micro-colonies and developing into biofilms. After TMC28 treatment, only scattered adherent bacterial or fungal cells could be observed, some of which were of abnormal shapes (Figure 7, B, D, and F). CLSM studies using nucleic acid specific SYTO 9 (fluoresce green for live cells) and propidium iodide (PI, fluoresce red for dead cells) staining indicated that the dusts/abnormal shapes were caused by dead/dying *S. epidermidis* (Figure 8, B) or *E. coli* (Figure 8, D). Similarly, fluorescence microscopic images (Figure 8, F) using Calcofluor White M2R for staining, which was widely used to identify *C. albicans*, suggested that the objects with abnormal shapes were indeed dead or dying *C. albicans*, confirming the potent biocidal effects of TMC28 against pre-attached bacterial and fungal cells.

2.5 *In vitro* Biocompatibility of TMC

The biocompatibility of TMC with mammalian cells was investigated by the MTT assay using rat skin fibroblast cells as a model. As shown in Figure 9, the viability of the fibroblast cells was not significantly affected by the presence of up to 1.0 mg/mL of TMC28 after 24 h of contact. At even higher TMC28 content, the viability was significantly decreased ($p < 0.05$), and the half maximal inhibitory concentration was estimated to be 3.10 mg/mL (figure not shown). Since 1.0 mg/mL of TMC28 could significantly reduce the level of pre-attached microorganisms in preexisting biofilms, these findings point to great potentials of the quaternized chitosans as biocompatible, biofilm-binding biocides for a wide range of biofilm-control/infection-reduction related applications.

3 Experimental

3.1 Materials

Chitosan samples were obtained from TCI America (C0831; viscosity: 200-600mPa·s, 0.5% in 0.5% acetic acid at 20°C; deacetylation value: min. 80.0%). Methyl iodide, polymethylmethacrylate (PMMA), phenylisocyanate, and N-methyl-2-pyrrolidinone (NMP) were purchased from Fisher Scientific (USA). All other chemicals were analytical grade and used as received.

Staphylococcus epidermidis (*S. epidermidis*; ATCC 35984), *Escherichia coli* (*E. coli*, ATCC 15597), *Candida albicans* (*C. albicans*, ATCC 10231) and the model mammalian cells, rat skin fibroblast cells (ATCC CRL-1213) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA).

3.2 Preparation of N,N,N-trimethylchitosans (TMC)

The quaternized chitosans, N,N,N-trimethylchitosans (TMC), were synthesized by reacting methyl iodide with chitosan following a previously reported procedure.³² Briefly, 4.0g chitosan were dispersed in 160 mL NMP under constant stirring at 90 °C for 24 h; 9.6g sodium iodide and 22.0 mL of 15wt % sodium hydroxide aqueous solution were added into the flask, followed by the addition of methyl iodide (1:1 to 3:1 molar ratio of amino groups on chitosan). The mixture was stirred at 90 °C for 18 h. The solution was filtered, and added into acetone. The precipitates were collected by filtration, dried at 50 °C under vacuum, and then dissolved in 10 wt% sodium chloride aqueous solution to exchange iodide ions with chloride ions. The final product (TMC) was precipitated with ethanol, dried at 50 °C under vacuum, and stored in a desiccator.

Viscosity average molecular weights of the TMC samples were calculated from intrinsic viscosity data (tested in 2% acetic acid/0.2 M sodium acetate at 25 ± 0.1 °C in triplicate) following published procedures.²⁸ FT-IR spectra of the samples were recorded using KBr discs on a Nicolet iS10 Mid-IR spectrometer. The structure and degree of quaternization (DQ) of the samples were determined with a 600 mHz ¹H-NMR spectrometer (Bruker, Switzerland) using D₂O as solvent.³³

3.3 Determination of the Minimal Bacterial Concentration (MBC) of TMC against the Bacterial and Fungal Cells

The microdilution method⁴⁸ was followed to determine the MBCs of the TMC samples against *S. epidermidis* (Gram-positive bacteria, ATCC 35984), *E. coli* (Gram-negative bacteria, ATCC 15597), and *C. albicans* (yeast, ATCC 10231). For *S. epidermidis*, a colony of the 3rd generation *S. epidermidis* grown on tryptic soy agar was inoculated into 5 mL tryptic soy broth and grew at 37 °C for 24 h. The *S. epidermidis* suspension was diluted with fresh broth solution to a 0.5 McFarland Standard.⁴⁸ One milliliter of the inoculum was incubated with different amounts of TMC at 37 °C for 24 h. Afterwards, 100 µL of the mixture was spread onto tryptic soy agar plates and incubated at 37 °C for 24 h. MBC was defined as the lowest concentration of TMC at which no visible microbial growth could be detected on the agar plates. Each assay was performed in triplicate. The MBCs of TMC against *E. coli* and *C. albicans* were determined similarly, except that in the testing of *E. coli*, EC agar and broth were used as the growth media; in the testing of *C. albicans*, YM agar and broth were used, and the yeasts were grown at ambient temperature for 48 h.

3.4 Bacterial and Fungal Biofilm Formation on PMMA Films

PMMA films were fabricated by dissolving PMMA powders in acetone to prepare 10% (w/v) polymer solutions. Acetone was slowly evaporated in a fume hood. Films were cut into small pieces (0.6 cm of diameter and 0.25±0.025 cm of thickness) and sterilized with UV light.

In the preparation of microbial biofilms on PMMA films, bacteria or yeast cells were grown in the corresponding broth solutions (see the section above) and adjusted to a density of 10⁸⁻⁹ CFU (colony forming unit)/mL. Sterile PMMA films were cultured with the microbial

cells at 37 °C for 3 days (for *S. epidermidis* and *E. coli*), or at ambient temperature for 6 days (for *C. albicans*) to allow microbial adhesion and biofilm formation.

3.5 Zeta Potential Measurements

S. epidermidis, *E. coli*, or *C. albicans* biofilms on PMMA films were gently washed with sterile PBS 3 times to remove non-adherent microbial cells. Some of the films were tested with a Beckman Coulter® Delsa™ NanoHc particle Analyzer (Beckman, Brea, CA) with a flat surface cell assembly to determine surface zeta potentials of the biofilms. Pure PMMA films (without preexisting bacterial or fungal biofilms) were tested under the same conditions to serve as controls. The rest of the biofilm-containing PMMA samples were placed in PBS solutions containing 0 - 6.0 mg/mL of TMC for incubation at 37 °C overnight. After gently washing with PBS, the surface zeta potentials of the TMC-treated biofilm-containing films were tested. Each measurement was repeated 4 times.

3.6 Preparation of Phenyl Group Labeled TMC (Ph-TMC)

Ph-TMC was synthesized to introduce UV absorbing function to TMC for the biofilm-binding kinetic studies. Briefly, 0.5 g TMC was dispersed in 50 mL NMP containing 0.1 mL phenylisocyanate. The reaction mixture was stirred at 90 °C for 8 h. The resulting Ph-TMC was precipitated from acetone, collected by filtration, and dried under vacuum at 50 °C for 24 h. The structures the Ph-TMC were confirmed by FT-IR and ¹H-NMR, and UV studies were performed on a Beckman Coulter DU® 520 UV/Vis spectrophotometer. The UV calibration curves of Ph-TMC aqueous solutions were obtained at the concentration range of 0~400 µg/mL.

3.7 Binding of Ph-TMC onto Preexisting Bacterial and Fungal Biofilms

Biofilms of *S. epidermidis*, *E. coli*, or *C. albicans* on PMMA films were prepared as described above, gently washed with sterile PBS 3 times to remove non-adherent microbial cells, and placed in PBS solutions containing 200 µg/mL of Ph-TMC. Concentrations of Ph-TMC in the solution were monitored over a 24h-period with a Beckman Coulter DU® 520 UV/Vis spectrophotometer. UV readings were used to calculate the binding amount of Ph-TMC onto the preexisting biofilms on the films. Each measurement was repeated 4 times. Ph-TMC incubated with pure PMMA films (without preexisting bacterial or fungal biofilms) were tested under the same conditions to serve as controls.

3.8 Effects of TMC on Pre-attached Bacterial and Fungal Cells

A series of freshly prepared *S. epidermidis*, *E. coli*, or *C. albicans* biofilms on PMMA were placed into PBS solutions containing 0-6.0 mg/mL of TMC. The vials were shaken gently at 37 °C in a water bath. At different time points, the PMMA films were removed from the immersing solutions with sterile forceps for microorganism culture and imaging assays. The films were gently washed 3 times with sterile PBS. A portion of the films was sonicated individually for 5 min using an ultrasonic cleaner (Branson 1510) and vortexed for 1 min to detach the adherent microorganisms into sterile PBS.^{16,49} The resulting solution was then serially diluted, and 100 µL of each diluent was placed onto the corresponding agar plates for overnight incubation at 37 °C (for *S. epidermidis* and *E. coli*) or for 48 h incubation at

ambient temperature (for *C. albicans*). The CFUs on each agar plate were counted, the level of recoverable adherent microorganisms on each sample was calculated, and the results were presented as CFU/cm². Each test was repeated 3 times.

Another set of the washed films was prepared for scanning electronic microscopy (SEM) studies by treating in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer at 4 °C overnight, serial dehydration with ethanol, and sputter coating with Pd-Au. SEM observation was performed on a JEOL JSM 7401 FE-SEM.

The remaining film samples were further subjected to confocal laser scanning microscopy (CLSM) or fluorescence microscopy examination. In the studies of *S. epidermidis* and *E. coli* biofilms, the biofilm-containing films were exposed to nucleic acid specific SYTO 9 and propidium iodide (PI) using *LIVE/DEAD*® *BacLight*™ Bacterial Viability Kits L7007 (Molecular Probes, Eugene, OR, USA) in dark for 15 min. CLSM observations were performed on a Olympus LSCM FV300 microscope (Tokyo, Japan) at 50 × magnification, using wavelengths of excitation 488 nm/emission 522 nm for SYTO 9 and excitation 568 nm/emission 605 nm for PI.⁵⁰ For *C. albicans* biofilms, the samples were stained with Calcofluor White M2R stock solution (Molecular Probes, Eugene, OR, USA) and incubated in dark for 30 min. Fluorescence microscopy images were taken at wavelengths of excitation 358 nm/emission 461 nm.

3.9 Biocompatibility Evaluation

The biocompatibility of the TMC samples was evaluated with the MTT tests using rat skin fibroblast cells (ATCC CRL-1213) as model mammalian cells. Rat skin fibroblast cells were cultured in a medium (DMEM with 10% FBS) at 37 °C in a humid atmosphere with 5% CO₂ and 95% air. The cells were trypsinized, and 100 µL of the cell suspension (5×10^5 cells/mL) was cultured in a 96-well plate at 37 °C with 5% CO₂ and 95% air for 24 h. Afterwards, cells were exposed to various amount of TMC (0-4.0 mg/mL) in fresh culture media. At the end of the 24 h incubation, 10 µL of MTT reagent (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) was added into each well and the mixture was incubated with cover in the dark for 4 h. After removing the growth medium, 100 µL DMSO was added to each well and the absorbance at 562 nm was measured on an Infinite® 200 PRO multimode reader. Cells incubated without the presence of TMC were tested under the same conditions as controls. Half maximal inhibitory concentration (IC₅₀) was estimated by putting percentage inhibition on the Y axis and log (TMC concentration) on the X axis. After determining the maximum and minimum inhibition levels, IC₅₀ was estimated as the TMC concentration that led to 50% inhibition of the rat skin fibroblast cells.

3.10 Data Analysis

Data were representative of at least three independent experiments and analyzed by student's *t* test. The *p* value of less than 0.05 was considered significant.

4 Conclusions

Quaternized chitosans, N,N,N-trimethylchitosans (TMC), were synthesized by reacting methyl iodide with chitosan. The TMC samples provided potent biocidal effects against

planktonic Gram-positive bacteria *S. epidermidis*, Gram-negative bacteria *E. coli*, and yeast *C. albicans*. More importantly, TMC bonded onto preexisting bacterial and fungal biofilms and eradicated the pre-attached microorganisms in the biofilms. Although more studies are needed to determine the exact biofilm-binding mechanisms, quaternized chitosans showed great potentials as a class of biofilm-binding biocides for a wide range of biomedical applications.

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Fig. 1.
a FT-IR spectrum of (A) TMC28 and (B) chitosan, and **b** ¹H-NMR spectrum of TMC28 in D₂O

Fig. 2.

Zeta-potentials of (A) pure PMMA film, (B) PMMA film with preexisting *S. epidermidis* biofilm, (C) PMMA film with preexisting *E. coli* biofilm, (D) PMMA film with preexisting *C. albicans* biofilm, (E) PMMA film with preexisting *S. epidermidis* biofilm, after overnight treatment with 1.0 mg/mL of TMC28, (F) PMMA film with preexisting *E. coli* biofilm, after overnight treatment with 1.0 mg/mL of TMC28, and (G) PMMA film with preexisting *C. albicans* biofilm, after overnight treatment with 1.0 mg/mL of TMC28.

Fig. 3. UV spectra of the aqueous solutions of (A) TMC28, and (B) Ph-TMC (TMC28 labeled with phenyl groups)

Fig. 4. Binding amounts of Ph-TMC onto biofilms produced by (A) *S. epidermidis*, (B) *E. coli*, and (C) *C. albicans*

Fig. 5.

Level of recoverable adherent (A) *S. epidermidis*, (B) *E. coli*, and (C) *C. albicans*, from the preexisting biofilms after treatment with different concentrations of TMC28. *S. epidermidis* and *E. coli* were treated for 24 h, and *C. albicans* were treated for 48 h. “#”, $p < 0.05$; “*”, $p < 0.005$; and “***”, $p < 0.001$ in comparison with the level of recoverable adherent microorganisms from the control, *i.e.*, TMC28 concentration = 0.

Fig. 6.

Level of recoverable adherent (A) *S. epidermidis*, (B) *E. coli*, and (C) *C. albicans*, from the preexisting biofilms after treatment with 1.0 mg/mL of TMC28 for different periods of time.

“#”, $p < 0.05$; “*”, $p < 0.005$; and “***”, $p < 0.001$ in comparison with the level of recoverable adherent microorganisms from the control, *i.e.*, TMC28 treatment time = 0.

Fig. 7. Representative SEM images of pre-attached *S. epidermidis*, *E. coli*, and *C. albicans* before (A, C, and E, respectively) and after (B, D, and F, respectively) treating with 1.0 mg/mL of TMC28 (*S. epidermidis* and *E. coli* were treated for 24 h, and *C. albicans* were treated for 48 h).

Fig. 8.

Representative CLSM images of pre-attached *S. epidermidis* and *E. coli* before (A and C, respectively) and after (B and D, respectively) treating with TMC28; and fluorescence microscopic images of pre-attached *C. albicans* before (E) and after (F) treating with TMC28. TMC28 concentration was 1.0 mg/mL. Treatment time for *S. epidermidis* and *E. coli* was 24 h, and for *C. albicans* was 48 h.

Fig. 9.
Effects of TMC28 concentration on the viability of rat skin fibroblast cells tested by the MTT assay

Tab. 1

The DQ, intrinsic viscosities ($[\eta]$), and viscosity average molecular weights (M_v) of the synthesized TMC*

TMC Samples	DQ (%)	$[\eta]$ (dl/g)	$M_v (\times 10^5 \text{ g/mol})$
TMC28	28	3.30 $\pm$ 0.26	4.09 $\pm$ 0.83
TMC47	47	2.47 $\pm$ 0.70	1.96 $\pm$ 1.37
TMC56	56	2.87 $\pm$ 0.18	2.86 $\pm$ 0.47

* $[\eta]$ was tested in 2% acetic acid/0.2 M sodium acetate at 25 ± 0.1 °C in triplicate.²⁸

Tab. 2

MBC values of the TMC samples with different DQ against bacterial and fungal cells

Microbial species	MBC of TMC with different DQ (mg/mL)		
	TMC28	TMC47	TMC56
<i>S. epidermidis</i>	0.5	0.25	0.25
<i>E. coli</i>	3.0	4.0	5.0
<i>C. albicans</i>	0.25	0.25	0.25