Synthesis, characterization and antimicrobial activities of quaternary chitosan-based materials

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Abstract. The emergence of new pathogenic strains together with continuous rise of antimicrobial resistance and the death of new antibiotics in the clinical pipeline raise an urgent call for the development of potent antimicrobial agents. There has been growing interest in the use of new agents, such as antimicrobial polymers, as alternatives for therapy and disinfection. Cationic chitosan derivatives, N-(2-hydroxypropyl)-3-trimethylammonium chitosan chlorides (HTCC), have been widely studied as potent antibacterial agents. However, their systemic structure-activity relationship (SAR), activity toward drug resistant bacteria and fungi, and mode of action are very rare. Herein, we investigated antibacterial efficacies of the HTCC polymer derivatives against multidrug resistant bacteria, including clinical isolates. The polymers were found to be active against a variety of bacterial pathogens (MIC = 32–128 μg/mL). Interestingly, these polymeric materials were active against Streptococcus Gram-positive bacteria, which are a predominant cause of hospital-acquired infections (HAIs).

Introduction
The emergence of antimicrobial resistance (AMR) and the steady decline of new antibiotics in the clinical pipeline is an increasing global health concern for humans and agriculture [1]. AMR causes a high minimal inhibitory concentration (MIC) of the current antibiotics, resulting in a decreased susceptible range for treatment and adverse outcomes which then calls for an urgent call for the development of potent antimicrobial agents [2]. Surfaces in clinical settings may act as reservoirs of microorganisms which could in turn lead to the spread of infection upon human contact, by either patients or healthcare workers. In cases where transmission by direct contact predominates, then surface-disinfection technologies should have a major impact in reducing infection rates. An obvious measure is therefore the development of medical devices with antimicrobial properties; either by application of antimicrobial coatings or by adding antibiotics to the material. The overuse of antibiotics has contributed to a rise in bacterial resistance, which then limits their applications in medical devices, leaving us with antimicrobial coatings as our last resort.

Chitosan, a biopolymer that is extracted from crustaceans, fungi and insects [3] is known to exert antimicrobial activity against both Gram-positive and Gram-negative bacteria together with fungi [4]. Unfortunately, the application of chitosan as antimicrobial polymers has been restricted by its lack of...
solubility in both organic solvents and neutral pH aqueous media. However, chitosan tested at lower pH values has revealed an increased activity against different microbes [5]. Quaternary chitosan derivatives, N-(2-hydroxypropyl)-3- trimethylammonium chitosan chlorides (HTCC), have been widely investigated as potent antimicrobial polymeric materials. A recent report by Jiaul Hoque et al showed that HTCC polymers are active against multidrug resistant bacteria including clinical isolates and pathogenic fungi (MIC = 125-250) and also displayed rapid microbicidal kinetics, killing pathogens within 60–120 min [6]. Micro- and nanomaterials have been recognised as an alternative to antibiotics for the treatment of diseases caused by antimicrobial resistant (AMR) microorganisms [7]. With a variety of metal based nanoparticles (NP), such as ZnO, TiO2, Au, Al, Ag, and Cu NPs, killing the bacteria using one or more mechanisms that damage cellular components, cell membrane, and wall or inhibit DNA synthesis and enzyme activity [8]. However, the application of metallic NPs in treating infectious diseases has been limited due to their potential environment toxicity. Herein, we report the facile synthesis and antimicrobial activity of chitosan derivatives against drug-resistant bacteria and pathogenic fungi. In addition, we also performed structure–activity studies, which is an essential part of the progress toward realizing the full potential of antimicrobial chitosan derivatives.

Experimental Section

Materials, Instruments and Bacterial Isolates
Chitosan (degree of deacetylation ≥93%), Allyl bromide, phthalic anhydride, glycidyl methacrylate, hydrazine hydrate, N-methyl-2-pyrrolidone, glycidyltrimethylammonium chloride (GTMAC), potassium dichromate, silver nitrate and deuterated water were obtained from Sigma Aldrich. All solvents and other chemicals were purchased from local commercial suppliers and were of analytical reagent grade, unless otherwise stated. The water used in all experiments was double distilled. Nuclear magnetic resonance spectra were recorded on a CSIR Inova-400 instrument. Infrared spectra of the chitosan derivatives were recorded on Perkin Elmer Spectrum 100 Fourier-transform infrared spectrometer. Bacterial isolates used in this study were sub-cultured twice from freezer stocks onto Mueller-Hinton agar (MHA) plates and incubated at 37 °C prior to the experiments. All subsequent liquid subcultures were derived from colonies isolated from the agar plates and were grown in cation-adjusted Mueller-Hinton broth (CAMHB) medium.

Synthesis of chitosan derivatives

N-(2-Hydroxy)propyl-3-trimethylammonium chitosan chloride (HTCC). Chitosan (0.3 g) was suspended in double distilled water (20 mL) and the suspension was stirred for 2 h at room temperature. GTMAC (3 equiv) was then added to the polymer solution in three portions at approximately 3 h intervals. The reaction temperature was raised to 85 °C for 24 h after the final addition of GTMAC. The chitosan derivative was precipitated in cold acetone and filtered through a sintered glass funnel and washed with acetone and ethanol copiously. The product was characterized by 1H NMR and FT-IR spectroscopy. The degrees of substitutions (DS) or degrees of quaternization (DQ) in the quaternary chitosan derivatives were determined by Mohr’s method of titration. For the derivative 2b, chitosan (100 mg, 0.62 mmol) was dispersed in distilled water (15 mL) at 85 °C, followed by the addition of GTMAC (1.2 mL) in three portions (0.4 mL each) at 2 h intervals. After 3 h, 1.2 mL of glycidyl methacrylate was added in situ, and the reaction was stirred for additional 18 h. The yellowish, clear reaction solution was precipitated in cold acetone (50 mL). The white product was then collected by filtration and washed freely with acetone and ethanol, yielding 2b.

N-Phthaloylation of chitosan. The synthesis procedure for the derivative is shown in Scheme 2. Chitosan (300 mg, 1.85 mmol) and phthalic anhydride (827 mg, 5.58 mmol) were suspended in 15 mL of DMF containing 5% v/v water and the mixture was heated to 120 °C while stirring under nitrogen atmosphere for 8 h. The reaction mixture was then cooled to room temperature before adding it to ice
water. A light brown precipitate was collected by vacuum filtration, washed with cold methanol and air dried for 24 h.

**Allylation of N-phthaloyl-chitosan.** In the second step, N-phthaloyl-chitosan O-allyl was prepared by reacting N-phthaloyl-chitosan (128 mg, 0.44 mmol) with allyl bromide (212 mg, 1.75 mmol) in DMSO (15 ml) at 80 °C under nitrogen atmosphere for 18 h. The product was then precipitated using a mixture of acetone and ethanol (1:1), collected by vacuum filtration and thoroughly washed copiously with acetone and ethanol.

**Deprotection and Quaternization.** The N-phthaloyl-chitosan O-allyl was deprotected by using 10% (v/v) ethanolic solution of hydrazine monohydrate at 40 °C and stirred for 24 h. The O-allyl chitosan was then quaternized using the method described above.

**Determination of Minimum Inhibitory concentration and Minimum Bactericidal Concentration (MIC/MBC) bacterial isolates.** The broth micro-dilution method was employed to determine the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) values, according to the Clinical and Laboratory Standards Institute (CLSI) guidelines.

**Results and Discussion**

**Synthesis and Characterization**

The ability of the C-2 primary amino group of chitosan to be protonated confers a vital distinguishing property onto it, i.e. solubility in aqueous acidic solutions. Cellulose, a closely related polysaccharide which differs structurally only in having a hydroxyl group at this position, is insoluble in aqueous solutions. Chemical modifications on chitosan’s primary amine are facile and have extended the applications of the polymer. N-alkyl substitutions are common and result in non-pH dependent quaternary cationic derivatives. The quaternary chitosan derivatives were synthesised by reacting high molecular weight chitosan with GTMAC in aqueous acidic conditions (scheme 1). Quaternary chitosan 2b was synthesized by using GTMAC as quaternizing agent, which was followed by an in situ epoxide ring opening reaction using glycidyl methacrylate.

The impact of protecting the hydroxyl groups on the antimicrobial activity of chitosan was investigated by introducing controlled modifications in the hydroxyl group (Scheme 2). The amino groups of chitosan were protected by N-phthaloylation and the product was confirmed by the FTIR band indicating the presence of phthalimide group. The N-phthaloylation facilitated the solubility of the protected chitosan in organic solvents which in turn was vital for the subsequent modifications. This was then followed by allylation at the C-6 position using allyl bromide in DMSO. The amino groups were then unblocked using hydrazine monohydrate, which was then followed by quaternazition using GTMAC.

![Schematic 1. Quaternization of chitosan](image)

The chemical modification of chitosan by a particular functional group is usually expressed as the degree of substitution (DS) of chitosan. During the chemical modification and antimicrobial evaluation of chitosan, structure–activity relationship (SAR) studies require precise characterization of the synthesized materials so that the contribution of the structural modification or the covalently
attached functional groups on the activity or toxicity can be correctly evaluated. In this study, the NMR and titration method were the only two techniques that were used to calculate the DS.

The DS of the HTCC was determined by Mohr’s method of titration. The titration of the chloride ions in HTCC was carried out with aqueous silver nitrate using potassium dichromate as an indicator. DS of HTCC was then calculated by the following equation 1:

\[
DS = \frac{V \times c \times 10^{-3}}{V \times c \times 10^{-3} + (W - V \times c \times 10^{-3} \times 314)} \times 100%
\]

Where \( c \) (mol/l) is the concentration of AgNO\(_3\) solution, \( V \) (ml) is the volume of AgNO\(_3\) solution, \( W \) (g) is the weight of HTCC.

The amount of AgNO\(_3\) solution used at the end point during the titration equals the amount of Cl\(^-\) ions present on the HTCC. The DS for 2a was found to be 0.62 when 3:1 molar ratio of GTMAC to chitosan, while the value increased to 0.98 when using the molar ratio of 6:1, which means that the amino groups of chitosan were fully substituted by quaternary ammonium salt groups.

FTIR studies of chitosan and its derivatives were performed in order to characterize their chemical structure. The presence of the phthalimide group was confirmed by the \( \text{C}=\text{O} \) stretching peaks at 1780 cm\(^{-1}\) and 1713 cm\(^{-1}\) of N-phthaloyl chitosan, which corresponds to the specific phthalimide group. The additional peaks at 1567 cm\(^{-1}\) (N-H deformation and C-N stretching), and 1398 cm\(^{-1}\) (coupled C-N and C-O stretching vibration from carbamate group) further confirms the protection of chitosan primary amines. The O-allylation of chitosan was confirmed by the presence of \( \text{C}=\text{C} \) stretch at 1689 cm\(^{-1}\). In addition, the \( \equiv\text{C}-\text{H} \) bending was observed at 940 cm\(^{-1}\). The deprotection of amino groups with ethanolic hydrazine monohydrate was confirmed by the disappearance of \( \equiv\text{C}-\text{H} \) out of plane deformation, \( \text{C}=\text{O} \) stretching and aromatic ring deformation at 1713, 720, and 530 cm\(^{-1}\) (figure 3). Finally, the quaternized O-allyl chitosan was characterized by the appearance of \( \text{C}-\text{H} \) bending of trimethylammonium salt group at 1481 cm\(^{-1}\) and the disappearance of the characteristic NH\(_2\) bending peak at 1754 cm\(^{-1}\).
Figure 1. $^1$H NMR spectrum of O- Allyl chitosan

The $^1$H NMR spectrum of O-allyl chitosan (Figure 1) was then used to calculate the degree of allylation. The total integral of seven hydrogens of the glucose monomers of the chitosan skeleton is designated as 7.00. The degree of allylation of O-allyl chitosan: $A$ equals $(A/3H) \times 100\%$, therefore 15%. $A$ refers to total integral of the chiral proton at 5.5 and 5.8 ppm, in which there is only three hydrogens. Degree of allylation $= (0.45/3) \times 100\% = 15\%$

Antibacterial Activity

We performed the antibacterial assays on the synthesized chitosan derivatives using the Alamar blue method and the MIC (µg/ml) results are summarized in table 1. Unmodified chitosan (CS) showed no activity at 512 µg/ml according the CLSI guidelines. The quaternized chitosan 2a showed activity against all Gram-positive bacterial strains (Entry 1-3), including a clinical isolate, *Streptotocus aureus*. Derivatives 2b and 3b also showed good antibacterial activity against Gram-positive bacteria, while 3a was not active at all. Therefore it is evident that the O-allylation of chitosan doesn’t improve the bacterial activity of chitosan. The best MIC values were obtained from materials 2a and 2b against *Bacillus subtilus* ATCC (32 µg/ml). The Gram-negative bacterial strains such as *E. coli* ATCC, *Pseudomonas aeroginasae*, *Klebsiella pneumoniae*, *Enterobacter cloaceae* NDM-1, *E. cloaceae* KPC-2, *E. cloaceae* GES-5, and *S. marcescens* IMP-1 were not susceptible towards the tested chitosan derivative (Entry 6-12). The degree of substitution for 2a had no effect on the MIC.

Table 1. Antimicrobial Activity of chitosan derivatives against Pathogenic Microorganisms

| Entry | Strains          | 2a | 2b | 3a | 3b | CS |
|-------|------------------|----|----|----|----|----|
| 1     | *S. aureus* ATCC 43300 | 48 | 64 | NI | 64 | NI |
| 2     | *B. subtilus* ATCC 6051 | 32 | 32 | NI | 64 | NI |
| 3     | *Salmonella* ATCC | 256 | 256 | NI | 512 | NI |
| 6     | *E. coli* ATCC 25922 | NI | NI | NI | NI | NI |
| 7     | *P. aeroginasae* | NI | NI | NI | NI | NI |
| 8     | *K. pneumoniae* | NI | NI | NI | NI | NI |
| 9     | *E. cloaceae* NDM-1 | NI | NI | NI | NI | NI |
| 10    | *E. cloaceae* KPC-2 | NI | NI | NI | NI | NI |
| 11    | *E. cloaceae* GES-5 | NI | NI | NI | NI | NI |
| 12    | *S. marcescens* IMP-1 | NI | NI | NI | NI | NI |

NI= No inhibitory activity at concentration of 512 µg/ml
NB: The highest concentration tested was 512 µg/ml (according the CLSI guideline).
The scope of Gram-positive bacterial strains was expanded by including clinical strains like *S. sanguinis* ATCC 10556, *S. aureus* ATCC 29213, *S. epidermidis* ATCC 12228, *Streptococcus mutants* ATCC25175 and *L. rhamnosus* ATCC 7469 (Table 2). Chitosan and its derivatives 2a, 2b and 3b were tested against these strains and compared to the positive controls tetracycline and ampicillin. Unmodified chitosan and derivative 3a did not show any activity, with the MIC greater than 512 μg/ml. The other derivatives (2a, 2b and 3b) showed good activity (64-128 μg/ml) against the *S. sanguinis*, *S. aureus* and *S. epidermidis* (Table 2, entry 1-3) while there was no activity against *Strep mutants* and *L. rhamnosus* (Table 2, entry 4-5).

**Table 2.** Antimicrobial Activity of Chitosan Derivatives and Antibiotics against Pathogenic Microorganisms

| Entry | Bacteria                  | 3b | 2a | 2b | Chitosan | Tetracycline | Ampicillin |
|-------|---------------------------|----|----|----|----------|--------------|------------|
| 1     | *S. sanguinis* ATCC 10556 | 128| 64 | 64 | >512     | <4           | 32         |
| 2     | *S. aureus* ATCC 29213    | 64 | 64 | 64 | >512     | <4           | <4         |
| 3     | *S. epidermidis* ATCC 12228 | 128| 64 | 64 | >512     | 32           | 16         |
| 4     | *Strep mutants* ATCC25175 | >512| >512| >512| >512     | 32           | <4         |
| 5     | *L. rhamnosus* ATCC 7469  | >512| >512| >512| >512     | <4           | <4         |

Previously it has been shown that chitosan microparticles interact with outer membrane protein A and lipopolysaccharide that results in bactericidal antimicrobial activity[9]. In the case of these functionalized chitosan derivatives, the minimum bactericidal concentrations (MBCs) were all greater than 3 folds, an indication that the materials are bacteriostatic and not bactericidal.

**Conclusion**

The water-soluble quaternary chitosan derivatives synthesized and tested against various drug-sensitive and drug-resistant bacterial strains showed improved inhibition of bacterial growth as compared to native chitosan. These derivatives were obtained by chemical introduction of quaternary ammonium salts in the main chain, enhancing the chitosan applicability in a large pH range, opening up a broad range of possibilities. The results of our study show great potential of quaternized chitosan derivatives to be used as safe antimicrobial agents in clinical settings for controlling the spread of infectious diseases caused by both drug sensitive and multidrug resistant microorganisms. The spectrum of activities is currently limited to Gram-positive bacterial strains.

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