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A novel strategy to control emerging drug resistant infections

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ABSTRACT

The increasing prevalence of antibiotic resistant bacteria in hospitals and the community has significantly limited the effectiveness of current drugs resulting in treatment failure. Moreover, bacterial cells growing within biofilms exhibit increased resistance to antibacterial agents, making it imperative that alternative approaches be explored to improve treatment strategies. The present study investigates the antimicrobial activity of chitosan (CS), quaternary ammonium chitosan derivative (QCS) and their nanoparticulate forms against clinically derived methicillin-resistant Staphylococcus aureus (MRSA) and extended-spectrum beta lactamase (ESBL) producing Escherichia coli. Chitosan nanoparticles (CS NP) and quaternary ammonium chitosan derivative nanoparticles (QCS NP) were prepared using the ionic gelation method and characterised using scanning electron microscope and zeta potential analyzer. The minimum inhibitory concentration and minimum bactericidal concentration of all the particles was determined. There was about 1.5 to 4 fold reduction in the minimum inhibitory concentration of CS NP and QCS NP for both the test organisms when compared to the parent CS and QCS particles. Potent invitro activity against biofilms of MRSA and ESBL-producing Escherichia coli was observed using CS and QCS nanoparticles. Our data demonstrates the antimicrobial efficacy of nanoparticulate forms of chitosan and its quarternized N-alkyl derivative and suggests their potential as novel therapeutic agents against emerging drug resistant bacteria.

Keywords: Chitosan, quaternary ammonium chitosan, nanoparticles, methicillin-resistant *staphylococcus aureus,* extended-spectrum beta lactamase.

INTRODUCTION

Control of the spread of infections caused by multidrug resistant and extended spectrum beta lactamase (ESBL) producing bacterial isolates is a major problem worldwide. ESBLs are defined as beta-lactamases capable of hydrolyzing oxyimino cephalosporins [1]. *E.coli*, an organism responsible for urinary tract infection, has the ability to produce ESBLs in large quantities [2, 3]. ESBL production among members of the family *Enterobacteriaceae* results from mutations within structural genes. These enzymes are plasmid borne and confer multiple drug resistance, making the infection difficult to treat [4]. ESBL-producing *E.coli* infections are growing globally

and are said to be harder to treat than infections caused by methicillin resistant *staphylococcus aureus* (MRSA). *Staphylococcus aureus* is a leading cause of both superficial and invasive infections in community and hospital settings, frequently resulting in chronic refractory disease. Certain *Staphylococcus aureus* strains have evolved resistance to methicillin, an antibiotic to which they were previously sensitive. Methicillin-resistant strains are becoming increasingly common and have been isolated from purulent skin and soft-tissue infections [5] . Studies have found that antibiotic resistant variants of organisms have enhanced ability to form biofilms [6]. Such biofilms are responsible for several chronic diseases that are difficult to treat and show much greater resistance to antibiotics than their free-living counterparts. With the dearth of new antibiotics coming to the market and the advance of multiple drug resistance, it is not difficult to see untreatable life-threatening bacterial infections becoming common. The growing resistance amongst organisms forewarns of the need to minimize selective pressure, making it imperative to examine innovative therapies, to which the bacteria are unlikely to evolve resistance. This would help to curtail associated morbidity and mortality and improve our capacity to treat these infections.

Chitosan is a linear polysaccharide that consists of (1, 4)-linked 2-amino-deoxy- D-glycan. It is a deacetylated form of chitin, the second most abundant polysaccharide found in nature after cellulose [7, 8]. The biocompatibility and biodegradable properties of this polymer along with its various biological properties such as antioxidative, antitumor and antimicrobial activity make it a promising candidate for various industrial and clinical applications [7]. However, chitosan is only soluble in organic acid solutions as it has a high molecular weight and this contributes to some limitation in its application.

Recently, investigators have studied the antibacterial activity of nanoparticulate forms of chitosan (CS) and quaternary ammonium chitosan (QCS) [9, 10]. Nanoparticles display unique physical and chemical features and the manipulation of materials towards the nanoscale can enhance antibacterial properties and increase their medical application [11]. Chitosan nanoparticles have improved solubility and the antibacterial activity of its aminoderivatized form has been reported to increase with increasing chain length of the alkyl substituent [10]. However, to our knowledge no studies have examined the in vitro activities of chitosan nanoparticles and quaternary ammonium chitosan nanoparticles for controlling the growth of emerging drug resistant organisms like ESBL producing *E.coli* and MRSA. Since CS and QCS nanoparticles can be easily administered intravenously, they could have great value for medical application. Hence in our present study we have explored the use of CS and QCS nanoparticles as alternative agents for the treatment of drug resistant infections.

EXPERIMENTAL SECTION

Culture & Growth conditions

Cultures of methicillin-resistant *staphylococcus aureus* (MRSA) and ESBL producing *Escherichia coli* were obtained from local hospitals. Yeast-dextrose broth containing 10 g/l peptone, 8 g/l beef extract,5 g/l sodium chloride, 5 g/l glucose and 3 g/l yeast extract (HiMedia) was used as growth medium for the two bacterial strains . All glasswares were sterilized in an autoclave at 120 ^{0}C for 20 min. Bacteria were incubated overnight at 37 ^{0}C with agitation in the growth medium. An aliquot (2 ml) of culture was then added to the yeast-dextrose broth and incubated for 6–8 h at 37 ^{0}C until the exponential growth phase was reached. This culture was then adjusted by spectrophotometric measurement at 600nm (ErmaInc.Colorimeter) to provide a final density of 10^{8} colony forming units (CFU)/ml.

Chitosan

CS with 86% deacetylation (Research Fine Lab Ltd.) was refined twice by dissolving it in dilute acetic acid (HOAc) solution. The solution was filtered, CS precipitated with aqueous sodium hydroxide and then dried in an oven for 24 h at 40°C.

Synthesis of QCS

1 g of CS was added to 50 ml N-methyl-2-pyrrolidinone (SRL chemicals) and suspended by stirring at room temperature for 12 h. The temperature of the suspension solution was lowered to 4^0C using an ice water bath. 15 ml 1.5N NaOH aqueous solution, 1.2 g potassium iodide (S.D. Fine Chemicals) and 13 g hexylbromide (S.D. Fine Chemicals) were added to this solution, its temperature raised to 45^0C and maintained at this value for 48 h while stirring. The reaction solution was then filtered using a mesh (120 meshes) to remove the insoluble portion. The filtrate was precipitated into a large excess of acetone and filtered using a filter paper. The precipitate was re-dispersed and washed with acetone 3 times and the resulting product was dried.

Preparation of chitosan nanoparticles (CS NP) and quaternary ammonium chitosan derivative nanoparticles (QCS NP)

CS NP was prepared using the ionic gelation method [3]. CS was dissolved in 1 v/v% HOAc solution at a concentration of 0.5 w/v% and the pH was raised to 4.6–5 with 10 N NaOH. CS NP was formed upon adding 5ml of 0.25% TPP (S.D.Fine Chemicals) in water to 15 ml CS solution under stirring at a speed of 1000 rpm. The nanoparticles were separated by centrifugation at 20,000 rpm for 30 min. The supernatant was discarded and the CS NP was extensively rinsed with water to remove any NaOH. QCS NP was obtained from QCS using the same method.

Characterization

The nanoparticles were examined using scanning electron microscope (SEM) and their size was determined .The dried nanoparticles were first suspended in dilute acetic acid and sonicated to obtain homogeneous suspension before the measurement. The zeta potential of the nanoparticles was measured by a zeta potential analyzer.

Determination of minimum inhibitory concentration and minimum bactericidal concentration

Using stock solutions of CS and CS NP (dissolved using 1% (v/v) acetic acid) and QCS and QCS NP (dissolved in distilled water) various dilutions were prepared in Mueller Hinton (MH) broth and inoculated with bacterial cultures (adjusted to 0.5 OD units at 540nm). After incubation at 37ºC for 24hrs, the lowest concentration that inhibited the growth of the microorganism being tested as detected by lack of visual turbidity, matching with a negative control included with the test, was reported as the Minimum Inhibitory Concentration (MIC). To evaluate the Minimum Bactericidal Concentration (MBC), a loopful of the sample was transferred from each tube without visible growth to MH agar plate and incubated at 37°C for another 24 h. The MBC was read as the lowest concentration without bacterial growth.

Activity against biofilm

Yeast-dextrose broth (containing 10 g/l peptone, 8 g/l beef extract, 5 g/l sodium chloride, 5 g/l glucose and 3 g/l yeast extract) was used as the growth medium for both the bacterial strains. Bacteria (adjusted to 0.5 OD units at 540nm) were inoculated and incubated overnight at 37°C in coplin jars containing the growth medium and sterile slides. After incubation different concentrations of CS, QCS and their nanoparticles were added and viable counts obtained after 48 hrs.

All experiments were performed in triplicate and repeated three times. Mean values of the results have been reported below.

RESULTS AND DISCUSSION

The extensive use of antimicrobial agents and the evolutionary antimicrobial resistance strategies of bacteria have resulted in the global increase of nosocomial infections making it necessary to embark on a continued search for new antimicrobial compounds. Several studies have reported on the development of multi-resistant planktonic strains of bacteria as well as on the therapeutic challenge of eradicating bacterial cells within biofilms. The present study was performed to assess the antibacterial activity of nanoparticles of chitosan and its quaternary ammonium derivative against MRSA and ESBL-producing *Escherichia coli* , both of which are important bacterial pathogens responsible for nosocomial infections. Often these bacteria are associated with infections of indwelling medical devices and implants, due to biofilm formation. Implanted devices are increasingly used in today's medical community to alleviate pain and improve mobility and function. As a result, the number of implant-associated infections due to biofilm formation by these organisms is also on the rise. Failure to treat biofilm associated infections makes it necessary to remove the implant, thereby increasing health care costs and risk of death. In the present study, nanoparticles were prepared by the ionic gelation method wherein an anionic cross-linking agent, TPP, was added to an aqueous solution of CS in acetic acid. CS or QCS NP were formed through interactions between the positively charged CS or QCS and negatively charged phosphate groups of TPP.

Figure 1 shows the SEM images of CS NP and QCS NP. The mean size of CS NP as determined by SEM was 70 nm, while that of the QCS NP was 100 nm.

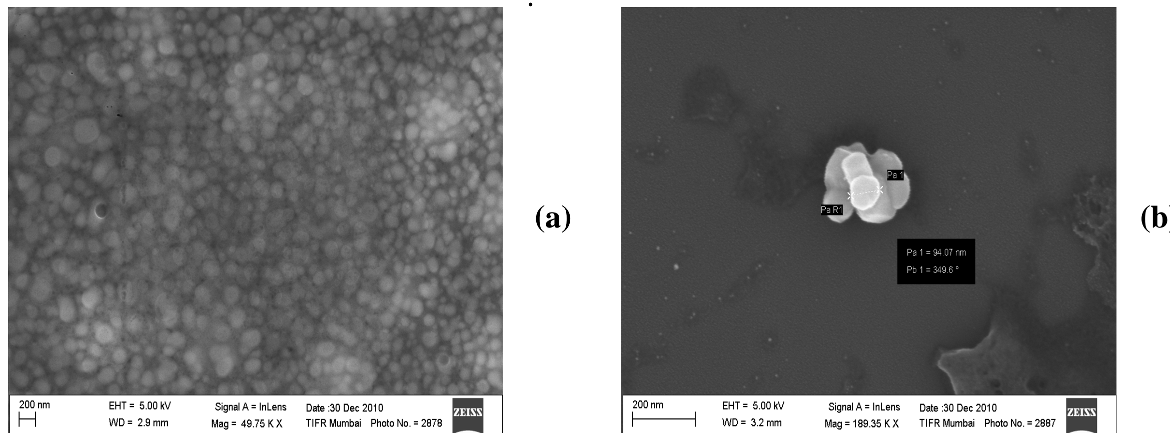

Figure1: **SEM images of CS NP (a) and QCS NP (b)**

The surface charge (zeta potential) of the CS and QCS are shown in **Table 1**. The zeta potential of the NP can greatly influence their stability in suspension through electrostatic repulsion between the particles, and it also determines the extent of interaction with the cell membrane of bacteria, which is usually negatively charged. As shown in Table 1, the zeta potential increases from 44.16 to 88.24 mV after quaternization.

Table 1. Zeta potential of CS NP and QCS NP

Nanoparticles	Zeta potential (mV)
CS	44.16
QCS	88.24

To analyze the in vitro activities of the particles, studies of antimicrobial activity were performed. All four particles demonstrated bactericidal activity at or near the MIC for both the cultures tested as shown in **Table 2**.

Table 2. In vitro activities CS, QCS and NP against MRSA and ESBL-producing *E. coli*.

Particles	MRSA		ESBL E.coli	
	MIC	MBC	MIC	MBC
Chitosan (CS) (mg/ml)	2	2	7	7
Chitosan nanopaticles (CS NP) (ppm)	500	500	4000	6000
Quaternary particles (QCS) (ppm)	250	250	2500	2500
Quaternary nanoparticles (QCS NP) (ppm)	150	200	1500	2000

It was noted that all the MIC concentrations were much below the toxicity level. *In vivo* studies have shown that chitosans have low toxicity and the oral mean lethal dose for chitosans in mice is in excess of 16 gm/day/kg of body weight, which is higher than that of sucrose [12]. The exact mechanisms of the antibacterial action of CS and its derivatives are still unknown, although different mechanisms have been proposed. Interactions between positively charged CS and negatively charged bacterial cell membranes lead to altered cell permeability, which prevents the transport of essential solutes into the cell [13,14] and results in leakage of proteinaceous and other intracellular components, thus killing the bacterial cells [15]. CS NP exhibits higher antibacterial activity than CS since the polycationic CS NP has higher surface area and charge density than CS and can interact to a greater degree with the negatively charged surface of the bacterial cell. Our results showed the MIC and MBC of QCS to be lower than that for CS for both the test organisms. Previous studies have also reported the antibacterial activity of QCS as being more pronounced than the parent chitosan, due to the increase in chain length of the alkyl substituent which help in disrupting bacterial cell membranes and causing cell lysis [16]. Further, it was seen that nanoparticles of QCS showed the highest antibacterial activity against the antibiotic resistant bacteria. This was due to the high surface charge density of the QCS NP (which increases the affinity for the negatively charged bacterial cell membrane) coupled with the effectiveness of the C6 alkyl substituent in penetrating this membrane. All the particles tested in the present investigation were found to be more effective against MRSA .This is in agreement with previous studies wherein ESBL producing *E.coli* has been reported to inactivate even those beta lactam antibiotics which are effective to treat MRSA infections, thus demonstrating a higher drug resistance.

The lowest concentration of the particles which resulted in complete elimination of all cells within the biofilm has been reported as biofilm killing concentration (BKC). Our results indicate QCS NP as significant an antibacterial agent against biofilms of both the test organisms. The mean BKC values of the particles against the test organisms are mentioned in **Table 3.**

Table 3 *In vitro* **activity against biofilms of MRSA and ESBL producing** *E.coli.*

Particles	MRSA	ESBL E.coli
Chitosan (CS)	5mg/ml	20 mg/ml
Chitosan nanopaticles (CS NP)	3mg/ml	12.5 mg/ml
Quaternary particles (QCS)	750Ug/ml	7.5mg/ml
Quaternary nanoparticles (QCS NP)	400Ug/ml	5 mg/ml

The above BKC results indicate that materials functionalized with the nanocomposites of QCS could help combat biofilm formation and may have many potential clinical applications in the future.

In conclusion, our study demonstrates nanoparticulate forms of chitosan and quaternary ammonium chitosan to be potent and broad spectrum antimicrobial agents against the drug resistant bacteria, MRSA and ESBL producing *E.coli* and our results warrant further detailed investigations by animal and clinical studies *in vivo*.

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