Antibacterial Activity of a Synthesized Chitosan-Silver Composite with Different Molecular Weights Chitosan against Gram-Positive and Gram-Negative Bacteria

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Abstract—Clean water is an essential part of everyday life. Nevertheless, wastewater is a common concern and rapidly increasing. Wastewater is contaminated with multiple waterborne pathogens which are the source of various diseases and therefore a major health concern. The potency of nanomaterial modified composites against pathogenic microorganisms has been reported, therefore, the polysaccharide chitosan, was used to treat wastewater bacteria (Gram-positive *Staphylococcus aureus* and *Bacillus subtilis* and Gram-negative *Escherichia coli* and *Salmonella typhi*). The effect of the chitosan molecular weight was investigated using chitosan of three molecular weights (low, medium, and high) with similar degrees of deacetylation. Additionally, the effect of silver nanoparticles and chitosan-silver composites were determined and compared with pure chitosan films. The results indicated the potency of the low molecular weight chitosan in the inhibition of the growth of Gram-positive bacteria, while the high molecular weight chitosan effectively inhibited the growth of both Gram-negative and -positive bacteria. The results further indicated that the MBC correlated with the MIC and, high, medium and low molecular weights chitosan were bactericidal at all concentrations, except against *B. subtilis* as the low molecular weight chitosan exhibited a bacteriostatic inhibition.

It was also found that the doping of chitosan with nanoparticles enhanced the antibacterial properties and improved the bactericidal efficiency. All four bacteria were susceptible to a much lower concentration of chitosan, irrespective of its molecular weights, when combined with the silver nanoparticles.

Index Terms— Antibacterial activity, Chitosan, Gram-positive bacteria, Gram-negative bacteria, silver nanoparticles.

I. INTRODUCTION

Wastewater is contaminated with multiple waterborne pathogens which are the source of various diseases. The presence of these pathogens in water is a major health concern as they are the source of morbidity and mortality [1]. Wastewater is traditionally treated by a combination of chemical, physical and biological processes aimed to remove solid and organic materials, which also removes nutrients [2]. These processes include methods such as coagulation, adsorption, osmosis and various membrane procedures, which are generally used to remove toxins from wastewater [3]. The problem with these conventional methods is that they are not sustainable and frequently poses health side effects. Another major problem with most of these treatment technologies are the fact that they are not economically feasible, especially when implemented on a large scale [2]. One effective method to remove microbes from wastewater without posing health side effects is filtration-based methods. In 2015, Dong & Yang [4] indicated that the application of nanomaterial modified filters has a promising future for the effective removal of pathogenic microorganisms and Rajendran *et al*. [5] further stated that the wide range of bio-applications in the field of nanotechnology is due to the strong antibacterial effect on a number of both Gram-positive and Gram-negative bacteria. Dong & Yang [4] further introduced the use of chitosan, a polysaccharide biopolymer, as an effective method to use against the growth of microbes, especially in water. Chitosan is known as a chitin derivative that can be found in the exoskeleton of crustaceans that are abundantly available [6-8]. Chitins, having a cationic nature, are able to absorb negatively charged particles which then forms a gel-like substance that settles in water, making it one of the easiest filtration based methods to apply. Chitosan also has the ability to inhibit the growth of bacteria and ultimately kill the bacteria [9-10]. Chitosan, which is relatively inexpensive, is also biodegradable and harmless in the human body which encourages it use in water treatment processes [11]. It was also revealed that the doping of chitosan with a nanoparticle further enhances the antimicrobial efficiency of chitosan and various researches were done on the type of nanoparticles that shows the best antimicrobial behaviour in accordance with chitosan [6]. One of the main factors that influences the antibacterial activity of chitosan are the molecular weight (MW) as well as the concentration. Furthermore, chitosan is not soluble in water but is soluble in an acetic acid

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environment. Acetic acid also has an antibacterial activity and can also influence the inhibition of bacterial growth [12]. It is indicated that noble metals such as silver and copper are among the finest antimicrobial agents available for prevention of microbial infection [10]. Honary and coworkers [13] found that silver-based compounds are more toxic to microbes compared to copper since silver shows a strong biocidal activity against as many as 12 bacterial species, including the Gram-negative bacteria, *E. coli*, compared to copper which only shows potency against 10 bacterial species [13]. Silver has also been used as an antimicrobial agent in various fields including, medical applications, air filtration, and water purification [15]. Gram-positive and Gram negative bacteria have different membrane structures and are thefore likely to respond differently to the antimicrobial compounds; it is therefore important to comparatively investigate their susceptibility to the synthesized antimicrobial compounds in this study.

II. METHODOLOGY

A. Chemicals and Cultures

Chitosan (75-85 % deacetylated) flakes of high, medium, and low molecular weight as well as Luria Bertani (LB) nutrient broth (10 g/L tryptone, 5 g/L yeast extract, and 5 g/L NaCl) was purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Agar powder, silver nitrate (AgNO$_3$), sodium citrate was purchased from Associated Chemicals Enterprises (ACE, Johannesburg, South Africa). All chemicals were used without any further purification. Bacteria cultures (*Escherichia coli, Salmonella typhi, Bacillus subtilis*, and *Staphylococcus aureus*) were supplied by the Department of Microbiology at the North West University, Potchefstroom Campus (South Africa).

B. Method

B.1 Chitosan preparation and composite formation.

A mass of 0.1 g chitosan flakes were mixed with 1 % acetic acid solution. Due to the poor solubility of chitosan, the solution was kept overnight. Silver nanoparticles were prepared from dissolved AgNO3 at 60°C and the addition of sodium citrate at a ratio of 1:10 for complete reduction. The solution was stirred at a constant temperature of 60°C until particle formation has taken place. The chitosan-silver complex were synthesized from 52 mM AgNO3 in 20 mL chitosan. The solution was kept at 95°C for 12 h.

B.2 Characterization.

The chitosan-silver composite morphology was characterized by means of scanning electron microscopy (SEM, FEI Quanta Quanta 250 FEG ESEM, Czech Republic).

B.3 Preparation of bacteria cultures.

Bacterial isolates were harvested and grown aerobically in sterilized LB nutrient broth at a shaking speed of 120 rpm and 30°C. When an OD of 0.1 was obtained, 1 ml of overnight culture was added to 50 ml un-inoculated broth in sterilized fermentation bottles. The new inoculated broth was grown for 24 h and applied for the determination of the bacterial growth curves as it was necessary to determine at what time it would be most susceptible to the compound. The growth curves were established by measuring the cell growth with a spectrophotometer (SHIMADZU) where aliquots of 3 mL were taken at specific time intervals up to 56 h. The results were verified by determining the colony forming units (CFUs).

Aliquots were collected and serially diluted in the growth medium and spread onto LB agar plates. After overnight incubation, the number of colonies were counted and assessed for the CFU determination.

B.4 Determination of the minimum inhibitory concentration (MIC).

The MICs were determined from disc diffusion technique. Bacteria species were grown aerobically for 16 hours in the LB broth at 30°C and a shaking speed of 120 rpm. 1 mL culture was spread across a 90 mm Petri dish containing 20 mL LB agar. 4 sterile 6 mm diffusion discs (Davies Diagnostics (Pty) Ltd, South Africa) were inoculated with 15 µL chitosan, silver nanoparticles, and chitosan-silver composite (concentrations include 150, 75, 37.5, 18.75, 9.33 µl/disc) separately. The discs were carefully placed on the agar plate with sterile forceps and the plates were incubated at 30°C for 24 h. The clear zone surrounding the diffusion discs were measured and compared to commercial antibiotics (100 µg Carbenicillin for Gram-negative bacteria and 30 µg Vancomycin for Gram-positive bacteria). The susceptibility diameter zone was reported as the mean value of 4 replicate measurements.

B.5 Determination of the minimum bactericidal concentrations (MBC).

The MBC was determined from the broth dilution analysis since it allows quantitative and qualitative results. Stock solutions of each tested bacteria species was grown under aerobic conditions for 16 hours in the LB broth at 30°C and a shaking speed of 120 rpm. 1 mL of the overnight culture was combined with 100 mL fresh broth and spiked with decreasing experimental concentrations (150, 75, 37.5, 18.75, 9.33 µl/mL) chitosan, silver, and chitosan composites to

determine the MBC. The highest dilution of a composite that killed >99.9% of bacteria was considered as the MBC. The percentage colony reduction was determined by Equation [1]. All experiments were carried out in sterilized 250 mL Erlenmeyer flasks and screw cap test tubes.

$$\% \ Inhibition = \frac{Colonies \ on \ control - Colonies \ on \ assay}{Colonies \ on \ control} \times 100$$

[1]

III. RESULTS AND DISCUSSION

A. Characterization

The characterization indicated that the Ag-NPs formed on the surface of the chitosan film. The NPs are also in close proximity to one another which may be due to the incomplete reaction time or poor solution of chitosan.
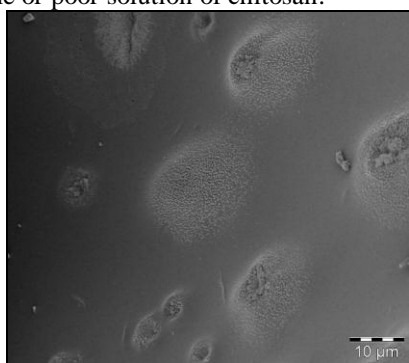

Fig. 1 Micrograph of silver nanoparticles on the chitosan film surface. The silver nanoparticles are not imbedded in the film, therefore it is more accessible to bacteria.

B. Growth curves

The growth curves were conducted at an OD of 0.1.The curves indicated that the lag phase for Gram-positive bacteria was longer than that of Gram-negative bacteria. Gram-negative bacteria also exhibited more aggressive growth with an increased gradient exponential growth phase. The antimicrobial composites was introduced at 8 hours and 12 hours for Gram-negative and Gram-positive bacteria, respectively.

C. Antibacterial activity

The antibacterial activity was measured by evaluating the clear inhibition zones around the diffusion discs. A strong antibacterial effect was observed from the HMW chitosan combined with silver (Figure 2). *E. coli* was also the most resistant bacteria, while *B. subtilis* was most susceptible to all compounds. LMW chitosan also exhibited the weakest activity against the bacteria, however it did produce a stronger effect in Gram-positive bacteria compared to Gram-negative bacteria.

TABLE I
THE REPRESENTATION OF THE MAXIMUM INHIBITION ZONES OBSERVED FROM THE DISC DIFFUSION METHOD. A AND B REPRESENTS *S. TYPHI* AND *E. COLI*, RESPECTIVELY. C AND D IS *B. SUBTILIS* AND *S. AUREUS*. THE DATA IS PRESENTED AS THE MEAN VALUE FROM TRIPLICATE ANALYSIS THAT HAS BEEN CONDUCTED ON THREE DIFFERENT OCCASIONS.

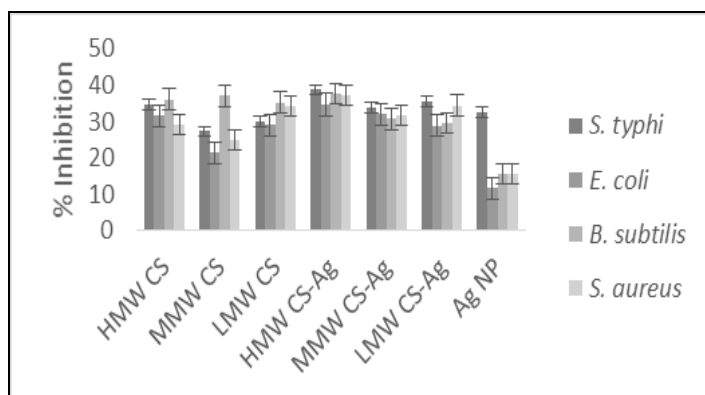| Complex	Zone of inhibition (mm)			
	A	**B**	**C**	**D**
Control	34.56	24.75	25.375	29.25
Ag-NPs	12.07	11.45	14.31	11.75
LMW chitosan	9.79	8.39	8.91	10.48
MMW chitosan	12.46	11.68	12.68	16.68
HMW chitosan	14.88	14.14	16.38	16.95
Ag-Chitosan (LMW)	14.98	16.98	14.18	16.07
Ag-Chitosan (MMW)	15.07	14.79	15.02	15.21
Ag-Chitosan (HMW)	15.38	15.07	16.80	15.49

Fig. 2 The percentage inhibition of various antimicrobial compounds tested from a reduction in colony forming units.

D. Minimum inhibitory and minimum bactericidal concentrations

The disc diffusion assay indicated that all bacteria were susceptible to the synthesized composites. The MICs of Gram-negative bacteria was overall higher than that of Gram-positive bacteria (Table 1). It is also noted that the antibacterial efficacy was higher at HMW chitosan, especially against Gram-negative bacteria and LMW chitosan produced good inhibition towards Gram-positive bacteria. The mechanism of inhibition was proposed by Helander and coworkers in 2001 where the deduction was made that the chitosan disrupts the outer membrane of the Gram-positive bacteria, leading to the leakage of cellular constituents and cell lysis [14] It is also suggested that the LMW chitosan penetrate the cell wall and combine with the DNA, thus inhibiting the synthesis of mRNA and reducing DNA transcription [16]. In the case of Gram-negative bacteria it is suggested that the chitosan binds to the outer membrane, affecting the barrier properties. It is also possible that the chitosan forms a film around the bacteria, which will inhibit nutrient absorption as described by Zheng & Zhu [17]. The mechanism of Ag NPs still remains to be fully understood, however studies suggest that the NPs attach to the cell membrane surface, disrupting the permeability and respirations of the cell [15].

TABLE II

THE ANTIMICROBIAL EFFECT OF CHITOSAN ON GRAM-NEGATIVE AND GRAM-POSITIVE BACTERIA. A AND B DENOTES *S. TYPHI* AND *E. COLI*, RESPECTIVELY. C AND D IS *B. SUBTILIS* AND *S. AUREUS*. THE CONCENTRATIONS ARE PRESENTED AS µG/DISC.

	MIC			
	Gram-negative		Gram-positive	
COMPLEX	A	B	C	D
Chitosan				
HMW	37.50	37.50	37.50	18.75
MMW	75.00	37.50	18.75	18.75
LMW	75.00	150.00	9.33	9.33
Chitosan-Silver				
HMW	18.75	18.75	37.50	18.75
MMW	9.33	37.50	9.33	9.33
LMW	75.00	18.75	9.33	18.75
Silver				
Ag-NP	100.00	25.00	50.00	50.00

HMW chitosan inhibited the growth of Gram-positive *B. subtilis* at a concentration of 37.5 µg/disc, however the same bacteria was inhibited at a MIC value of 9.325 µg/disc for LMW chitosan. For Gram-negative bacteria, the opposite was observed. The antibacterial effect of the chitosan-silver complex indicated a clear reduction in MIC values, with Gram-positive bacteria being the most susceptible. In the presence of HMW and LMW chitosan, inhibition was found at concentrations of 9.325 µg/disc and 18.75 µg/disc for B. *subtilis* and *S. aureus*, respectively. The variations of chitosan molecular weight did not affect the antibacterial activity in a chitosan-silver environment, but the antibacterial effect increased with increasing Ag concentration on the chitosan film.

The MBC values were determined from the broth dilution method (Table 2). The broth dilution tests follow a similar trend to the disc diffusion where high concentrations strongly inhibited the growth of the bacteria and lower quantities of colonies were observed. When the effect of concentration was tested, it was found that the chitosan exhibited a dose-dependent growth inhibitory effect that decreased the number of cells drastically. This phase was, however, followed by a regrowth phase where smaller colonies formed after longer incubation.

TABLE III

THE MINIMUM BACTERICIDAL CONCENTRATIONS OF THE COMPOSITES ON TESTED BACTERIA. A AND B REPRESENT *S. TYPHI* AND *E. COLI*, RESPECTIVELY. C AND D IS *B. SUBTILIS* AND *S. AUREUS*. THE CONCENTRATIONS ARE PRESENTED AS µG/ML.

	MBC			
	Gram-negative		Gram-positive	
COMPLEX	A	B	C	D
Chitosan				
HMW	150.00	150.00	75.00	75.00
MMW	75.00	0.00	1.17	1.17
LMW	75.00	1.17	1.17	1.17
Chitosan-Silver				
HMW	2.34	1.17	1.17	75.00
MMW	9.38	1.17	1.17	1.17
LMW	4.69	1.17	75.00	2.34
Silver				
Ag-NP	2.34	2.34	2.34	2.34

The MBC to MIC ratio was determined to evaluate if the extent of inhibition is bactericidal or bacteriostatic. The antibacterial activity of chitosan varied greatly against all the tested bacteria, but it was concluded that the effect was overall bacteriostatic rather than bactericidal, since regrowth was possible after cell damage has occurred.

IV. CONCLUSION

In summary, the antimicrobial activity of varied molecular weight grades of chitosan was studied and compared to a chitosan-silver composite. Silver nanoparticles were synthesized by reducing silver nitrate with non-hazardous chitosan. The morphology of the chitosan-silver composite produced finely dispersed NPs with a narrow size distribution and the obtained complex demonstrated high antibacterial activity against both Gram-positive and Gram-negative bacteria. As the concentration of chitosan increased, the antimicrobial effect was strengthened for Gram-negative bacteria at a high molecular weight chitosan, and the effect weakened at low molecular weight chitosan. The antimicrobial efficacy of low molecular weight chitosan was found to be stronger against Gram-positive bacteria, however the effect was increased for all bacteria in the presence of the chitosan-silver complex at lower concentrations. It can thus be stated that the synthesized complex had an increased antibacterial effect and is more effective than pristine chitosan. The results also allow to conclude that the presence of a small amount of silver nanoparticles in the chitosan film was enough to significantly enhance the antibacterial efficacy of all tested bacteria as compared to the original chitosan. A similar trend was observed from Helander and coworkers [14].

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