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Chitosan - Silver Nanocomposites and Their Antimicrobial Activity

Mohamed Rafiullah*, Mohammed Akbardeen, Sanjay Jayavanth, Khalid Al-Rubeaan

Strategic Center for Diabetes Research, University Diabetes Center, College of Medicine King Saud University, Saudi Arabia

***Corresponding author:** Mohamed Rafiullah, Strategic Center for Diabetes Research, University Diabetes Center, College of Medicine King Saud University, PO 245, Riyadh 11411, Saudi Arabia. Tel: +96614724179; +96614722049 Ext: 3347; Fax: +966114724179 Ext:1111; Email: mrafiullah@ksu.edu.sa; rafimmc@gmail.com

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Abstract

Chitosan (CS) and silver (Ag) are considered as effective wound healing components owing to their antimicrobial activity. In the development of wound healing dressings involving CS and Ag, it is necessary to determine their appropriate concentrations to obtain maximal antimicrobial potency. In this paper, we have developed three types of CS-based nanocomposites (viz., Chitosan Beads, CS -Ag nanoparticles, CS- Ag microbeads) and evaluated their antimicrobial activity with incremental values of chitosan and silver concentrations. We observed that for a given concentration of CS (0.8% w/v in 1% v/v acetic acid), the antimicrobial activity of the CS -Ag nanoparticles (CS-AgNP) linearly increased by25% with the increasing concentration of silver (from 0.01M to 0.16M), against *Staphylococcus aureus*. Similarly, the antimicrobial activity of the CS-AgNPs increased by 28% in case of *Pseudomonas aeruginosa*. When the concentration of the CS was increased (from 0.2 to 0.8% w/v), there was only a marginal increase in antimicrobial activity for a given concentration of silver. Further, the antimicrobial efficacy was found to be 50% higher than CS-AgNPs when rendered in the form of microbeads. These results indicate that AgNPs play a dominant role in determining the antimicrobial effectiveness of CS-Ag nanocomposites. At the same time when the CS-AgNPs are rendered in a micro-bead form their combined antimicrobial activity is highly effective in comparison with either chitosan beads or chitosan-silver nanoparticles.

Keywords: Antimicrobial; Chitosan; Silver nanoparticles; Silver microbeads

Introduction

Silver nanoparticles (AgNP) are well known antimicrobial agents, and this activity is attributed to their adhesion and accumulation to the surface of the bacterial cell wall. It damages the bacterial cell wall causing structural changes and increased permeability[1]. With a very high surface area, AgNPs will have increased contact with bacteria and can interact more. Development of bacterial resistance to silver is rare and also it does not adversely affect the mammalian cells [2]. These unique features make silver nanoparticles an ideal antimicrobial for developing scaffolds for wound dressings. However, the production of AgNP often involves using reducing agents such as hydrazine[3], N,N-dimethyl formamide [4] and sodium borohydride [5], which are toxic themselves, while the reaction process requires organic solvents. Hence, chemically synthesized AgNPs cannot be used for biomedical applications. To overcome this hurdle, many scientists have attempted to use biocompatible polymers to produce silver nanoparticles from silver precursors. This process not only reduces the silver precursor to silver nanoparticles but also disperses the produced AgNPs in the polymer matrix, thereby increasing the stability of nanoparticles. Chitosan, a natural polysaccharide obtained from chitin has been found to be a good reducing and stabilizing agent for producing AgNPs [6].

Chitosan has become one of the most promising polymers for wound dressing scaffolds due to its unique properties such as biocompatibility, biodegradability, antibacterial and wound healing activity etc., [7,8]. Chitosan's ability to produce and stabilize the silver nanoparticles without the requirement of any additional chemical or the use of any toxic solvents has been exploited to produce biocompatible antimicrobial scaffolds. Silver-containing chitosan wound dressings have been reported to be very effective in increasing the wound healing rates [9]. As both chitosan and AgNP exhibit different degrees of antimicrobial activity, it is necessary to optimize the concentrations and proportions of chitosan and silver in order to obtain a best performing antimicrobial composite. Although there is limited information regarding concentrations of chitosan and silver necessary to produce the maximal antimicrobial

performance, this study is meant to investigate the antimicrobial performance of various combinations of CS and Ag using different concentrations.

Materials and Methods

Materials

Chitosan (~90% DDA, NAQUA, Saudi Arabia), silver nitrate (DF Goldsmith Chemical and Metal Corp, USA), acetic acid (Qualikems, India), Sodium tripolyphosphate (Sigma, USA) and De-Ionized (DI) water (Nanopure, Barnstead).

Nanomaterials Synthesis

Chitosan-Silver Nanoparticles

Chitosan solution was prepared by stirring the specified amount (as given in Table 1) in 1% v/v acetic acid in DI water overnight. Silver nitrate solution was prepared by dissolving in DI water. Varying concentrations of chitosan and silver nitrate mixed in a 4:1 volume ratio. The mixture was sonicated for two hrs at 20 KHz and 20% energy (~10000 J) using a probe sonicator (Wiseclean, WUC N30H). The reaction mixture was left at room temperature for 48 hrs for the reaction to be completed. The resulting brown colored solutions were taken for further analysis. When the concentration of CS was increased beyond 0.8% the resulting AgNPs suspensions tend to become gel, and hence were avoided.

Chitosan concentrations	Silver nitrate concentrations
0.20%	0.01 M
0.40%	0.02 M
0.80%	0.04 M
	0.08 M
	0.16 M
For each chitosan concentration, all the five concentrations of silver nitrate were used to produce the chitosan silver nanoparticles	

Table 1: Concentrations of chitosan and Silver nitrate.

Chitosan Beads

About 1%of w/v Chitosan dissolved in 1%v/v acetic acid and 10% w/v sodium tripolyphosphate (TPP) dissolved in DI water were prepared separately. The pH of the TPP solution was adjusted to 4.0 by using 1% v/v acetic acid. Chitosan solution was pumped using a syringe pump into TPP solution to produce chitosan beads.

Chitosan-silver Beads

A 1% w/v Chitosan and 0.1M silver nitrate were mixed in 4:1 ratio and sonicated to produce chitosan-silver nanoparticles as described above. The resulting brown colored chitosan-silver nanoparticles suspension was pumped into 10% w/v TPP at pH 4.0 using a syringe pump. The resulting suspension was left overnight for curing.

Characterization

Surface Plasmon Resonance (SPR) spectra of silver nanoparticles were recorded using a UV-Visible spectrophotometer (Nanodrop 2000c, Thermoscientific) over 190 nm-890 nm wavelengths at room temperature.

Antimicrobial Activity

Antimicrobial activity was evaluated by measuring the zone of inhibition against *S. aureus* and *P.aeruginosa* using disk diffusion method. Inocula were prepared for the *S. aureus* and *P.aeruginosa* and suspensions of the appropriate microorganism were spread on the Mueller-Hinton Agar plates. A 10 µL of the suspension of the different antimicrobial test materials were dispensed and spread uniformly onto the disk papers and dried. The sample loaded disks were lightly pressed onto Mueller-Hinton agar surface. After incubation (at 35^{o}C for 16-18 h), the microbial growth around each disc was observed and their zones of inhibition of microbial growth were measured.

Results

The formation of the silver NPs and the variation in their concentration was further verified by measuring the UV spectral absorbance (Nanodrop 2000C, Thermo scientific). Figures 1 and 2 show the spectral absorbance for CS-AgNPs obtained from varying concentration of silver nitrate (0.01, 0.02, 0.04, 0.08, 0.16M) solution at 0.4% and 0.8% of chitosan concentration (data not shown for 0.2%). The wavelength at which the peak occurs (λ~ 421 nm) corresponds to the typical surface resonance peak representing the silver nanoparticles formation, and the peak intensity represents the concentration of the NPs. Higher the peak, greater the absorption at that peak showing a greater concentration of silver in NP formed. It was observed that AgNPs concentration was higher when 0.8% CS was used. The higher concentration of AgNP at 0.16 M showed an absorbance of 0.32 AU in 0.8% CS, while the same concentration of Ag (0.16M) showed 0.17 AU with 0.4% CS.

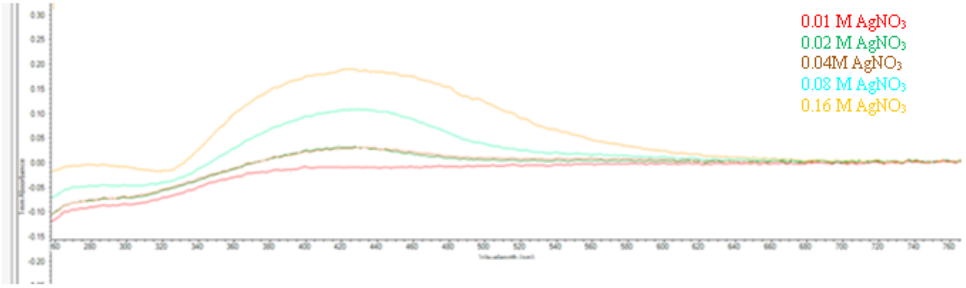


Figure 1: UV spectral absorbance of silver nanocomposites obtained from 0.4% chitosan at different $AgNO_3$ concentrations.

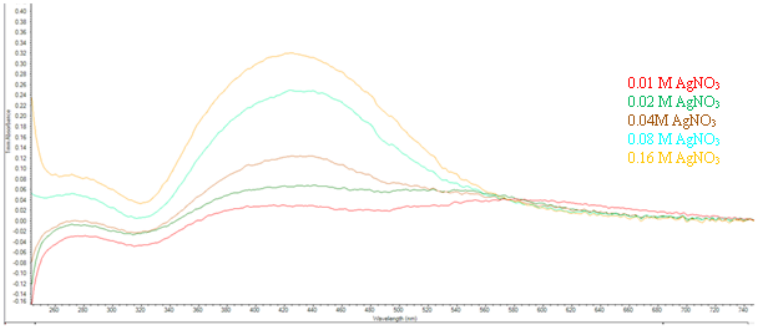


Figure 2: UV spectral absorbance of CS-silver nanocomposites obtained from 0.8 % chitosan at different $AgNO_3$ concentrations.

Figures 3a & 3b show the plot of inhibition zone of CS-AgNP versus concentration of silver nitrate solutions used to prepare the nanoparticles (0.01, 0.02, 0.04, 0.08, and 0.16 M) against *S. aureus* and *P. aeruginosa* respectively. With the increase in the concentration of silver nitrate, the size of the inhibition zone increased. Increasing the chitosan concentration also resulted in the increase of inhibition zone. However, the maximal inhibition zone is observed at both 0.4% and 0.8% of chitosan concentrations with silver nitrate at 0.16 M. It was also observed that the inhibition zone at the lowest CS concentration was lower for *S. aureus* than that for *P. aeruginosa*.

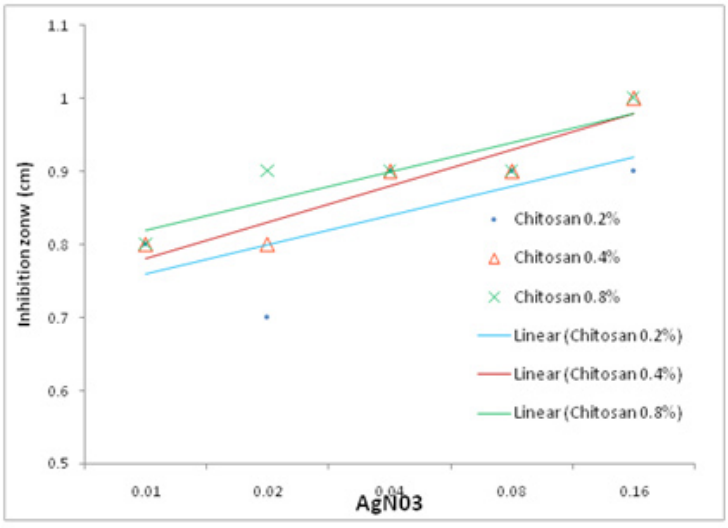


Figure 3a: Inhibition zone of chitosan-silver nanoparticles against *S. aureus*.

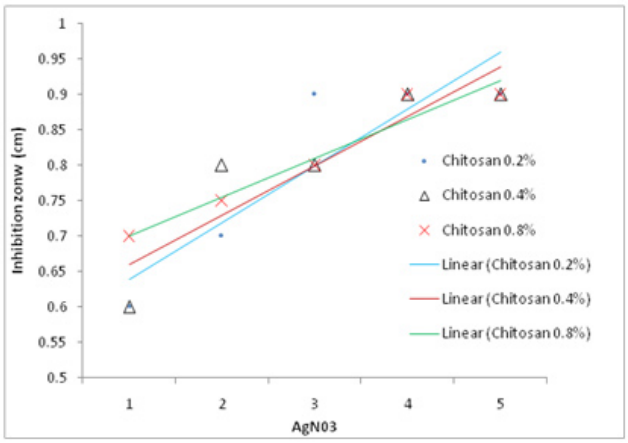


Figure 3b: Inhibition zone of chitosan-silver nanoparticles against *P. aeruginosa.*

Table 2 shows the antimicrobial activity of CS-AgNPs and CS-AgNP beads in comparison to CS bead and control. It demonstrates that the control did not have any inhibition zone, whereas the chitosan beads, chitosan-AgNPs, and chitosan-AgNP beads showed antimicrobial activity. The CS-silver nanoparticle beads when compared to CS beads or CS-AgNPs achieved the maximal inhibition zone. It is interesting to note that CS-AgNPs in the bead form exhibited the highest antimicrobial effect than in the nanoparticles form.

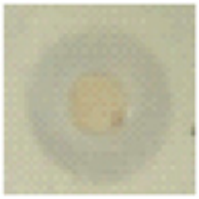
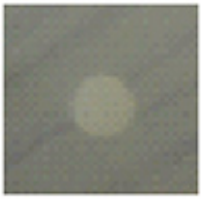
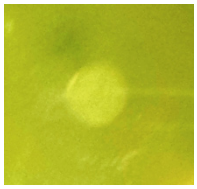
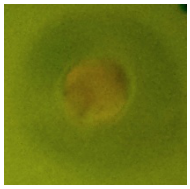
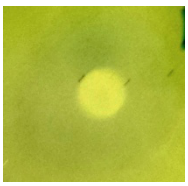
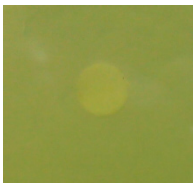
S. aureus				
P. aeruginosa				
Organism	Chitosan beads (%)	Chitosan-AgNP (%)	Chitosan-AgNP beads (%)	Control
S. aureus	16.67	150	200	
P. aeruginosa	0	116.67	150	

Table 2: Antimicrobial activity of the chitosan nanocomposites. Inhibition zone is expressed as the percentage ratio of the difference between the diameter of the individual zone of inhibition and the diameter of the control to the diameter of the control.

Discussion

CS acts as an excellent reducing and stabilizing agent for production of silver nanoparticles. Therefore, a chosen concentration of CS can be treated with silver nitrate to obtain the silver NPs and thereafter the resulting composite of CS-AgNPs. Production of silver nanoparticles in chitosan media requires the application of some form of external energy to induce the reduction. Several techniques such as gamma-ray irradiation, UV irradiation, microwave irradiation and ultrasonication can be used to induce the synthesis of AgNP. In the present study, we adopted the ultrasonication technique to reduce the silver nitrate into silver nanoparticles in the presence of chitosan solution. Results indicate the color of the reaction mixtures turned brown immediately after sonication indicating the formation of silver nanoparticles. The intensity of this brown color increased in the reaction mixtures that had higher silver nitrate as well as chitosan concentrations due to the increased production of AgNP. The formation of AgNP was confirmed by the appearance of their characteristic SPR peak from 410-430 nm in the UV-Visible spectra (figure 1&2). The

increase in the intensity of peaks corresponded with the increased concentrations of silver nitrate and chitosan. This substantiates the concentration-dependent production of AgNP through the ultrasound-mediated reduction in chitosan solution.

Various concentrations of CS-AgNP composites showed a clear inhibition zone against both gram-positive (*S. aureus*) and gram-negative (*P. aeruginosa*) bacteria. Higher concentrations showed higher inhibition zones. The increase in inhibition zone was more prominent in samples with higher silver precursor (silver nitrate) concentration when compared to the concentration of chitosan. As expected the antibacterial activity of chitosan alone was lower than the chitosan-silver nanocomposites. While we have not tested the antimicrobial activity of AgNPs per se, the presence of AgNPs in chitosan solution augmented the overall antimicrobial activity, in other words, the increased AgNP concentration in the composite produced enhanced the antimicrobial effect. This upholds the use of a biocompatible polymer such as chitosan with silver NPs as an effective antimicrobial material for biomedical applications.

To enhance the trapping of silver nanoparticles in the chitosan matrix, we tried to cross-link the chitosan in the mixture after the formation of silver nanoparticles. When TPP was used to cross-link the CS, it resulted in the formation of silver nanoparticles loaded chitosan beads. The UV-Vis spectra of the supernatant from the beads suspension showed no characteristic peak of silver nanoparticles (data not shown), indicating trapping of all of AgNPs in the suspension into chitosan beads. The neutralization of some of the positive charges of chitosan by TPP is expected to throw AgNPs outside the chitosan matrix. However, in this case, it did not happen. As the CS-AgNP suspension was pumped into the TPP solution, beads were formed by solidification of chitosan because of cross-linking. This instantaneous solidification might have trapped the AgNPs in the beads. Results showed a superior antimicrobial effect of CS-AgNP beads in comparison to the CS-AgNPs (1% CS and 0.1 M $AgNO_3$ was used in both preparations). This could be attributed to the accumulation of silver nanoparticles in the beads, facilitating a better interaction of AgNPs with the bacterial surface. To the best of our knowledge, incorporating the silver nanoparticles into TPP cross-linked chitosan in a bead form has not been reported so far. Other studies reported loading of metal ions (including silver ions) into prefabricated chitosan nanoparticles [10], or using an external reducing agent to reduce silver precursor preloaded into chitosan beads [11]. In one study, chitosan-TPP nanoparticles were used to reduce silver nitrate to synthesize AgNP loaded chitosan nanoparticles [12]. Antimicrobial activity of these AgNP loaded chitosan nanoparticles was found to be higher than the individual components [12,13].

In conclusion, the chitosan-silver nanocomposites were successfully produced using chitosan as a reducing and stabilizing agent under sonication. Chitosan-AgNP nanocomposites produced from 0.16 M silver nitrate and 0.4% chitosan exhibited the maximum antibacterial activity in *S. aureus* and *P. aeruginosa*. AgNPs when loaded into TPP cross-linked chitosan beads, showed a higher antibacterial activity when compared with the CS-AgNP nanocomposites. This new form of AgNP loaded chitosan beads with a higher antimicrobial activity may be useful for various antimicrobial applications. However, the duration of action of AgNPs loaded chitosan beads needs to be evaluated.

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