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Chitosan Silver Nano Composites (CAgNCs) as Potential Antibacterial Agent to Control *Vibrio tapetis*

Dananjaya SHS[1], Godahewa GI[2], Jayasooriya RGPT[2], Chulhong OH[3], Jehee Lee[2,4]* and Mahanama De Zoysa[1,4]*

[1]*College of Veterinary Medicine (BK21 Plus Program) and Research Institute of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon, 305-764, Republic of Korea*
[2]*Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea*
[3]*Korea Institute of Ocean Science Technology, Ansan 426-744, Republic of Korea*
[4]*Fish Vaccine Research Center, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea*

Abstract

Nanocomposites exhibit high performance on antimicrobial activities and have the potential to be developed alternative antibiotics. In this study, antibacterial effects of chitosan silver nano composites (CAgNCs) were investigated using pathogenic *Vibrio tapetis* as a bacterial model. Agar disc diffusion and turbidimetric assay results showed that CAgNCs could inhibit the growth of *V. tapetis* in concentration depended manner. The minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of CAgNCs against *V. tapetis* were 50 µg/mL and 100 µg/mL, respectively. The field emission scanning electron microscopy (FE-SEM) images analysis of *V. tapetis* showed severe structural damage to cells after treating CAgNCs at 75 µg/mL compared to un-treated bacteria. Moreover, CAgNCs induce the intracellular ROS level, leakage of nucleic materials (RNA and DNA), decrease the cell viability and protein level in *V. tapetis* cells. Herein, we demonstrate that CAgNCs as effective antibacterial agent with capability to disrupt cell membrane, de-stabilize the membrane permeability, induce the oxidative stress and inhibit the expression of protein or synthesis of macromolecules. Overall results from this study suggest that mode of action of CAgNCs may be associated with excessive generation of ROS, loss in membrane integrity and inhibiting protein synthesis that cause the bacterial cell death.

Keywords: Antimicrobial agents; *Vibrio tapetis;* Chitosan-silver nanocomposite; Reactive oxygen species

Introduction

In recent years, various nanoparticles (NPs) have been developed with broad spectrum of antimicrobial properties. Silver (Ag) is considered as one of the most prominent and effective inorganic bactericidal agent in ionic form as well as in nanoparticle level [1]. One of the important characteristic features of any antimicrobial agent is that it should avoid the generation of antibiotic resistant to given target organism, hence the major advantage of Ag over the conventional antibiotics is that microbes are less likely to develop resistance against Ag. However, application of silver nanoparticles (AgNPs) should be strictly regulated due to possibility of their accumulation in various tissues over the time and cause higher toxicities to normal cells [2]. Regiel et al. [3] reported that composite form of AgNPs with polymer based matrix could be a better option to enhance antimicrobial properties at low concentrations that reduces the toxic effects. Therefore, it is important to develop biodegradable nanocomposites to get multiple benefits in controlling pathogens.

Chitosan is a cationic biocompatible polysaccharide derived after deacetylation of chitin and it has been used to synthesize wide array of composites with higher antibacterial activities [4]. Essential function of the composite is that positively charged chitosan matrix can capture negatively charged bacteria on its surface. Then small size AgNPs create pores on bacterial cell wall which leads to rapid disintegration of bacteria and change the cell permeability [5]. Chitosan is also used as a stabilizer instead of chemical reducing agent for protecting AgNPs from agglomeration [6]. Due to unique characteristics of chitosan Ag nano composites (CAgNCs), they have become attractive candidates as antimicrobial materials and various biomedical applications such as anticancer, wound healing and vaccine delivery agents [7]. Therefore, application of CAgNCs would be a better choice for development of efficient antimicrobial agents as well as other therapeutic applications. Also, it is important to improve the physiochemical properties, efficacy, safety, and pharmacodynamic properties of CAgNCs. Moreover, understanding its functional events on bacterial inhibiting is essential to use it effectively.

Several *Vibrio* species are considered as important pathogens in aquaculture species and cause human infections associated with the consumption of contaminated food and drinking water [8]. Among them *Vibrio tapetis* is a Gram negative marine bacterium responsible for the brown ring disease (BRD) in the manila clam [9]. Therefore, we have used *V. tapetis* as a model bacterium to understand the antibacterial effects of CAgNCs and preliminary investigation of its mode of action.

In this study, stable CAgNCs were synthesized by reduction method and preliminary investigation of its antimicrobial effects and mode of action was carried out using *V. tapetis* as a model bacterium. Antibacterial activities of CAgNCs were investigated

***Corresponding authors:** Jehee Lee, Department of Marine Life Sciences, School of Marine Biomedical Sciences, Jeju National University, Jeju Self-Governing Province 690-756, Republic of Korea, Tel: +82647543472; Fax: +82647563493; E-mail: jehee@jejunu.ac.kr

Mahanama De Zoysa, College of Veterinary Medicine (BK21 Plus Program) and Research Institute of Veterinary Medicine, Chungnam National University, Yuseong-gu, Daejeon, 305-764, Tel: +82428216795; Fax: +82428218903; E-mail: mahanama@cnu.ac.kr

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using agar diffusion and turbidimetric assays and MIC and MBC of CAgNCs were determined. Additionally, FE-SEM image analysis was conducted to confirm the structural damage in cells. Change of cell membrane permeability, ROS production, and protein expression levels were investigated to understand the functional role of CAgNCs on bactericidal effects.

Materials and Methods

Preparation of CAgNCs

Stable CAgNCs were synthesized by reduction method as described previously [4]. Briefly, 4 mL of freshly prepared 0.01 M $AgNO_3$ (Sigma Aldrich, USA) and 200 µl of 0.3 M NaOH (Biosesang, Republic of Korea) were mixed with 100 mL of 0.1% (w/v) chitosan (Showa, Japan) while stirring at 95°C. The heating was stopped after 10 min and resulted suspension was filtered, washed with deionized water. Final product of CAgNCs was dried and stored at room temperature until further use. The particle size and zeta potential of newly synthesized CAgNCs were measured by Zetasizer Nano-ZS90 (Malvern Instruments, UK). The surface morphology of CAgNCs and silver nano particle size was observed using a FE-SEM (Hitachi S-4800, Japan).

Bacteria strain and *in vitro* antibacterial activity of CAgNCs

Antimicrobial activity of CAgNCs was investigated by agar disc diffusion assay described by Meena and Sethi [10] and modified liquid growth inhibition assay according to method of Cai and Wu [11] using pathogenic *V. tapetis* (KCTC12728) as a bacterial model. In an agar disc diffusion assay, *V. tapetis* was inoculated on marine agar plates by spreading overnight culture using sterile cotton tipped swabs under aseptic conditions. Subsequently, sterilized Whatman filter paper discs (8 mm in diameter) were immediately impregnated with 20 µL of different amounts (12.5, 25, 50, and 75 µg/disc) of CAgNCs in 0.25% (v/v) acetic acid followed by air-drying and placed on seeded marine agar plates. The same volume of 0.25% acetic acid (solvent) and chloramphenicol of 100 µg/disc were used as negative and positive controls, respectively. All plates were incubated at 25°C for 24 h. After incubation, antibacterial activity was determined by measuring the diameter of inhibition zone (DIZ) in millimeter scale (including paper disc). Each assay was carried out in triplicates. In the liquid growth inhibition assay, a single colony of *V. tapetis* was grown in marine broth at 25°C with shaking at 160 rpm overnight. Following day, culture was re-inoculated into 20 mL of fresh marine broth at 1:100 dilution and again incubated at 25°C supplemented with CAgNCs in concentration of 25 to 100 µg/mL. Bacterial growth was measured at 600 nm at 2 hour time interval up to 12 hours using spectrophotometer (Optizon Bio, Korea). MIC and MBC of CAgNCs were determined according to methods described by Doughari et al. [12]. Different concentrations of CAgNCs were used for MIC determination. The lowest concentration of CAgNCs that inhibits visible growth of *V. tapetis* was considered as MIC. To determine the MBC, 100 µL of bacterium was collected from culture tubes of MIC or higher concentrations and sub-cultured onto marine agar plates followed by incubation at 25°C for 24 h. The lowest concentration of CAgNCs with no visible growth (bacterial colonies) of *V. tapetis* was considered as MBC.

Characterization of morphological changes

The morphological change of *V. tapetis* after CAgNCs treatment was investigated by FE-SEM (S-4800, Hitachi, Japan) analysis. Bacteria cell fixation was performed according to the method described by Kim et al. [1]. Briefly, CAgNCs treated cells and control cells were washed with PBS and fixed with 2.5% glutaraldehyde for 30 min followed by a dehydration series (10-100%) using ethanol. Then cells were dried in a desiccator and sputter-coated with osmium. Samples were then analyzed using FE-SEM (S-4800, Hitachi, Japan).

Detection of intracellular ROS in *V. tapetis*

Generation of ROS in *V. tapetis* cells were measured using 2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA). Bacterial culture (OD 600 nm=0.5) was treated with different concentrations of CAgNCs and incubated at 25 °C for 3 h under shaking at 100 rpm. Then, H_2DCFDA (30 µg/mL) was added into bacterial culture and incubated for 30 min. Cells were harvested by centrifugation (13000 rpm for 2 min) and re-suspended in 500 µl of PBS. Time series (1 to 4 h) and concentration (CAgNCs) dependent intracellular ROS generation in *V. tapetis* was measured using the FACScaliber flow cytometer (Becton Dickinson, USA).

Cell viability assay

Effect of CAgNCs on viability of *V. tapetis* was determined by colorimetric MTT (3-[4,5-dimethylthiazol-2-yl]-2, 5 diphenyltetrazolium bromide) assay. Bacteria (OD 600 nm=0.3) were treated with CAgNPs (6.25, 12.5, 18.75, 25, 50, and 75 µg/µL) and incubated 25 °C for 24 h under shaking at 200 rpm. The pre-treated cells were allowed to react with 20 µL of MTT (5 mg/mL in PBS) for 30 min and then cells were harvested after centrifugation (1500 rpm × 4 °C for 5 min). The formazan product (formed in live cells) was dissolved in DMSO (200 µg/µL). Cell viability was evaluated by comparing the amount of formazan in samples by measuring the OD at 570 nm.

Integrity of cell membrane

Membrane integrity correlates to release of cytoplasmic constituents of cells. It was investigated by measuring the amount of DNA released by CAgNCs treated *V. tapetis* according to method of Chen & Cooper [13]. Bacterial culture (OD 600 nm=0.5) was treated with different concentrations of CAgNCs and incubated at 25 °C under shaking at 160 rpm. Bacteria samples (1.5 mL) were collected from CAgNCs treated (50 and 75 µg/mL) culture at different time intervals (0, 20, 40, 60, 80, and 100 min). After centrifugation supernatant was collected and filtered (0.2 µm filter) to remove residual bacteria. Absorbance of the supernatant was measured at 260 nm using Nanodrop 2000 UV-Vis spectrophotometer (Thermo Scientific, USA). Data were compared with the respective control bacteria sample without CAgNCs.

Determine the effect of CAgNCs on protein expression

SDS – PAGE analysis was conducted to investigate the effect of CAgNCs on protein expression according to method described by Tao et al. 2011 [14]. Bacterial culture (OD 600 nm=0.5) was treated with different concentrations of CAgNCs and incubated at 25 °C for 1 h under shaking at 160 rpm. Aliquots of 1.5 mL bacterial culture were pelleted from 50 µg/mL CAgNCs treated and control samples and then subjected to 12% SDS-PAGE analysis.

Results

Particle characterization of CAgNCs

Synthesized CAgNCs were characterized for particle size distribution and zeta potential. Results showed that CAgNCs were in the size range of 100-500 nm and +31.20 mV zeta potential. Additionally, size distribution and shape of the AgNPs were assessed and majority of AgNPs embedded in CAgNCs were in spherical shape and well dispersed in chitosan matrix (data not shown). The average

size of the AgNPs was determined in the dry state and it was ranged from 15-35 nm.

Antibacterial activity of CAgNCs

Antibacterial activities of CAgNCs were evaluated using agar disc diffusion, growth dynamics in liquid growth, MIC, and MBC values against a Gram negative bacterium *V. tapetis*. Figure 1 clearly shows the gradual increase of clear zone (inhibitory zone) diameter of *V. tapetis* with respect to concentration (12.5-75 µg/mL) of CAgNCs. The lowest and highest inhibition zones were observed in 0.25% (V/V) acetic acid (control) and 100 µg/mL chloramphenicol (positive control). Effect of CAgNCs on bacterial growth dynamics was also monitored in liquid marine broth medium with starting *V. tapetis* culture of OD 600 nm at 0.05 and 25, 50, 75, 100 µg/mL of CAgNCs for 12 h (Figure 2). CAgNPs at 25 µg/mL had moderate inhibition or growth delay compared to un-treated control. However, 50, 75 and 100 µg/mL treated bacteria showed complete (100%) inhibition from 2 h post treatment and which inhibition was continued to maintain at more or less same level until

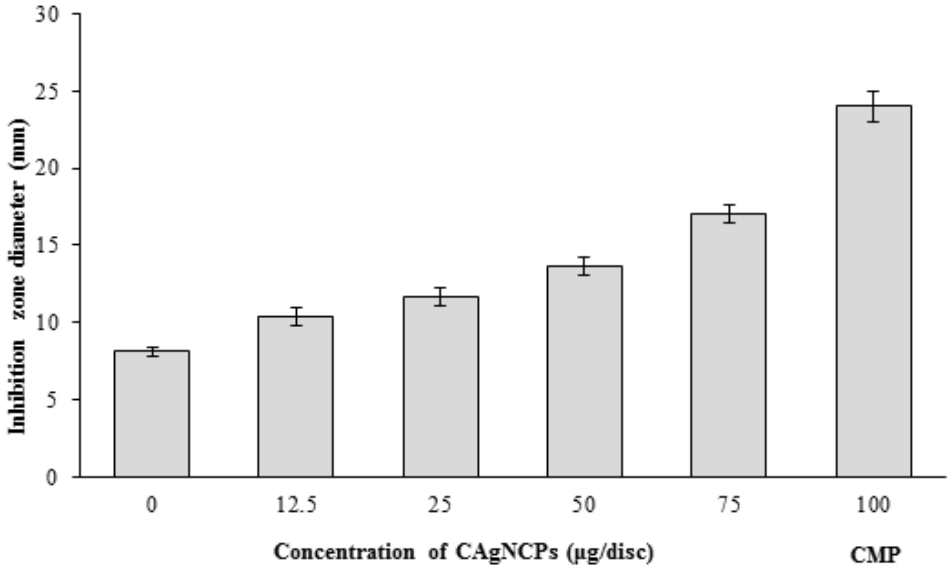

Figure 1: The inhibition zone of CAgNCs against *V. tapetis* in an agar disc diffusion assay, different concentrations (0-75 µg/disc) of synthesized CAgNCs was added on paper discs and plates were incubated at 25°C for 24 h. The discs with 0.25 % (V/V) acetic acid and chloramphenicol (100 µg/disc) were used as negative and positive controls, respectively. The diameter of the clear zone was measured in mm with diameter of disc (8 mm) for each treatment. The bars indicate the mean ± S.D (n=3).

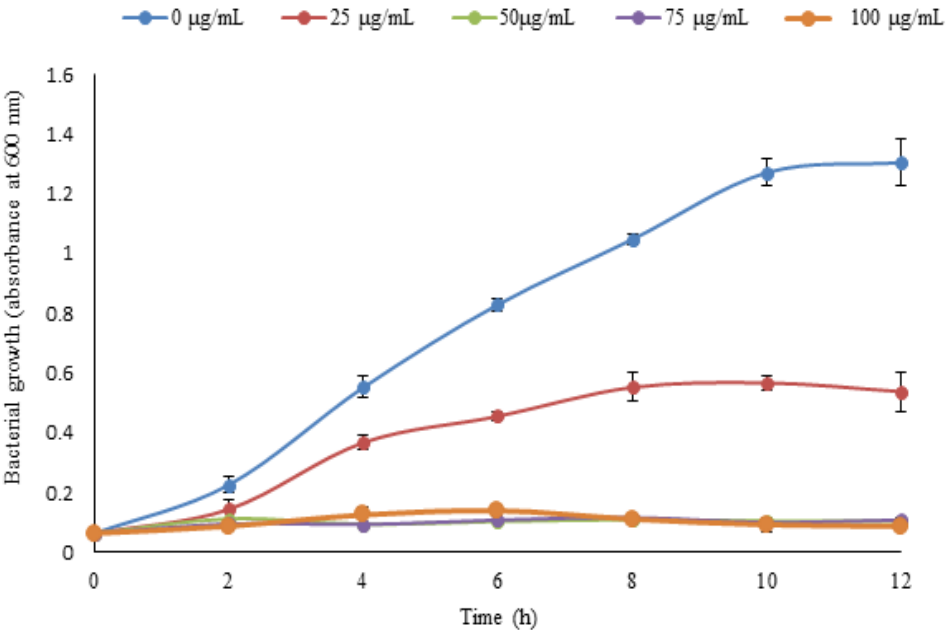

Figure 2: Growth curves of *V. tapetis* exposed to different concentrations of CAgNCs. In growth inhibition assay, cell growth was assessed after treatment with CAgNCs (12.5, 25, 50 and 75 µg/mL) at 25 °C by measuring the OD at 600 nm at every 2 h intervals. The bars indicate the mean ± S.D (n=3).

12 h. Using the same liquid growth dynamics, the MIC of CAgNCs towards *V. tapetis* was examined at 50 µg/mL, incubated at 25°C for 24 h with complete inhibition of visible growth. No single colony was observed on marine agar plates which was streaked with 100 µg/mL of CAgNCs treated *V. tapetis* culture (100 µL). Therefore, MBC of CAgNCs was considered as 100 µg/mL.

To understand the physical effects and changes of surface morphology of *V. tapetis* after CAgNCs treatment, the FE-SEM analysis was conducted. Most of cells in un-treated sample were not shown cell surface damage and consisted with uniform cell size (Figure 3A). However, CAgNPs treated *V. tapetis* showed gradual increase of damage on cell surface and significant change of cell morphology with increasing concentrations from 25 to 75 µg/mL for 6 h (Figure 3B-3D). At 75 µg/mL CAgNCs showed complete destruction of cell wall and

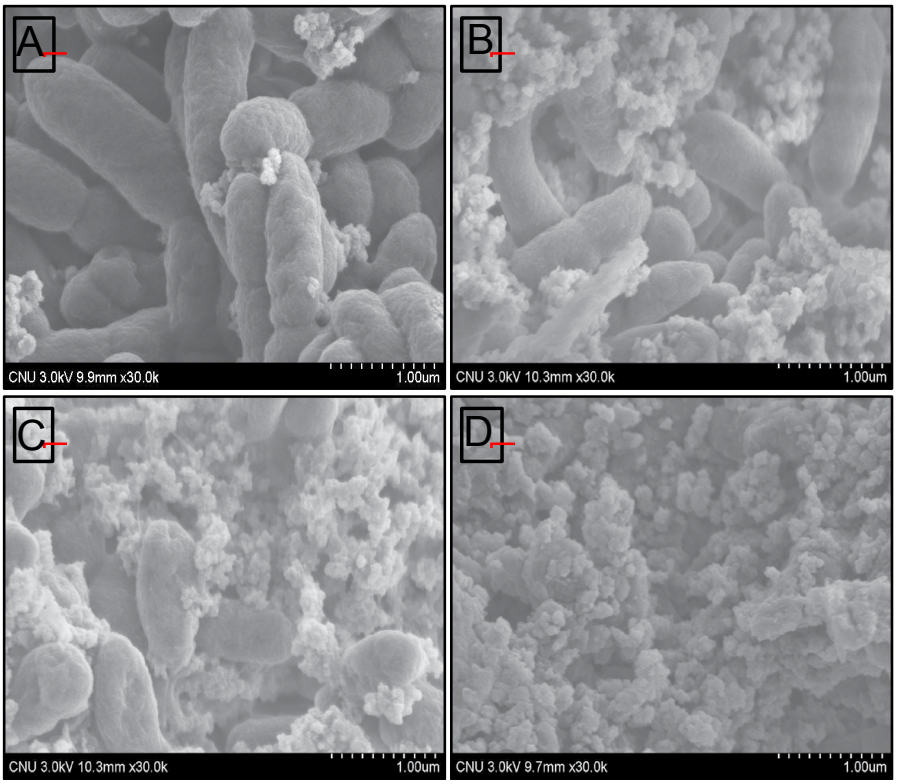

Figure 3: FE-SEM images of CAgNCs treated and native *V. tapetis* incubated at 25 °C for 6 h. A: Un-treated *V. tapetis* (control); B: 25 µg/mL; C: 50 µg/mL; D: 75 µg/mL.

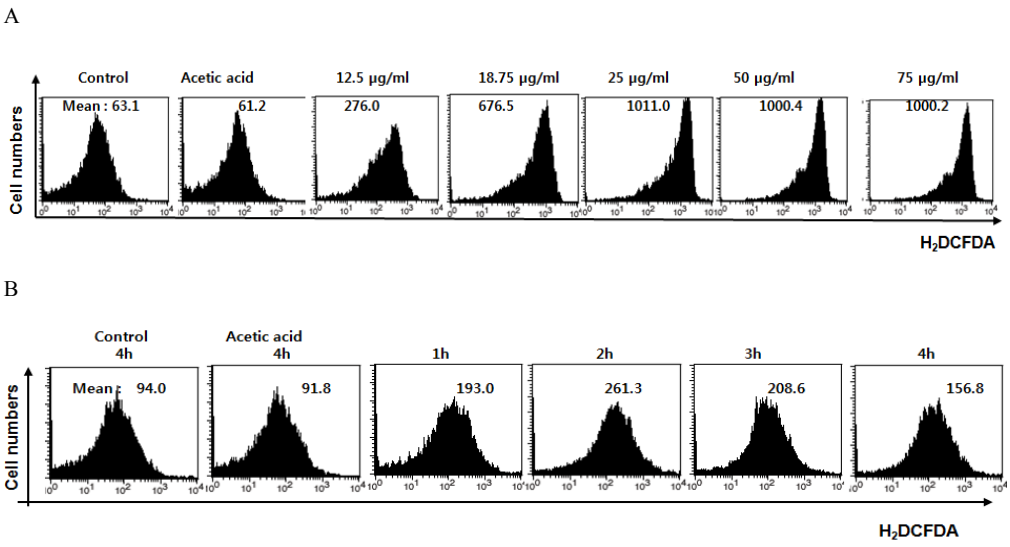

Figure 4: Effect of CAgNCs on ROS production in *V. tapetis* cells. Intracellular ROS generation was determined by the flow cytometry using H_2DCFDA. **A:** concentration dependent ROS production; **B:** time dependant ROS production.

a greater degree of cell lysis which has formed no visible cells in the sample.

Analysis of ROS production in *V. tapetis* with CAgNPs

To understand the possible mode of action of CAgNPs related to cellular events of *V. tapetis*, intracellular ROS was investigated. Flow cytometry analysis results revealed that CAgNCs generate higher ROS level in dose and time dependent manner (Figure 4). Cells treated with 12.5-25 µg/mL of CAgNCs were highly increased the level of ROS after 3 h. However, there was no further increase of ROS at 50 and 75 µg/mL. ROS production was reached to maximum at 3 h when cells exposed to 25 µg/mL CAgNCs. To investigate the time dependent ROS production, cells were treated at 25 µg/mL for 4 h and result were shown that maximum generation of ROS at 2h.

Effect of CAgNPs on cell viability

V. tapetis exposed to CAgNPs showed clear effect on cell viability as evident by the decrease in the formation of formazan in MTT assay (Figure 5). The number of live cells was decreased with the increasing CAgNPs concentration. At MIC level at 50 µg/mL, cell viability was observed as 55% whereas at 75 µg/mL, it was approximately 10%. Cells without CAgNCs treatment had highest cell viability of 100%.

Effect of CAgNPs on cell membrane permeability

To confirm the effect of CAgNPs on permeability change of cell membrane, level of nucleic acids in bacterial suspension was determined by spectrophotometry at 260 nm. Results revealed that 50 and 75 µg/mL CAgNCs treatment dramatically increased the OD at 260 nm up to 100 min compared to that of in un-treated cells which indicates the release of nucleic acids due to change of membrane permeability (Figure 6). It further shows the rate of permeability correlates with the concentration of CAgNCs and the time. Un-treated cells showed almost constant level of nucleic acids during the time of analysis.

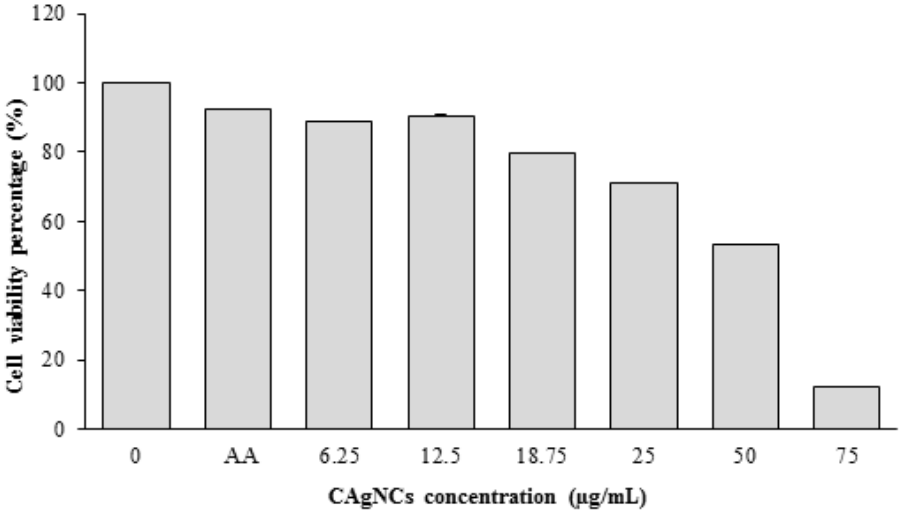

Figure 5: Effect of CAgNCs on cell viability of *V. tapetis*. Cell viability was determined by MTT assay after treatment with different concentrations of CAgNCs (6.25, 12.5, 18.75, 25, 50 and 75 µg/mL). AA: 0.25% acetic acid. Bars represent the standard deviations (n = 3).

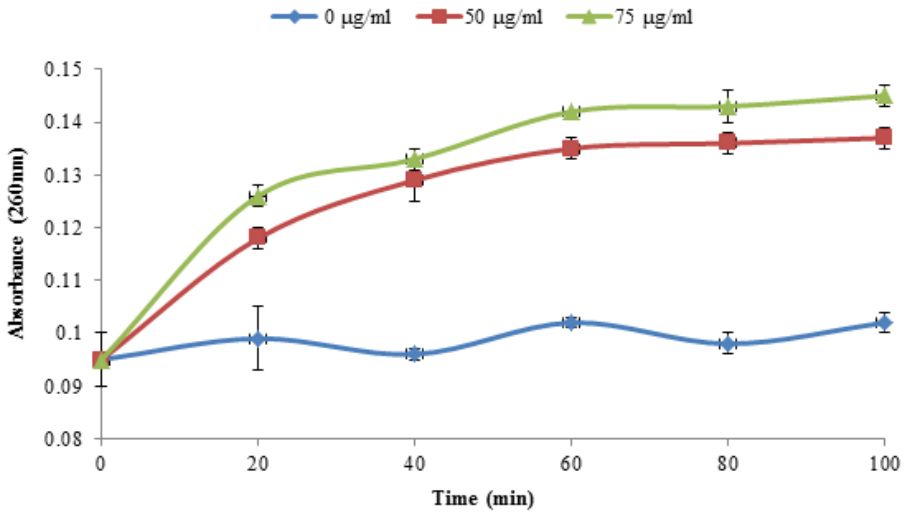

Figure 6: Effect of CAgNCs on membrane integrity of *V. tapetis*. The cell membrane integrity was determined by measuring residual nucleic acids in bacterial supernatant. Two CAgNCs treatments i) MIC (50 µg/mL) and ii) above MIC (75 µg/mL) are compared with un-treated *V. tapetis* cells at 25 °C by measuring at 260 nm for 20 min interval. The bars indicate the mean ± S.D (n=3).

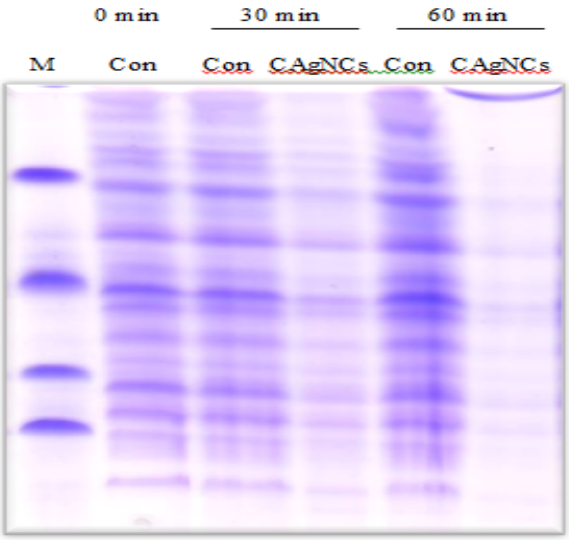

Figure 7: Protein expression analysis of CAgNCs treated (MIC-50 µg/mL) *V. tapetis* cells by SDS–PAGE. Cells were harvested 30 and 60 min after treatment and analyzed for total protein. M: protein marker; Con: un-treated cells.

Effect of CAgNPs on protein expression

Amount of protein in *V. tapetis* cells with respect to CAgNCs treatment is shown in Figure 7. It was very clear that 50 µg/mL of CAgNCs (MIC) treatment has greatly reduced the level of protein in cells after 30 min and 60 min compared to respective control samples which had strong protein expression. Change of protein level with disappearing the specific bands with the time after CAgNCs treatment also evident as it had lowest protein level in 60 min than 30 min.

Discussion

Numerous studies have shown antimicrobial properties of individual treatments of chitosan and silver nanoparticles [6,15,16]. Moreover, several attempts have been made to develop chitosan and Ag based nano products with higher antibacterial properties at a low concentration lower than that of individual components i.e. chitosan and Ag NPs [4]. Sanpui et al. [17] describe that MIC and MBC of CAgNCs against *Escherichia coli* are 100 and 120 µg/mL, respectively. MIC and MBC of the CAgNCs in the present study against *V. tapetis* are 50 and 100 µg/mL which suggests that CAgNCs may have stronger bactericidal effects against Vibrio species than *E. coli* strains. Two other reports show that CAgNPs inhibits pathogenic *E. coli, Salmonella choleraesuis, Salmonella typhimurium,* and *Staphylococcus aureus* [18] and *S. aureus*, *Pseudomonas aeruginosa* and *Salmonella entrica* [19]. However, no MIC and MBC values available to compare the present results of CAgNCs against Vibrio species and this is first report of CAgNCs antibacterial activity with *V. tapetis.*

Inoue et al. [20] has described that group of ROS such as superoxide anions, hydrogen peroxide, hydroxyl radicals, and singlet oxygen contributed to the antibacterial activity against *E. coli.* To understand the mode of action of CAgNCs, the possible involvement of ROS production was investigated. Our result clearly shows that CAgNCs treatment generate the higher ROS level in *V. tapetis* even at very low concentration 12.5 µg/µL. It is very clear that ROS generation is concentration dependent from 12.5 µg/µL to 25 µg/µL. Also, ROS level maintains at higher and constant level at 50 and 75 µg/µL which concentration correlates with the MIC (50 µg/µL) of the CAgNCs. The results suggest that CAgNCs triggers the oxidative stress by generating ROS which causes various damages to macromolecules such as DNA, RNA, and protein and other cellular components. The production of ROS could be caused by the impeded electronic transport along the respiratory chain in the damaged plasma membrane [21].

The bacterial cell membrane is a structural component that separates the cell interior from the outside environment. Exposure to antibacterial agents usually changes the bacterial cell membrane integrity and permeability [22]. Therefore, leakage of intracellular components is a classic indication of membrane damage. Large molecules such as DNA, RNA and proteins as well as small ions like potassium and phosphate tend to release depend on the level of cell permeability change [23]. Present study clearly shows that CAgNCs treatment (50 and 75 µg/µL) cause increase of nucleotides in bacterial suspensions suggesting this could be due to change of membrane integrity and structural damage. This damage is further supported by the results obtained from FE-SEM analysis which showed damage and irregular shape *V. tapetis* cells under same treatment of CAgNCs (50 and 75 µg/µL).

SDS-PAGE results shows lower level of protein content in CAgNPs treated cells at 30 min and it was further decreased at 60 min. However, we did not observe higher protein content in the supernatant as a result of permeating and disrupting cell membrane. Cui et al. [24] proposed that chitosan decreases the protein level in *S. aureus* and which may be due to inhibited protein synthesis or control gene expression. Similarly we propose that CAgNCs may involve in the inhibition of protein synthesis or control of gene expression in *V. tapetis* and which exact mechanism needs to be elucidated in future.

Conclusion

This study shows CAgNCs have superior antibacterial activity against *V. taptis* with lower MIC (50 µg/mL) and MBC (100 µg/mL). However, further modification of CAgNCs towards lowering the particle size may increase the bactericidal effects by following latest nanotechnology techniques. Herein, we demonstrate that CAgNCs are effective antibacterial agents with capability to disrupt cell membrane, de-stabilize the membrane permeability, induce the ROS production, and inhibit the expression of protein or synthesis of macromolecules. Overall results from this study suggest that excessive generation of ROS, loss in membrane integrity and inhibiting protein synthesis by CAgNCs may cause the bacterial cell death. However, further studies with different groups pathogenic bacteria (eg. Gram positive and gram negative) are necessary in order to fully understand its potential as a new bactericidal agent.

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