Silver coated carbon nanotubes regulate virulence gene expression in *Salmonella* Typhimurium

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ABSTRACT

Resistance of bacterial pathogens to existing antibiotics is a serious concern. Over the past several years, use of nanoparticles as an effective alternative to antibiotics is becoming popular. Of relevance, silver coated carbon nanotubes (AgCNTs) have several biomedical applications and their antibacterial activity against several multi-drug resistant pathogens is well documented. However, the exact molecular mechanism for their antibacterial activity is yet to be explored. In the present study, the antibacterial effect of AgCNTs against *Salmonella enterica* serovar Typhimurium was investigated using minimum inhibitory concentration (MIC) assay, quantitative growth analysis of *Salmonella* exposed to AgCNTs, electron microscopy (EM) and molecular studies using qRT-PCR. The MIC of AgCNTs was 62.5 µg/ml as characterized by no reduction of resazurin dye. Quantitative analysis showed the bactericidal effect of AgCNTs at 50 µg/ml whereas the concentrations such as 25, 12.5 and 6.25 µg/ml showed modest bactriostatic effect. EM analysis further revealed that when exposed to AgCNTs, bacterial cells internalized AgCNTs which resulted in lysis of the cells. The molecular studies using qRT-PCR showed that the expression of some of the major virulence genes of salmonella such as cigR (inner membrane protein), crp (virulence regulatory protein), ompF (outer membrane integrity), safC (outer membrane structure) and ybeF (DNA transcriptional activator) were downregulated several folds compared to the non treated bacteria (~2-3 folds). Our results thus indicate that AgCNTs enter bacteria through damage to the cell membranes and may regulate virulence gene expression in Salmonella Typhimurium.

Keywords: Silver coated carbon nanotubes, mechanism, virulence, gene, electron microscopy

1 INTRODUCTION

Increasing resistance of various pathogenic bacteria to existing antibiotics is of serious concern [1]. In the past few years, the use of metallic nanoparticles and their nanocomposites are gaining poularity as an effective resistance free approach due to their strong antimicrobial properties [2-4]. In particular, silver nanoparticles (AgNPs) have been reported to kill both gram-positive and gram-negative bacteria [5-8].

However, due to their agglomeration properties, AgNPs tend to aggregate into large particles, thus affecting their bactericidal properties [9]. To form a stable nanocomposite, carbon nanotubes (CNTs) are widely used as a host nanomaterial for AgNPs [8, 10, 11]. Silver coated CNTs (AgCNTs) have stronger antibacterial activity against Gram-positive as well as Gram -negative bacteria compared to commercially available AgNPs and CNTs alone [8]. However, the antibacterial action of AgCNTs is yet to be explored. Despite their significant role as antibacterials, very few studies have reported their antibacterial mechanism.

Some of the mechanisms implicated include dissolution of silver ions and contact mediated cell membrane damage through the generation of reactive oxygen species (ROS) [9]. However, the exact molecular mechanism of action still remains unclear. In case of pathogenic bacteria, expression of virulent genes is important to cause overwhelming disease [12]. There are several putative and well known gene-encoded virulence factors which have been identified for the virulence of bacteria [12]. It is of interest to investigate whether AgCNTs regulate the expression of the genes regulating virulence of bacteria.

Accordingly, the present study was designed to investigate whther AgCNTs regulate virulence genes' expression in a gram negative food borne pathogen, *Salmonella enterica* serovar Typhimurium. The antibacterial activity was investigated using the minimum inhibitory concentraion (MIC) assay, the quantitative growth curve assay, and electron microscopy (EM). Virulence gene expression studies were performed using quantitative reverse transcriptase polymerase chain reaction (qRT-PCR).

2 MATERIALS AND METHODS

2.1 Preperation of AgCNTs suspension

AgCNTs (1-5 nm, 95% pure), were purchased from NanoLab, Inc. (Waltham, MA, USA). 1 mg of AgCNTs were suspended in 1ml of water, sonicated for 1-2h and shaken for 30 min to obtain 1mg/ml suspension.

2.2 Determination of MIC.

The MIC values of AgCNTs were evaluated using a broth microdilution assay in sterile 96-well microtiter plates with or without using the redox reagent resazurin as described previously [13]. Briefly, 1×10^5 colony forming units (cfu/ml) of *Salmonella* Typhimurium were exposed to AgCNTs (62.5- 1.9 µg/ml). All plates were incubated at 37°C for 24 h. Upon incubation, the inhibition of bacterial growth was determined by measuring absorbance at 600 nm with a TECAN Sunrise™ enzyme-linked immunosorbent assay (ELISA) plate reader (Tecan US, Inc Morrisville, NC, USA). In resazurin dye added plates, MIC values were identified at 24 h as a color shift from purple to pink at the lowest concentration.

2.3 Quantitative growth curve of *Salmonella*

Salmonella Typhimurium (1×10^5 cfu/ml) growth was quantified in a timely manner (0, 4, 8, 16 and 24 h) after exposure to 50, 25, 12.5 and 6.25µg/mL of AgCNTs. The cultures were incubated at 37 °C with shaking at 250 rpm and the optical density at 600 nm (OD600) was determined at each time point. The growth curve was plotted as O.D vs. time point on the Y and X axis, respectively. For quantification of bacteria at each time point, 1ml of bacterial culture was subjected to serial 10-fold dilutions and then plated on PCA to quantitate their cfu/ml.

2.4 Electron microscopy

Scanning electron microscopy (SEM, Zeiss EVO 50, Carl Zeiss Meditec, Oberkochen, Germany) and transmission electron microscopy (TEM, Zeiss EM 10C 10CR, Carl Zeiss Meditec, Oberkochen, Germany) was used to examine AgCNTs and to observe the ultrastructural changes in *Salmonella* Typhimurium treated with AgCNTs. The non-treated bacterial cells were used as a control. SWCNTs-Ag and pSWCNTs-Ag samples for SEM were prepared as described previously with minor modifications [8].

2.5 qRT-PCR

The mRNA levels of some virulence factor-associated genes of *Salmonella* Typhimurium were investigated using qRT-PCR. The primer sets and the function of each gene are described in Table 1. The bacteria (1×10^5 cfu/ml) were treated with 12.5µg/mL of AgCNTs for 16h in a shaking incubator (250 rpm) at 37 °C, followed by total RNA extraction using RNeasy Mini kit (Qiagen, Germany). DNA was amplified and quantified in the Applied Biosystems® ViiA™ 7 real time PCR system (Life Technologies) using SYBR® Select Mastermix (Life Technologies, Grand Island, NY).

3 RESULTS AND DISCUSSION

AgCNTs, were visualized using electron microscopy. SEM and TEM of AgCNTs showed 1-5nm AgCNTs and the silver coating on CNTs was clearly evident (Figure 1)

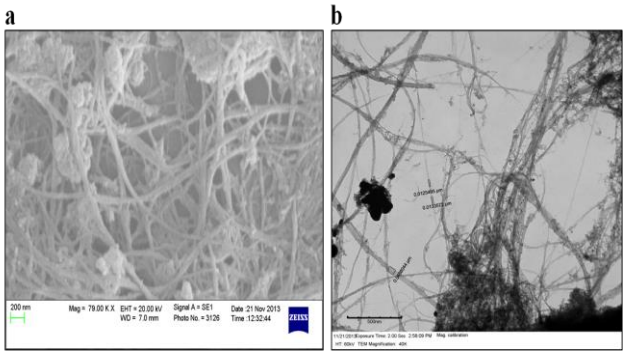

Figure 1: (a) Scanning electron microscope image of AgCNTs; (b) Transmission electron microscope image of AgCNTs

3.1 MICs

The MIC of AgCNTs against *Salmonella* Typhimurium was 62.5 µg/ml as indicated by no visible growth at this concentration. Based on the resazurin dye added to the plate, there was no color shift from purple ro pink at 62.5 µg/ml, whereas all other concentrations showed a color shift indicating that other concentrations of AgCNTs did not have bactericidal effect (Figure 2).

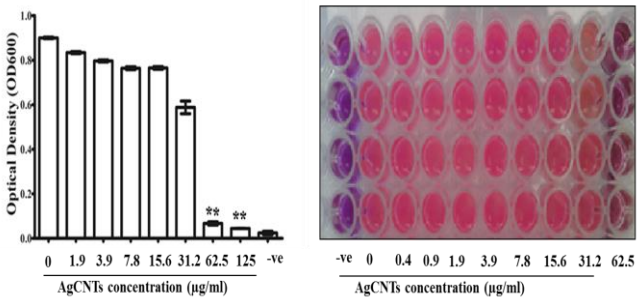

Figure 2: MIC concentration assay. (a) AgCNT without resazurin; (b) AgCNT with resazurin

3.2 Quantitative growth analysis

Salmonella growoth was monitored and quantified in a time and concentration dependent manner. Bacteria exposed to 50 µg/ml of AgCNTs did not show any bacterial growth at 8, 16 and 24 h of exposure. All other concentrations (25,

12.5 and 6.25 µg/ml) lower than 50 µg/ml did not show any inhibitory or antibacterial effect (Figure 3).

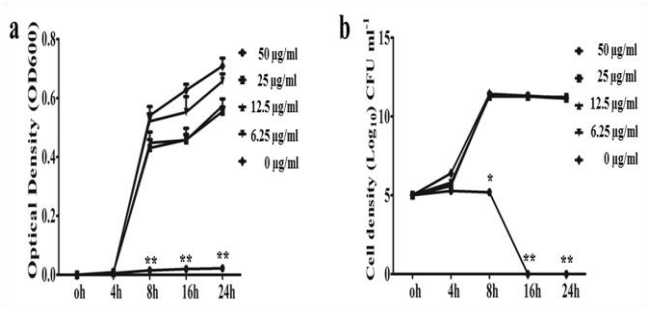

Figure 3: Growth curve and quantitative analysis. (a) Bacterial growth curve exposed to AgCNTs; (b) cfu/ml of survived bacteria upon exposure to AgCNTs

3.3 Ultrastructral changes in *Salmonella*

Salmonella Typhimurium were exposed to 12.5 µg/ml of AgCNTs and the ultrastructral effects on the bacteria were observed using SEM and TEM. SEM analysis revealed that AgCNTs damaged bacterial membranes and caused lysis of the bacterial cells (Figure 4b) compared to non treated healthy cells (Figure 4a). These findings were further confirmed by TEM analysis, which further showed the intereaction of the nanomaterial with the bacterial cell membrane and damaging the cell membrane (Figure 4d) comapred to non-treated cells (Figure 4c). TEM findings showed that the nanoparticles were internalized by the bacterial cells via damage to the membranes and caused explusion of the cytoplasmic conents (Figure 4d).

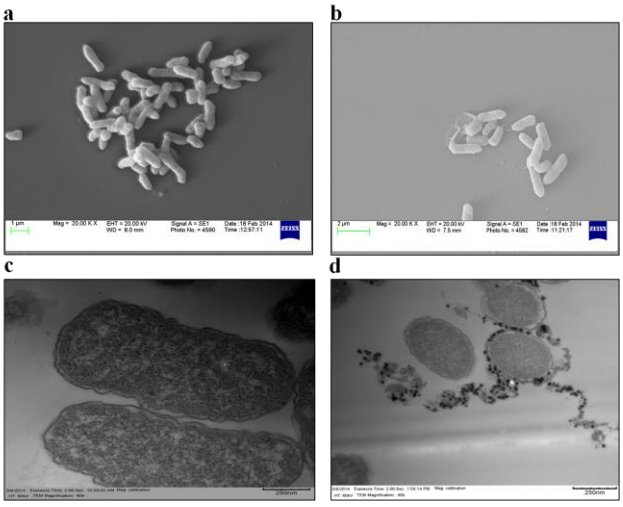

Figure 4: EM images of *salmonella*. (a) SEM of non treated; (b) SEM of AgCNTs treated; (c) TEM of non treated; (d) TEM of treated.

3.4 Virulence genes expression

In order to explore the molecular mechanism for the antibacterial activity of AgCNTs against *Salmonella* Typhimurium, expression of few, yet important, virulence associated genes were investigated. The virulence associated genes such as *cigR* [*Salmonella* pathogenicity island-3 (SPI)-associated], *crp* (SPI-1 regulation), *ompF* (outer membrane integrity), *safC* (SPI-6) and *ybeF* (transcriptional activator) were significantly downregulated several folds as compared to the non-treated cells (Figure 5).

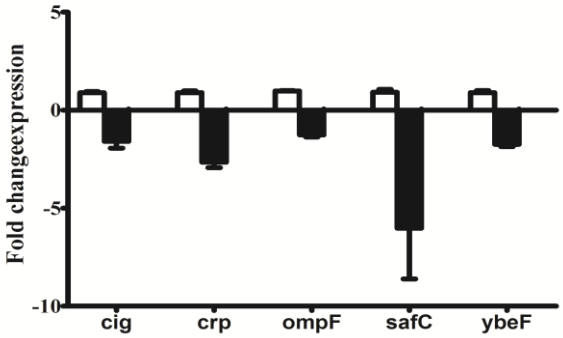

Figure 5: Virulence gene expression using qRT-PCR

Salmonella have several pathogenicity islands through which the bacteria regulate their virulence mechanisms [14]. *Salmonella* is an intracellular pathogen and requires various SPIs for its adhesion to the host cells, survival inside the host and then its replication [14]. In the present study, our results show that some of the major virulence associated (SPIs) genes were downregulated uopn AgCNTs treatement in *Salmonella* Typhimurium. In addition, a gene regulating the outer membrane integrity (*ompF*) was also significantly downregulated. This corroborates our TEM findings wherein the nanomaterial damages bacterial membranes causing cell lysis.

In conclusion, our results show that AgCNTs enter bacteria through damage to the cell membranes and AgCNTs may regulate virulence gene expression in *Salmonella* Typhimurium.

Table 1. Primers used in this study.

Gene	Forward (5`-3`)	Function
cigR	CCCACATCAGAAGGGCAATATC GCCATTACCATTTCCCGGA	SPI-3 associated
safC	ATGGTAGCGCCATTCCTTTC CCGCCAAACCAGTGAGATAAA	SPI-6 associated
ompF	CGTTGGCGCGAAATATGATG GTTTGCCATTTCCACGGTATC	Outer membrane protein F
ybeF	TCCCGATCCGCTGTTTATTC GCTTATCATAGCTGCCCGTAA	DNA transcription activator
crp	GTGGCAGTGCTGATCAAAGA CAGGCCCAGTTCACCAATAAA	SPI-1 regulation
16s rRNA	CAGAAGAAGCACCGGCTAAC AATGCAGTTCCCAGGTTGAG	Endogenous control

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REFERENCES

[1] L.M. Durso and K.L. Cook, Impacts of antibiotic use in agriculture: what are the benefits and risks?, Current opinion in microbiology, 19C, 37-44, 2014.

[2] R.Y. Pelgrift and A.J. Friedman, Nanotechnology as a therapeutic tool to combat microbial resistance, Adv Drug Deliv Rev., 65, 1803-15, 2013.

[3] S. Chatterjee, A. Bandyopadhyay and K. Sarkar, Effect of iron oxide and gold nanoparticles on bacterial growth leading towards biological application, J Nanobiotechnology, 9, 34, 2011

[4] A.J. Huh and Y.J. Kwon, "Nanoantibiotics": a new paradigm for treating infectious diseases using nanomaterials in the antibiotics resistant era, J Control Release, 156, 128-45, 2011.

[5] P. Dallas, V.K. Sharma and R. Zboril, Silver polymeric nanocomposites as advanced antimicrobial agents: classification, synthetic paths, applications, and perspectives, Adv Colloid Interface Sci, 166, 119-35, 2011.

[6] M. Guzman, J. Dille and S. Godet, Synthesis and antibacterial activity of silver nanoparticles against gram-positive and gram-negative bacteria, Nanomedicine, 8, 37-45, 2012.

[7] A. Kurek, A.M. Grudniak, A. Kraczkiewicz-Dowjat and K.I.Wolska, New antibacterial therapeutics and strategies, Pol J Microbiol, 60, 3-12, 2011.

[8] V.K. Rangari, G.M Mohammad, S. Jeelani, A. Hundley, K. Vig, S.R.Singh and S.R. Pillai, Synthesis of Ag/CNT hybrid nanoparticles and fabrication of their nylon-6 polymer nanocomposite fibers for antimicrobial applications, Nanotechnology, 21, 095102, 2010.

[9] R. Su, Y. Jin, Y. Liu, M. Tong and H. Kim, Bactericidal activity of Ag-doped multi-walled carbon nanotubes and the effects of extracellular polymeric substances and natural organic matter, Colloids Surf B Biointerfaces, 104, 133-39, 2013.

[10] S. Brahmachari, S. Duttaa and P.K. Das. Biotinylated amphiphile-single walled carbon nanotube conjugate for target-specific delivery to cancer cells, J Mater Chem B, 2, 1160-73, 2014.

[11] J.H. Jung, G.B. Hwang, J.E. Lee and G.N.Bae, Preparation of airborne Ag/CNT hybrid nanoparticles using an aerosol process and their application to antimicrobial air filtration, Langmuir : the ACS journal of surfaces and colloids, 27, 10256-264, 2011.

[12] A.A. Chaudhari and S. Kariyawasam. Innate immunity to recombinant QseC, a bacterial adrenergic receptor, may regulate expression of virulence genes of avian pathogenic *Escherichia coli*, Vet Microbiol, 171, 236-41, 2014.

[13] M.K. Soehnlen, M.E. Kunze, K.E. Karunathilake, B.M. Henwood, S. Kariyawasam, D.R. Wolfgang and B. Jayarao, *In vitro* antimicrobial inhibition of *Mycoplasma bovis* isolates submitted to the Pennsylvania Animal Diagnostic Laboratory using flow cytometry and a broth microdilution method, J Vet Diagn Invest, 23, 547-51, 2011.

[14] S.L. Marcus, J.H. Brumell, C.G. Pfeifer and B.B. Finlay, *Salmonella* pathogenicity islands: big virulence in small packages, Microbes Infect, 2, 145-56, 2000.