

Sequential interactions of Silver-silica nanocomposite (Ag-SiO₂NC) with cell wall, metabolism and genetic stability of *Pseudomonas aeruginosa*, a multiple antibiotic resistant bacterium

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Running title: Antibacterial silver nanoparticles

Significance and Impact of the Study: Although the synthesis, structural characteristics and biofunction of silver nanoparticles are well understood, their application in antimicrobial therapy is still at its infancy as only a small number of microorganisms are tested to be sensitive to nanoparticles. A thorough knowledge of the mode of interaction of nanoparticles with bacteria at subcellular level is mandatory for any clinical application. The present study deals with the interactions of Ag-SiO₂NC with the cell wall integrity, metabolism and genetic stability of *Pseudomonas aeruginosa* which would contribute substantially in strengthening the therapeutic applications of silver nanoparticles.

Abstract

The study was carried out to understand the effect of silver-silica nanocomposite (Ag-SiO₂NC) on the cell wall integrity, metabolism and genetic stability of *Pseudomonas aeruginosa*, a multiple drug-resistant bacterium. Bacterial sensitivity towards antibiotics and Ag-SiO₂NC was studied using standard disc diffusion and death rate assay, respectively. The effect of Ag-SiO₂NC on cell wall integrity was monitored using SDS assay and fatty acid profile analysis while the effect on metabolism and genetic stability was assayed microscopically, using CTC viability staining and comet assay, respectively. *P. aeruginosa* was found to be resistant to β -lactamase, glycopeptidase, sulfonamide, quinolones, nitrofurantoin and macrolides classes of antibiotics. Complete mortality of the bacterium was achieved with 80 μgml^{-1} concentration of Ag-SiO₂NC. The cell wall integrity reduced with increasing time and reached a plateau of 70 % in 110 min. Changes were also noticed in the proportion of fatty acids after the treatment. Inside the cytoplasm, a complete inhibition of electron transport system was achieved with 100 μgml^{-1} Ag-SiO₂NC, followed by DNA breakage. The study thus demonstrates that Ag-SiO₂NC invades the cytoplasm of the multiple drug-resistant *P. aeruginosa* by impinging upon the cell wall integrity and kills the cells by interfering with electron transport chain and the genetic stability.

Key words: Silver nanoparticles, *Pseudomonas aeruginosa*, antibacterial, Comet assay, VBNC

Introduction

Emergence of multiple antibiotic resistant pathogenic bacteria due to the indiscriminate use of antibiotics has necessitated the search for alternate strategies for controlling these pathogens. Antimicrobial properties of silver nanoparticles (AgNPs) have been demonstrated against a wide range of microorganisms (Sondi 2004; Panacek et al. 2006; Kim et al. 2007; Pal 2007; Srivastava 2007; Choi 2008; Lut et al. 2008; Ayala-Nunez et al. 2009; Rai et al. 2009; Kalishwaralal et al. 2010; Lara et al. 2010; Mandal et al. 2011; Beer et al. 2012; Li 2010), and no documented report on the emergence of bacterial resistance to silver nanoparticles is available. However, it is not clear whether AgNPs function similar to silver ions or differently. Silver ions damage the membrane proteins, interrupts electron cycle and induce DNA dimerization through the interaction with thiol (S-H) groups of aminoacids and other compounds (Feng et al. 2000; Gunawan et al. 2011). It was proposed that AgNPs upon contact with the cell wall of *Escherichia coli* initiates a chain of reactions, including the expression of a number of envelop proteins (OmpA, OmpC, OmpF, OppA, and MetQ), resulting in the formation of permeable pits (Vara 1992; Lok et al. 2006). A major prerequisite for achieving maximal antimicrobial activity of AgNPs is to maintain their sensitive surface chemistry, colloidal stability and nanosize (Jana et al. 2007). However, in many cases the metal nanoparticles showed aggregation in aqueous solutions, resulting in retarded biological activity (Jana et al. 2007). Though several strategies have been reported for producing biofunctional AgNPs with enhanced water solubility and monodispersity, silica coating is the widely accepted one (Jana et al. 2007; Thomas et al. 2008).

In this study, we investigated the effect of silver-silica nanocomposite (Ag-SiO₂NC) on the cell wall integrity, metabolism and genetic stability of a multiple antibiotic resistant strain of *Pseudomonas aeruginosa*. *P. aeruginosa* is an aerobic, non fermenting Gram negative rod bacterium that is important in the etiology of nosocomial infections. This bacterium is notorious for its ability to colonize various sites of the human body, including sputum, cornea, nasal mucosa and wet skin, causing a range of diseases, from simple skin infection to fulminant septicemia (Lee et al. 2012). Being a nosocomial pathogen, it is possible that *P. aeruginosa* may be co-evolving with the antibiotics, thus requiring more efficient strategies for controlling the infections. Coating the surgical devices with AgNPs has been proposed as one of the efficient methods for preventing the transmission of this pathogen (Furno et al. 2004). Here, we report how Ag-SiO₂NC impinges upon the cell wall integrity and interferes with electron transport chain and genetic stability of a multiple antibiotic resistant strain of *P. aeruginosa*.

Results and Discussion

Continuous evolution of antibiotic resistant mechanisms among nosocomial pathogens demands the identification of alternate strategies for countering their threat to both humans and animals. The experimental strain of *P. aeruginosa* showed resistance to all the cell wall- (β -lactamase and glycopeptidase classes) and nucleic acid synthesis (sulfonamide, quinolones and nitrofurantoin classes) - inhibiting antibiotics used in the study (Table S1). It was also resistant to the Macrolid class protein synthesis inhibitor Azithromycin, but was sensitive to the Aminoglycosides (Amikacin and Gentamycin), Tetracyclines (Tetracyclin and Oxytetracyclin) and Chloramphenicol class of protein synthesis inhibiting antibiotics. Recent reports on co-evolution of antibiotic resistance among microorganisms reiterate the importance of silver compounds with AgNPs as a promising substitute (Rai et al. 2012). Compared to silver compounds, Ag-SiO₂NC has better antibacterial properties owing to their large surface area, positive surface charge and monodispersity. Net positive charge of SiO₂ facilitates more number of nanoparticles to interact with negatively charged surface of bacteria, resulting in highly efficient antimicrobial activity (Jana et al. 2007). Interestingly, we observed that the death rate of *P. aeruginosa* increased with increasing concentration of Ag-SiO₂NC (13.5 % on exposure to 20 $\mu\text{g mL}^{-1}$) and attained 100 % mortality with 80 $\mu\text{g mL}^{-1}$ (Figure 1).

Although the antimicrobial mechanisms of silver compounds are well documented, the exact mode of action of AgNPs remains unclear (Gunawan et al. 2011; Rai et al. 2012). Here, we observed sequential mode of interaction of Ag-SiO₂NC with the test bacterium at sub-cellular level. Continuous exposure of *P. aeruginosa* to Ag-SiO₂NC for 110 min reduced the cell wall integrity to 70 % (Figure 2). Cell wall integrity is expressed as a function of its sensitivity towards detergent mediated cell lysis (Lok et al. 2006). Cell wall with damaged proteins will be highly prone to detergents, showing sharp decline in the optical density (OD₆₀₀) due to the leakage of cell content. Sondi et al (2004) observed the localization of AgNPs on the surface of *E. coli*, while particles below 10 nm size penetrated the cell membrane. Cell membrane is the first site of action of AgNPs, where they initiate a cascade of reactions, including the expression of a number of envelope proteins (Lok et al. 2006) and disruption of barrier compounds (Vara 1992; Lok et al. 2006). We also observed major changes in the proportion of fatty acids such as 3OH-10:0, 2OH-12:0, 3OH-12:0, 16:0, Cyclo-17:0, Cyclo w8c – 19:0, w7c-18:1 and w6c-18 in *P. aeruginosa* after treating with Ag-SiO₂NC, but no significant changes in their profiles were seen (Table 1). Bacteria maintain homeostasis between the external environment and cell physiology by

adjusting the membrane fluidity via altering the proportions of fatty acids. However, severe alterations may impinge upon the cell wall integrity, resulting in the dissipation of transmembrane gradients of protons and ions (Mrozik et al. 2004). In the present case, we presume that the alteration in the proportion of fatty acids may be responsible for the translocation of Ag-SiO₂NC in to the cytoplasm.

The bacterial metabolism was measured as the ability of electron transport chain of metabolically active cells to reduce 5-cyano-2,3-ditolyl tetrazolium chloride (CTC) into red fluorescent formazan. A gradual decrease in the metabolically active cells against increasing concentrations of Ag-SiO₂NC was recorded (Figure 2). When all the cells are metabolically active, the ratio of metabolically active: total cells will be close to 1. High ratio of 0.8 was observed in the control (without Ag-SiO₂NC) whereas in presence of Ag-SiO₂NC, the ratio decreased close to zero (100 µgmL⁻¹). Though metabolically active, the cultivability of the bacteria was completely lost at 80 µgmL⁻¹ concentration of Ag-SiO₂NC. This discrepancy could be attributed to cells being viable but nonculturable (VBNC) due the bacteriostatic activity of Ag-SiO₂NC as was observed in the difference in bacterial counts between CTC and death rate assay. Evidently, many bacteria are capable of entering into VBNC state to protect themselves from natural stresses (Oliver 2005; Griffitt et al. 2011).

It was observed that DNA of 68 % of cells was damaged after 1 h exposure to Ag-SiO₂NC, whereas DNA of all the cells in untreated group remained intact (Figure 4). This can be attributed to the reactive oxygen species (ROS) generated through the release of Ag⁺ ions by Ag-SiO₂NC on contact with cytoplasmic proteins (AshaRani 2009). ICP-MS analysis recorded 0.136 and 0.126 ppm of available silver ions from 80 and 100 µgmL⁻¹ concentrations of Ag-SiO₂NC respectively (Figure S1). ROS are a group of highly reactive species formed via the activation/ reduction of molecular oxygen and includes singlet oxygen (¹O₂), superoxide anion (O₂^{-*}), hydroxyl radical (HO^{*}), and hydrogen peroxide (H₂O₂). Among the different propositions describing the antibacterial properties of AgNPs, the release of silver ions and subsequent induction of ROS are the prominent ones (Choi et al. 2008; Navarro et al. 2008; Wigginton et al. 2010; Badawy et al. 2011). Although minimum concentrations of ROS are necessary for maintaining the regular metabolism of cells, elevated levels can induce DNA damage and breakage (Anas et al. 2008). Short lifespan (250 ns) and narrow diffusion power (45 nm) of ROS (Asok et al. 2012) demand AgSiO₂NC placed in the proximity of DNA to attain a substantial damage. Silver ions form strong adducts with purine and pyrimidine bases of DNA (Wang et al. 2010), and therefore the substantial DNA damage observed here is a possibility. Ag-SiO₂NC induced DNA breakage in *P.*

aeruginosa was confirmed in the comet assay. In conclusion, the study proposes that Ag-SiO₂NC is a potential antibacterial agent, and follows sequential interaction with the cell wall, metabolism and genetic stability of the multiple antibiotic resistant *P. aeruginosa*. Ag-SiO₂NC intrudes the cytoplasm by inducing damages to the cell wall and kills the cells by interfering with electron transport chain and genetic stability. An extension of this work with species specific marker conjugated Ag-SiO₂NC and targeting of specific pathogens is proposed which will have considerable positive impact on antimicrobial therapy.

Experimental procedures

Bacteria and nanoparticles

P. aeruginosa strain was obtained from the Marine Microbial Reference Facility (MMRF) of CSIR- National Institute of Oceanography- Regional Centre Cochin India. *P. aeruginosa* was inoculated into Luria Bertani (LB) media and grown overnight at 28 ± 2 °C on a shaker at 120 rpm. Working cultures of *P. aeruginosa* were maintained on LB agar slants and subcultured every 2-3 weeks. The purity of the culture was confirmed by the fatty acid profile.

Ag-SiO₂NC was prepared using Ag-SiO₂ sol and employing tetraethyl orthosilicate -(TEOS), ethanol, distilled water and silver nitrate as precursors. The detailed procedure of nanoparticles synthesis and its characterization using X-ray powder diffractometer and Transmission Electron Microscopy techniques are discussed elsewhere (Thomas et al. 2008). Histogram of the particle sizes obtained from transmission electron microscope showed that the mean particle size was 5-6 nm. The available Ag in Ag-SiO₂NC phosphate buffered saline (PBS) was measured using inductively coupled plasma mass spectrometry (ICP-MS).

Bacterial sensitivity towards antibiotics and Ag-SiO₂NC

Sensitivity of *P. aeruginosa* to twelve antibiotics was tested following standard disc diffusion technique. LB agar plates were swabbed with overnight grown *P. aeruginosa* suspension cultures and commercially available antibiotic discs (HiMedia, India) were placed on the agar and incubated at 28 ± 2 °C for 24 h. The zone of inhibition was recorded at the end of the incubation period. The antibiotics tested were ampicillin 25 mcg, azithromycin 15 mcg, amikacin 10 mcg, chloramphenicol 30 mcg,

ciprofloxacin 10 mcg, gentamycin 30 mcg, vancomycin 10 mcg, nalidixic acid 30 mcg, nitrofurantion 100 mcg, oxytetracycline 30 mcg, trimethoprim 10 mcg and tetracycline 10 mcg.

Sensitivity of *P. aeruginosa* against Ag-SiO₂NC was tested following standard death rate assay (Asok et al. 2012). Briefly, the overnight grown *P. aeruginosa* cells (10⁶ cells mL⁻¹) were mixed with different concentrations of Ag-SiO₂NC (0, 20, 40, 60, 80 and 100 µg mL⁻¹) and incubated at room temperature for 1 h. Subsequently, 100 µL aliquots were plated in triplicate over the surface of a nutrient agar plate, incubated at 28± 2°C for 24 h before enumerating total number of colonies. Death rate ‘ε’ was calculated, by using the following equation:

$$\text{Death rate } (\epsilon) \% = \frac{(N_1 - N_2)}{N_1} \times 100$$

Where, N₁ and N₂ are the number of colonies grown on the control and experimental plate, respectively.

Also, the number of metabolically active and dead cells in the experimental groups were counted microscopically after staining with BacLight Redoxsensor CTC viability kit (Molecular Probes, Invitrogen USA), following the protocols suggested in the product brochure.

Effect of Ag-SiO₂NC on cell wall integrity and genetic stability of *P.aeruginosa*

The effect of Ag-SiO₂NC on cell wall integrity and genetic stability of *P. aeruginosa* was investigated by SDS assay (Lok et al. 2006) and comet assay (Singh et al. 1999), respectively. For SDS assay, *P. aeruginosa* cells from an overnight culture were washed copiously with sterile phosphate buffered saline and dispensed in the wells of a sterile microplate to a volume of 200 µL. After measuring the initial absorbance, the test solution was supplemented with Ag-SiO₂NC and SDS (0.1%). Control wells without Ag-SiO₂NC also were maintained. The absorbance at 600 nm was recorded every 15 min for a period of 2 h. The decrease in absorbance compared to the initial reading was plotted against time. All the experiments were carried out three times in duplicate. The fatty acid profile of *P. aeruginosa* before and after treatment with Ag-SiO₂NC was analyzed. The log phase cells of *P. aeruginosa* were treated with Ag-SiO₂NC for 3 h and whole cell fatty acids were extracted, methylated according to standard protocols and analyzed using a gas chromatography system (Agilent GC 6950).

For comet assay, 10^6 bacterial cells before and after Ag-SiO₂NC treatment were mixed with 100 μ l of 0.5% low melting point agarose prepared in TAE buffer containing RNase (5 gmL^{-1}), SDS (0.25 %) and lysozyme (0.5 mgmL^{-1}). Bacterial cells impregnated in agarose solution were spread over a microscopic slide pre-coated with thin layer of agarose (0.5 %). The cells on the slides were lysed at 37 °C for 1 h, by immersing in a lysis solution, followed by incubation in an enzyme solution for 2 h at 37 °C. Subsequently, the slides were equilibrated with 300 mM sodium acetate and subjected for electrophoresis at 25 V for 1 h. Following electrophoresis, the slides were immersed in 1 M ammonium acetate in ethanol for 30 min, and then in absolute ethanol for 1 h, air dried at 25°C, immersed in 70 % ethanol for 30 min and air dried. The slides were stained with SYBR green and comets were observed under a fluorescent microscope. The nucleic acids were classified based on comet length as intact (0- 10 μ m) and damaged ($>10 \mu$ m) groups and expressed as % of incidence in the histogram. The assay was repeated three times in duplicates.

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Tables

Table 1. Comparison of the fatty acid composition of *Pseudomonas aeruginosa* before and after treatment with Ag-SiO₂NC

Fatty acid	Proportion of fatty acid (%)	
	<i>Before treatment</i>	<i>after treatment</i>
10:0 3OH	4.42	2.66
12:00	3.96	3.62
11:0 3OH	0.1	ND
12:0 2OH	4.89	4.35
12:1 3OH	0.53	0.27
12:0 3OH	5.47	4.71
14:00	0.8	0.85
15:0 anteiso	ND	0.1
16:00	33.91	35.08
17:1 iso w5c	ND	0.97
17:0 iso	0.11	0.13
17:1 w8c	0.2	0.18
17:0 cyclo	2.21	1.68
17:00	0.22	0.24
18:00	0.74	0.81
19:0 cyclo w8c	3.68	2.84
Summed Feature 3*	8.58	8.66
Summed Feature 8*	30.17	32.84

Results are presented as percentage of the total fatty acids. *Summed features represent groups of two or three fatty acids that cannot be separated by GLC with the MIDI system. Summed feature 3 contains 16:1 w7c/16:1 w6c, Summed feature 8 contains 18:1 w7c and 18:1w6c.

Figure Legends

Figure 1. Responses of *Pseudomonas aeruginosa* towards increasing concentrations of Ag-SiO₂NC. Results are expressed as death rate \pm SD

Figure 2. Effect of Ag-SiO₂NC on metabolic activity of *Pseudomonas aeruginosa*. Results are expressed as ratio of active: total cells observed in dead/live staining

Figure 3. Cell wall integrity of *Pseudomonas aeruginosa* on exposure to Ag-SiO₂NC for different time intervals. Results are expressed as cell wall integrity (%) \pm SD

Figure 4. Comet assay histogram of *Pseudomonas aeruginosa* (a) before and (b) after treatment with Ag-SiO₂NC. Comets are classified based on tail length into intact (0-10 μ m) and damaged (>10 μ m) groups

Figure 1:

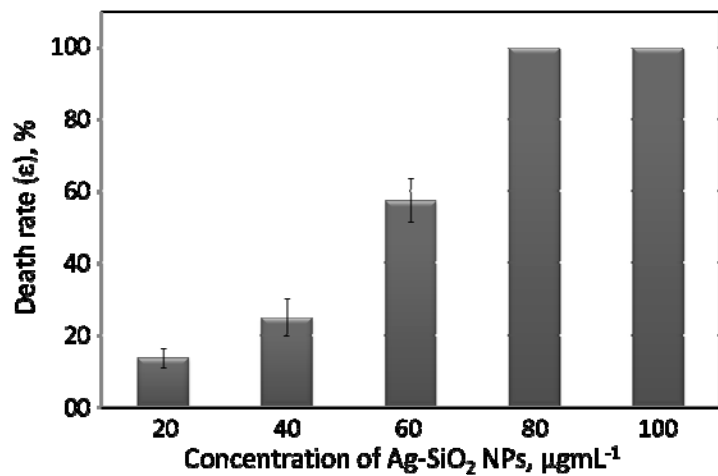


Figure 2:

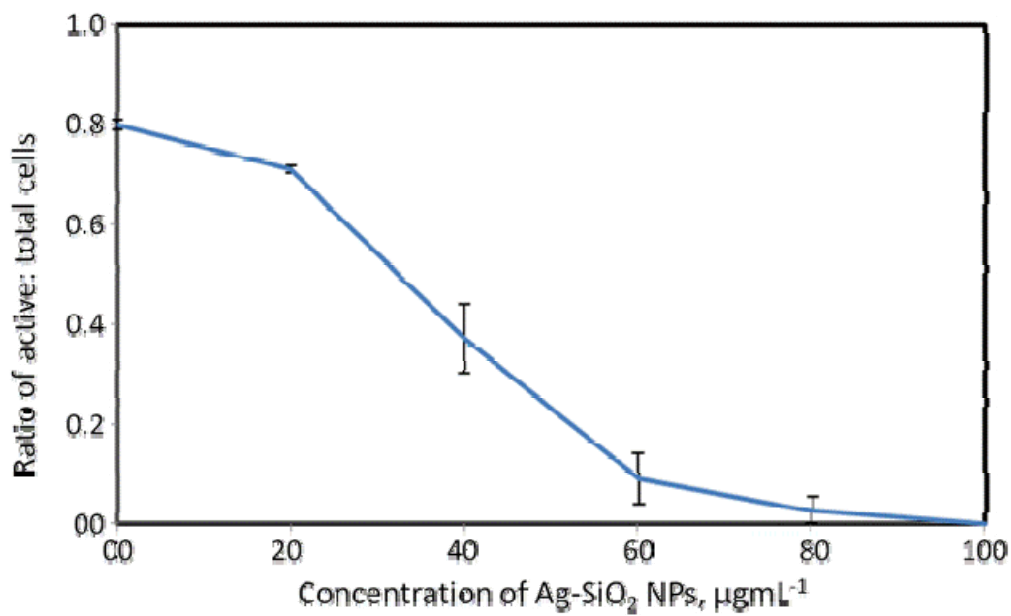


Figure 3:

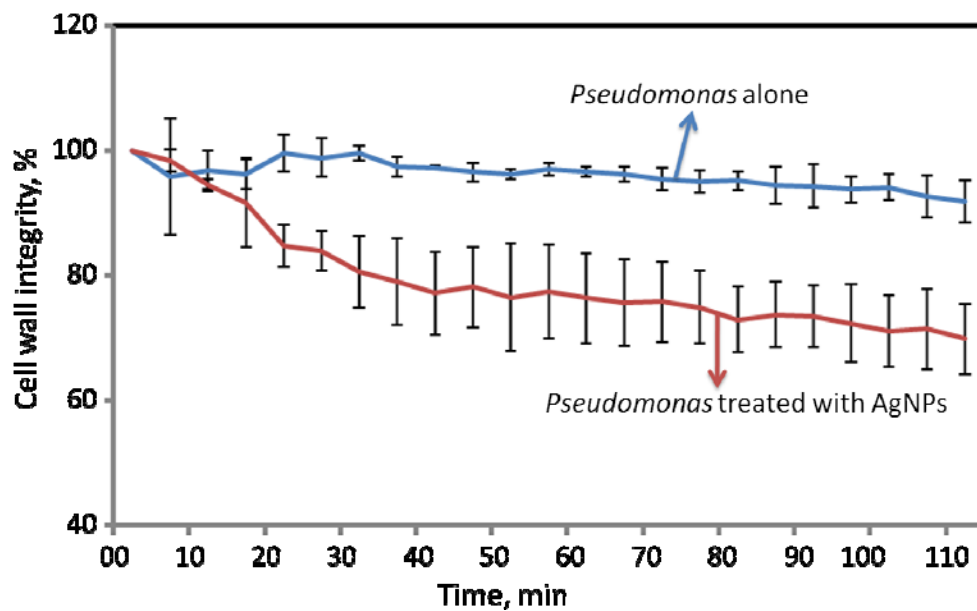


Figure 4:

