

# Silver-coated carbon nanotubes downregulate the expression of *Pseudomonas aeruginosa* virulence genes: a potential mechanism for their antimicrobial effect

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**Abstract:** The antimicrobial activity of silver-coated carbon nanotubes (AgCNTs) and their potential mode of action against mucoic and nonmucoic strains of *Pseudomonas aeruginosa* was investigated in vitro. The results showed that AgCNTs exhibited antimicrobial activity against both strains with minimum inhibitory concentrations of approximately 8 µg/mL, indicating a high sensitivity of *P. aeruginosa* to AgCNTs. AgCNTs were also bactericidal against both strains at the same minimum inhibitory concentration. Scanning and transmission electron-microscopy studies further revealed that a majority of the cells treated with AgCNTs transformed from smooth rod-shape morphology to disintegrated cells with broken/damaged membranes, resulting in leakage of cytoplasmic contents to produce ghost cells. The molecular effects of AgCNTs on *P. aeruginosa* genes involved in virulence and pathogenicity, stress response, and efflux pumps were evaluated for changes in their expression. Quantitative real-time PCR (qRT-PCR) showed that after exposure to AgCNTs, the expression levels of the *rpoS*, *rsmZ*, and *oprD* genes were significantly downregulated in both strains of *P. aeruginosa* compared to the untreated samples. These results suggest that the mechanism of action of AgCNTs may be attributed to their effect on cell-membrane integrity, downregulation of virulence-gene expression, and induction of general and oxidative stress in *P. aeruginosa*.

**Keywords:** *Pseudomonas aeruginosa*, AgCNT, antimicrobial activity, qRT-PCR

## Introduction

One area of rapidly growing interest in the development of new antimicrobials is the use of nanoparticles coupled with metals. Silver, for example, has shown antimicrobial activity against a wide range of microorganisms.<sup>1</sup> Therefore, silver-based nanomaterials are being used for their bactericidal activity.<sup>2,3</sup> Although the antimicrobial activity of silver is well known, its mechanism of action is not yet fully understood. Several mechanisms have been proposed, such as a loss of the replication ability of DNA<sup>4</sup> and changes in membrane structure.<sup>5</sup>

Carbon nanotubes (CNTs) are used in many nanocomposites, because they possess distinct mechanical and electronic properties.<sup>6</sup> CNTs also possess antimicrobial activity, possibly resulting from physical damage to bacterial cells that inhibits the growth of biofilm-forming microbes on surfaces. However, drawbacks to their antimicrobial applications include instability, aggregation, and lack of bioavailability of the NTs.<sup>7</sup> Some of these issues may be mitigated by hybrid nanocomposites of silver and CNTs, which may have enhanced antimicrobial activity while reducing aggregation. Silver-coated carbon nanotubes (AgCNTs) are highly attractive as water-disinfecting materials, due

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to their ability to remain insoluble. Other antimicrobial hybrid materials, such as dendritic poly(amidoamine) combined with silver, are soluble in water and contaminate treatment feeds when used as antimicrobials.<sup>8</sup>

*Pseudomonas aeruginosa* is a Gram-negative bacterium that causes a wide array of human infections. Normally, it is harmless; however, if the immune system is compromised, it gains access to submucosal tissues, becoming a persistent opportunist pathogen.<sup>9</sup> *P. aeruginosa* is a human pathogen of major public health significance, causing approximately 16% of nosocomial pneumonia infections, 12% of hospital-acquired urinary tract infections, and 8% of surgical wound infections.<sup>10</sup> It is one of the most fatal pathogens. Mortality rates among patients with *P. aeruginosa* bacteremia range from 33% to 61%, and bacteremia associated with this pathogen is responsible for 50% of deaths in HIV-infected patients.<sup>11</sup> Also, it colonizes the respiratory tracts of cystic fibrosis patients, contributing to morbidity and mortality in this disease,<sup>12–15</sup> and it is associated with ventilator-acquired pneumonia in intubated patients.<sup>16</sup> Treatment of *P. aeruginosa* is problematic, due to its high intrinsic resistance to antibiotics, such as  $\beta$ -lactams and fluoroquinolones.<sup>17</sup>

This resistance is coordinated by a variety of virulence genes that enable its persistence and pathogenicity in vivo. A well-known mechanism enabling antibiotic resistance in *P. aeruginosa* is the outer-membrane protein OprD, the narrow channels of which reduce outer-membrane permeability.<sup>18</sup> The OprD protein is the main reason for resistance to carbapenems, due to bacterial alteration in the expression of the *oprD* gene. Other systems of virulence include quorum sensing (QS) and efflux pumps.<sup>19</sup>

QS is a major determinant of persistence and pathogenicity in *P. aeruginosa*, and inhibitors of this system may act as antimicrobial agents by enabling the bacterium's susceptibility to antibiotics. The efflux pumps, MexAB-OprM and MexEF-OprN, which are regulated by the *mexR* and *mexT* genes, respectively, are recognized as significant antibiotic-resistance complexes, involved in removing toxic substances, such as antibiotics, from the bacterium.<sup>20</sup> In addition to the inherent difficulty in treating *P. aeruginosa* infections, the organism also has the ability to convert from a nonmucoid to a mucoid state. The mucoid matrix enables the formation of protected biofilm microcolonies,<sup>21–23</sup> and provides increased resistance to opsonization, phagocytosis, and digestion.<sup>24</sup> The mucoid phenotype is characterized by the synthesis of a large quantity of alginate exopolysaccharide, which allows consistent infections<sup>25</sup> and poor prognosis for infected patients,<sup>26,27</sup> because this phenotype is rarely eradicated.

To our knowledge, the effects of AgCNTs on the virulence genes of *P. aeruginosa* involved in pathogenicity and persistence have not been determined. Therefore, the objectives of this study were to evaluate the antimicrobial activity of AgCNTs against *P. aeruginosa* and to determine their effects on the expression of virulence genes of *P. aeruginosa*.

## Materials and methods

### Silver-coated single-wall nanotubes

AgCNTs were purchased from NanoLab Inc (Waltham, MA, USA). The AgCNTs had an outer diameter of 1–5  $\mu\text{m}$  and a length of 1–2  $\mu\text{m}$ . AgCNTs were dispersed in a mixture of sterile distilled water and dispersant (Nanosperse AQ; NanoLab). Portions (1 mg) of AgCNTs were suspended in 1 mL aliquots of water and dispersant mixture according to the manufacturer's instructions, and then immediately sonicated for 1 hour to obtain AgCNT dispersion of 1 mg/mL.

### Bacteria, media, and antibiotics

Mucoid (ATCC 39324) and nonmucoid (ATCC 27318) strains of *P. aeruginosa* were purchased from the American Type Culture Collection (Manassas, VA, USA). Cation-adjusted Müller–Hinton broth CA-MHB from BD (Franklin Lakes, NJ, USA) was used to grow *P. aeruginosa* for determination of in vitro antimicrobial activity and time-kill assays. Todd–Hewitt media supplemented with yeast extract was used for scanning electron microscopy (SEM), transmission electron microscopy (TEM), and quantitative real-time polymerase chain reaction (qRT-PCR) analysis. Gentamicin sulfate and polymyxin B were purchased from Thermo Fisher Scientific (Waltham, MA, USA).

### Antimicrobial activity

Minimum inhibitory concentrations (MICs) against both strains of *P. aeruginosa* were measured according to the Clinical and Laboratory Standards Institute (CLSI) M7-A7 method.<sup>28</sup> Briefly, serial twofold dilutions of AgCNTs in dispersant, starting at 64  $\mu\text{g/mL}$ , were added into Costar untreated polystyrene 96-well plates, and each well was inoculated with 20  $\mu\text{L}$  ( $1 \times 10^6$  CFU/mL) of either strain of *P. aeruginosa* in CA-MHB to a final concentration of approximately  $1 \times 10^5$  CFU/mL. Positive control wells were inoculated with bacteria alone, whereas negative control wells had only media to ensure sterility. The MIC was determined as the lowest concentration of AgCNTs or antibiotic (polymyxin B and gentamicin sulfate) that showed no visible

growth after 24 hours of incubation at 37°C. The dilution representing the MIC and two concentrations above the MIC were then plated on Luria agar plates and viable CFU/mL were enumerated to obtain the minimum bactericidal concentration (MBC).<sup>29</sup> The MBC was determined as the lowest concentration of the AgCNTs yielding a 99.9% reduction in the initial colony count after 24 hours' incubation.

### Time-kill analysis

Time-kill studies of AgCNTs against each strain of *P. aeruginosa* were carried out based on the M26-A guidelines of the CLSI.<sup>30</sup> The assay was conducted by incubating an initial inoculum of approximately  $1 \times 10^5$  CFU/mL of either strain into Costar untreated polystyrene 96-well plates with AgCNTs at their respective MICs, two dilutions above the MIC (2× and 4× MIC) and one dilution below (0.5× MIC) in CA-MHB. Viable cell counts were determined after 2, 4, 8, and 24 hours of incubation at 37°C by plating serially diluted samples on Luria agar. Antimicrobial activity was defined as  $\geq 3 \log_{10}$  decrease in CFU compared to the initial inoculum.<sup>30</sup>

### Scanning electron microscopy

Exponential phase bacteria ( $1 \times 10^9$  CFU/mL) were exposed to AgCNTs at approximately 4× MIC for 4 hours at 37°C, before being centrifuged at  $2,000 \times g$  (Sorvall ST 40R; Thermo Fisher Scientific) for 10 minutes. The pellets were then washed in 0.1% phosphate-buffered saline (PBS) and fixed overnight in a mix of 2.5% glutaraldehyde and 1% formaldehyde in 0.1% PBS. Samples were treated with 1% osmium tetroxide in 0.1% PBS, before stepwise dehydration in increasing concentrations of ethanol in water. The dehydrated samples (5  $\mu$ L each) were then placed on SEM stubs (Electron Microscopy Sciences, Hatfield, PA, USA), and air-dried and sputter-coated with gold in a sputter-coat device (EMS 550X; Electron Microscopy Sciences) prior to performing SEM (EVO 50 variable pressure scanning; Carl Zeiss Meditec AG, Jena, Germany) analysis.

### Transmission electron microscopy

For TEM sample preparation, a similar processing method was used as described earlier for SEM, until achieving dehydration in 100% ethanol. After dehydration, the samples were passed through propylene oxide and infiltrated with Embed 812 resin (Electron Microscopy Sciences) and polymerized overnight. Ultrathin sections were collected on copper grids stained with 2% uranyl acetate and lead citrate, and imaged using the TEM (EM10; Carl Zeiss Meditec AG).

## RNA extraction and quantitative real-time PCR

For qRT-PCR analysis, untreated and treated bacteria ( $1 \times 10^9$  CFU/mL) were exposed to either 4× MIC of AgCNTs or gentamicin for 4 hours at 37°C. Total RNA was purified using an RNeasy Mini Kit (Qiagen NV, Venlo, the Netherlands), and quantified using a spectrophotometer (NanoDrop 2000c; Thermo Fisher Scientific). The complementary DNA synthesis was carried out in 20  $\mu$ L reaction volumes using the Applied Biosystems High-Capacity cDNA Reverse Transcriptase Kit (Thermo Fisher Scientific). A total of 1  $\mu$ g of RNA was used to amplify the *oprD*, *lasA*, *creD*, *lecA*, *lecB*, *rpoS*, *creB*, *rsmZ*, *ampD*, *ampP*, *ampG*, *mexT*, and *mexR* genes of *P. aeruginosa* using the Applied Biosystems ViiA 7 real-time PCR system. Primer pairs for each gene are shown in Table S1. The amplification conditions used were one cycle of initial denaturation at 95°C for 2 minutes, followed by 40 cycles of 95°C for 15 seconds, 56°C for 25 seconds, and 72°C for 30 seconds. The relative changes in gene expression were calculated using the equation  $2^{-\Delta\Delta CT}$ , where all values were normalized with respect to the 16S messenger RNA levels. Each real-time PCR assay was performed in triplicate from three independent cultures, and the results are expressed as means  $\pm$  standard deviation.

### Statistical analysis

Data were analyzed by Student's *t*-test using SigmaPlot software. Significance was established at  $P < 0.05$  and  $P < 0.01$ .

## Results

### Antibacterial activity of AgCNTs

Susceptibility of the mucoid and nonmucoid strains *P. aeruginosa* to AgCNTs was determined by the micro-broth dilution assay. Both strains displayed susceptibility to AgCNTs at an MIC of 8  $\mu$ g/mL compared to gentamicin or polymyxin B controls, with MICs between 0.25 and 0.5  $\mu$ g/mL (Table 1). The MIC and MBC concentrations were the same for both

**Table 1** Antimicrobial susceptibility of *Pseudomonas aeruginosa*

	MIC/MBC ( $\mu$ g/mL)	
	Mucoid	Nonmucoid
AgCNTs	8/8	8/8
Gentamicin sulfate	0.5/0.5	0.25/0.25
Polymyxin B	0.5/0.5	0.5/0.5

**Note:** MIC and MBC of AgCNTs, positive control gentamicin sulfate and polymyxin B against the mucoid and nonmucoid strains of *P. aeruginosa*.

**Abbreviations:** AgCNTs, silver-coated carbon nanotubes; MIC, minimum inhibitory concentration; MBC, minimum bactericidal concentration.

strains. We further conducted time-kill experiments to evaluate microbial reduction by AgCNTs over time.

### AgCNTs are bactericidal at the MBC and above

Time-kill analysis showed rapid killing occurred at the MBC and above, as the viable cell count decreased by 5 log<sub>10</sub> CFU/mL in the first 2 hours (Figure 1A and B). The MBC was at the MIC. These results show that AgCNTs are effective in killing both mucoid and nonmucoid strains of *P. aeruginosa*.

### AgCNTs cause morphological changes to the cell membrane of *P. aeruginosa*

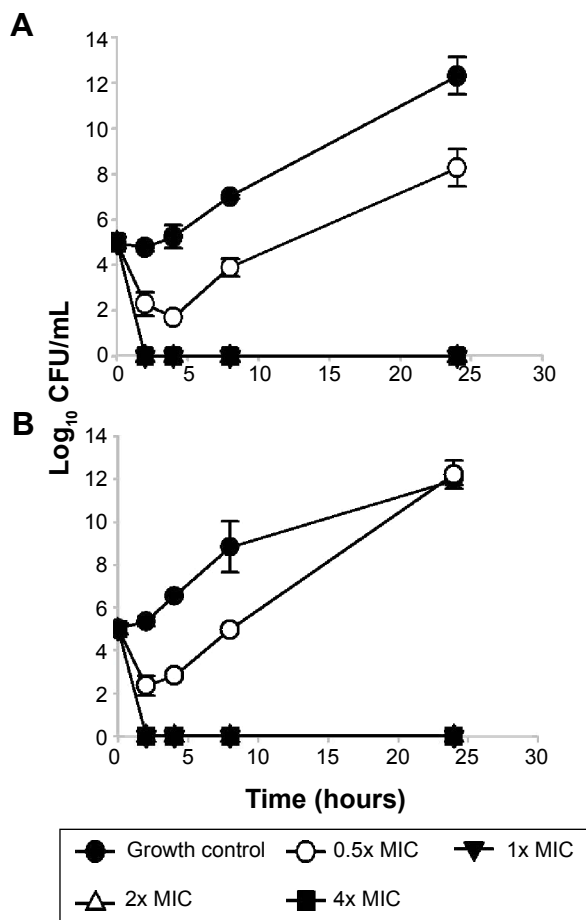
We employed both SEM and TEM to understand the mechanisms by which AgCNTs exert their antimicrobial

effects against *P. aeruginosa*. The effect of AgCNTs on *P. aeruginosa* cell morphology was examined after 4 hours' treatment of 5 mL of exponential phase bacteria (1×10<sup>9</sup> CFU/mL) of either strain of *P. aeruginosa* with 4× MIC of AgCNTs. Both strains showed broken cell membranes, cell-membrane disintegration, and the formation of irregular cell surfaces, in contrast to the smooth surfaces of the untreated samples (Figure 2). These images suggest a probable mechanism of action whereby AgCNTs interact with the cell membrane, causing membrane permeabilization and degradation, with accompanying changes in cell morphology.

TEM analysis confirmed the SEM findings, with treated cells showing complete loss of cell membranes (Figure 3), along with dispersed and disintegrated intracellular material of both strains. Together, these results suggest that AgCNTs caused loss of membrane integrity with lethal effects on both strains of *P. aeruginosa*.

### Virulence-gene expression in response to AgCNT treatment

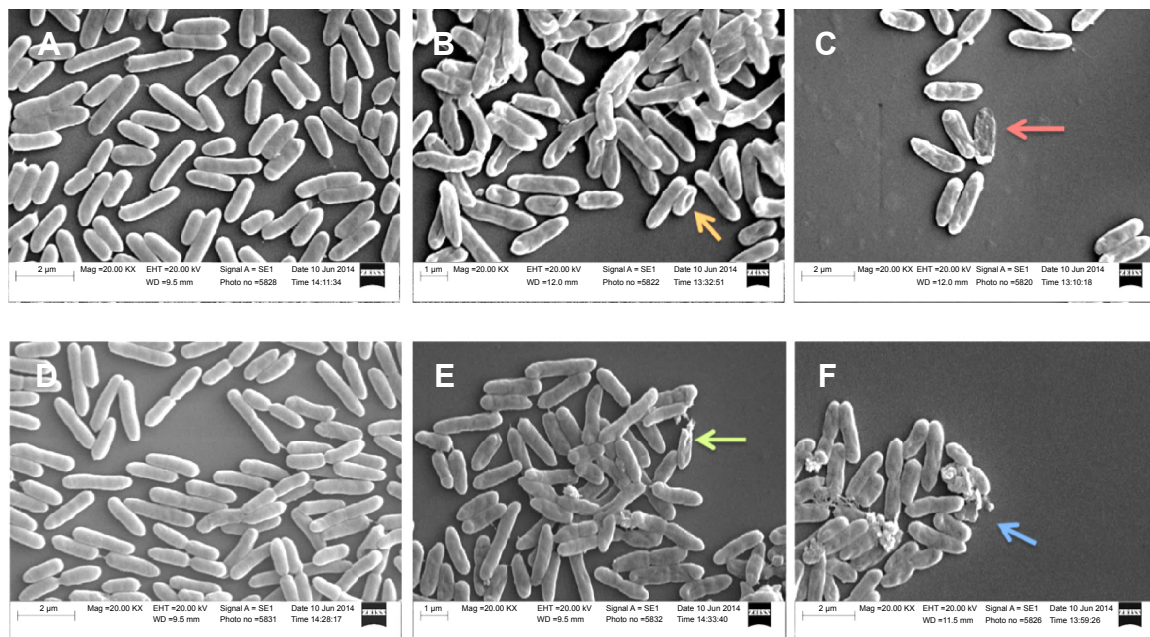
To study the molecular effects of AgCNTs against the mucoid and nonmucoid strains of *P. aeruginosa*, virulence genes involved in resistance, stress response, motility, pathogenicity, attachment, and redox regulation were selected for quantification of their transcriptional expression using qRT-PCR, and then compared to the molecular effects of gentamicin. Both strains exhibited similar gene-expression patterns (Figure 4A and B) for the AgCNT samples, except for a few exceptions, such as downregulation of expression of *lasA* and *creD*, which are essential for attachment to eukaryotic cells and the development of resistance, respectively. Their expression was significantly downregulated in the nonmucoid strain, but remained unchanged in the mucoid strain. By contrast, the expression of the *mexT* gene was downregulated in the mucoid strain, but showed no significant change in the nonmucoid strain. As expected, the presence of AgCNTs significantly downregulated expression of the outer-membrane porin *oprD* gene by ten- and elevenfold in the mucoid and nonmucoid strains, respectively. The *rpoS* gene expression (encoding sigma factor RpoS, a regulator of cellular response to oxidative stress) was also significantly downregulated: six- and fourfold for the mucoid and nonmucoid strains, respectively. The *lasA* gene expression (encoding a QS-regulating factor LasA, an endopeptidase) was also downregulated four- and 14-fold for the mucoid and nonmucoid strains, respectively. Other virulence factors that were downregulated in both strains included the *priR* (encoding a negative regulator of pyocin genes) and *mexR*



**Figure 1** Time-dependent effect of AgCNTs on the growth of the mucoid (A) and nonmucoid (B) strains of *Pseudomonas aeruginosa*.

**Note:** The assay was conducted by incubating an initial inoculum of approximately 10<sup>5</sup> CFU/mL of either strain into Costar untreated polystyrene 96-well plates with AgCNTs at their respective MICs, two dilutions above the MIC (2× and 4× MIC), and one dilution below (0.5× MIC) in CA-MHB. Viable cell counts were determined after 2, 4, 8, and 24 hours of incubation at 37°C by plating serially diluted samples on Luria agar.

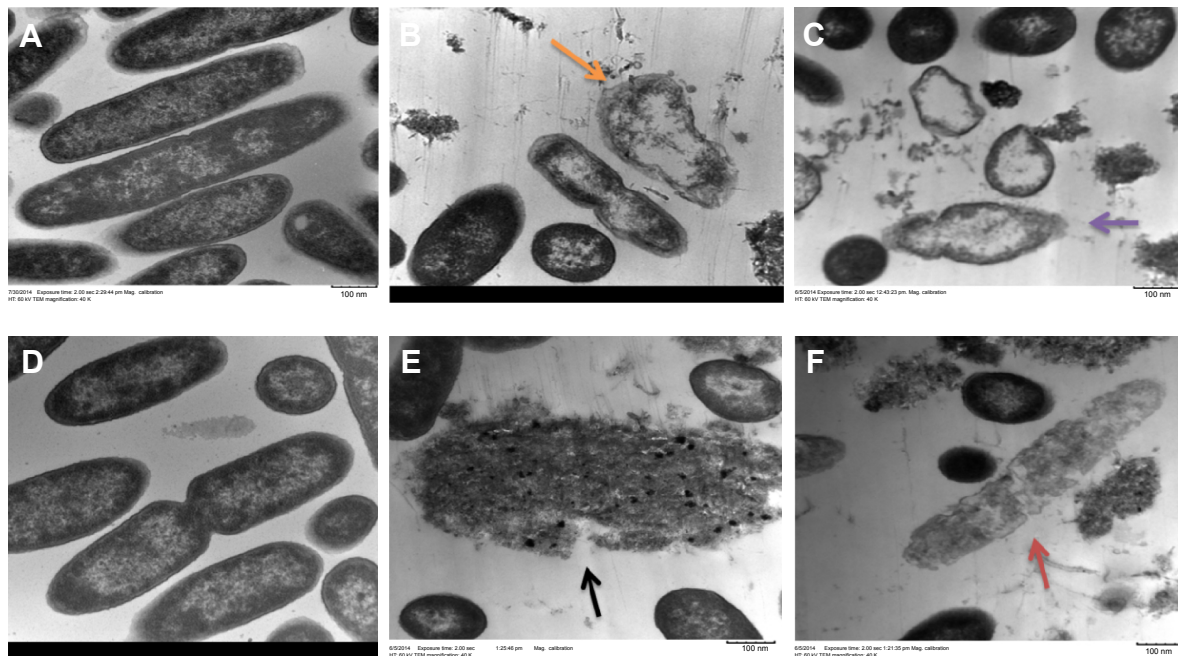
**Abbreviations:** AgCNTs, silver-coated carbon nanotubes; MIC, minimum inhibitory concentration; CA-MHB, cation-adjusted Müller–Hinton broth.



**Figure 2** SEM images of mucoid and nonmucoid *Pseudomonas aeruginosa*.

**Notes:** Exponential phase bacteria were treated with 4× MIC of AgCNTs for 4 hours at 37°C and centrifuged at 2,000× g for 10 minutes. The pellets were then washed in 0.1% PBS and fixed overnight in a mix of 2.5% glutaraldehyde and 1% formaldehyde in 0.1% PBS. Samples were treated with 1% osmium tetroxide in 0.1% PBS, before stepwise dehydration in increasing concentrations of ethanol in water. The dehydrated samples (5 µL each) were then placed on SEM stubs and air-dried and sputter-coated with gold in a sputter-coat device prior to performing SEM analysis. (A) and (D) show images of untreated mucoid and nonmucoid cells, respectively. Bacterial cells have intact cell membranes with uniform shape. (B) and (C) show disintegrated and irregularly shaped mucoid cells, and (E) and (F) show AgCNT-treated nonmucoid bacterial cells. Treated samples show cell disintegration (red and blue arrows), dents or kinks on cell membranes (orange arrow), and broken cells (green arrow).

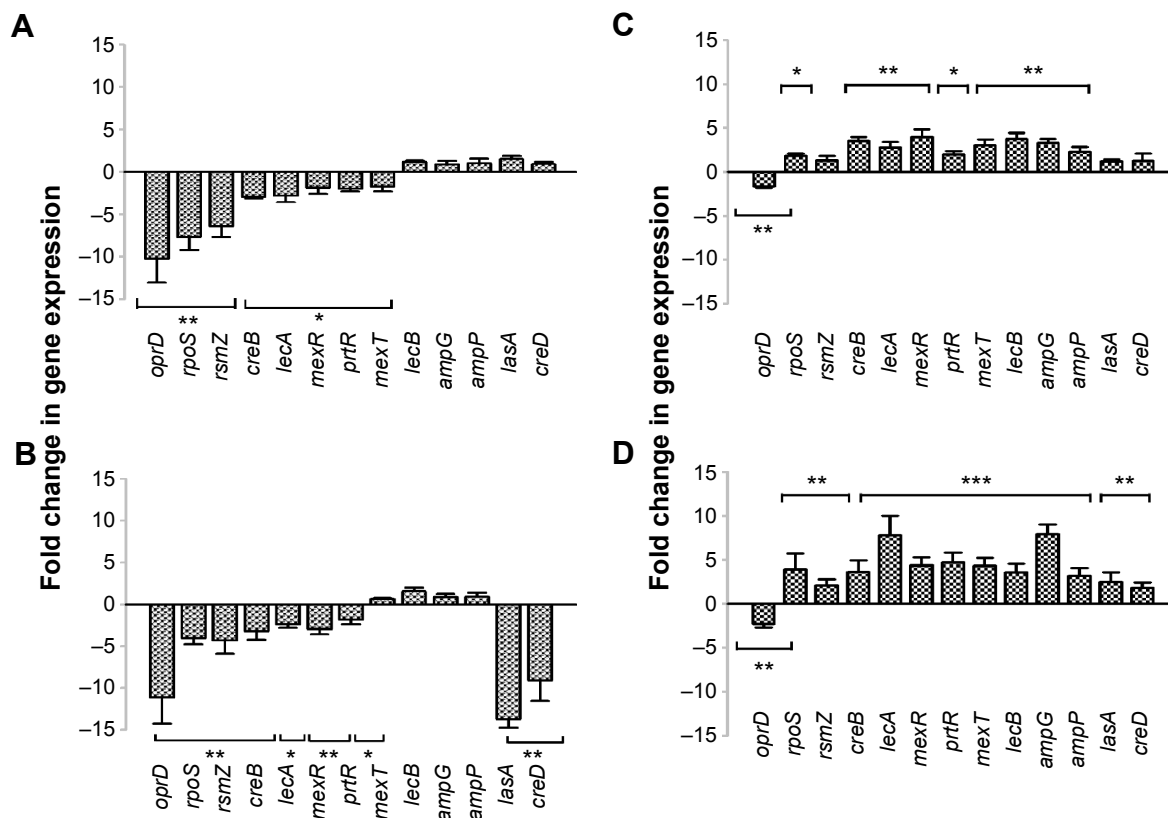
**Abbreviations:** AgCNTs, silver-coated carbon nanotubes; MIC, minimum inhibitory concentration; PBS, phosphate-buffered saline; SEM, scanning electron microscopy.



**Figure 3** TEM of mucoid and nonmucoid strains of *Pseudomonas aeruginosa*.

**Notes:** Exponential phase bacteria were treated with 4× MIC of AgCNTs for 4 hours at 37°C, and centrifuged at 2000× g for 10 minutes. The pellets were then washed in 0.1% PBS and fixed overnight in a mix of 2.5% glutaraldehyde and 1% formaldehyde in 0.1% PBS. Samples were treated with 1% osmium tetroxide in 0.1% PBS, before stepwise dehydration in increasing concentrations of ethanol in water. After dehydration, the samples were passed through propylene oxide and infiltrated with Embed 812 resin and polymerized overnight. Ultrathin sections were collected on copper grids stained with 2% uranyl acetate and lead citrate and imaged using the TEM. (A) and (D) show images of untreated mucoid and nonmucoid cells, respectively, with intact cell membranes. (B) and (C) show AgCNT-treated mucoid cells with efflux of cellular material and empty ghost cells (orange and purple arrows), whereas (E) and (F) show cells with intracellular material completely disintegrated (black and red arrow) and the absence of cell membranes.

**Abbreviations:** AgCNTs, silver-coated carbon nanotubes; MIC, minimum inhibitory concentration; SEM, scanning electron microscopy; TEM, transmission electron microscopy.



**Figure 4** Relative gene-expression levels of AgCNT-treated *Pseudomonas aeruginosa*.

**Notes:** \* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ . Untreated and either AgCNT- or gentamicin-treated exponential phase bacteria were exposed to  $4 \times$  MIC for 4 hours at  $37^\circ\text{C}$ . Total RNA was purified using an RNeasy Mini kit and quantified. cDNA synthesis was carried out in  $20 \mu\text{L}$  reaction volumes using the Applied Biosystems High-Capacity cDNA Reverse Transcriptase Kit. A total of  $1 \mu\text{g}$  of RNA was used to amplify the virulence genes of *P. aeruginosa* using the Applied Biosystems ViiA 7 real-time PCR system. The amplification conditions used were one cycle of initial denaturation at  $95^\circ\text{C}$  for 2 minutes, followed by 40 cycles of  $95^\circ\text{C}$  for 15 seconds,  $56^\circ\text{C}$  for 25 seconds, and  $72^\circ\text{C}$  for 30 seconds. The relative changes in gene expression were calculated using the expression  $2^{-\Delta\Delta\text{CT}}$ , where all values were normalized with respect to the 16S mRNA levels. (A) and (C) are AgCNTs- and gentamicin-treated mucooid *P. aeruginosa*, respectively; (B) and (D) are AgCNTs- and gentamicin-treated nonmucooid *P. aeruginosa*, respectively.

**Abbreviations:** AgCNTs, silver-coated carbon nanotubes; MIC, minimum inhibitory concentration; cDNA, complementary DNA; PCR, polymerase chain reaction; CT, cycle threshold; mRNA, messenger RNA.

(negative regulator of MexAB-OprM) genes. In contrast to the AgCNT-treated samples, gentamicin-treated samples of both strains showed upregulation of gene-expression patterns for all genes analyzed, except for downregulation of *oprD* gene expression (Figure 4, C and D). Collectively, these results show that AgCNTs deregulate a number of virulence genes of *P. aeruginosa*, which may be linked to their antimicrobial properties, which appear to be different from the mechanism of action of the gentamicin. Virulence genes and function are shown in Table 2.

## Discussion

AgCNT hybrid nanoparticles have a broad spectrum of activities against microbial pathogens, such as *Staphylococcus aureus*, *Streptococcus pyogenes*, *Escherichia coli*, and *Salmonella enterica* serovar Typhimurium.<sup>31</sup> In this study, we investigated the antimicrobial activity and mechanistic effect of AgCNTs against mucooid and nonmucooid strains of *P. aeruginosa*, a major opportunist pathogen of

immunocompromised patients.<sup>32</sup> To our knowledge, this is the first investigation of the antimicrobial activity of AgCNTs against a mucooid variant of *P. aeruginosa*, a more virulent phenotype, which fosters the formation of biofilms and enables resistance against antimicrobials.<sup>33</sup>

Our results showed that AgCNTs are bactericidal against both strains of *P. aeruginosa*, with no recovery of AgCNT-treated cells on Luria agar plates at MIC concentrations or higher. Although the antimicrobial activity of AgCNTs against *P. aeruginosa* has been investigated,<sup>3</sup> their mechanism of action is not well established. However, the antimicrobial effect has been attributed to membrane damage caused by direct or electrostatic interaction between AgCNTs and cell membranes. An examination of the effects of AgCNTs on cell morphology by SEM revealed obvious membrane damage, as evidenced by changes from a smooth morphology to irregular cell surfaces with membrane disintegration, suggesting membrane damage as a probable mechanism of action. TEM confirmed the effect of AgCNTs on *P. aeruginosa*

**Table 2** Genes, gene product, and product function

Gene	Gene product	Product function
<i>oprD</i>	Outer-membrane porin OprD	Resistance
<i>rpoS</i>	Alternative sigma regulator RpoS	General stress regulator
<i>rsmZ</i>	Small regulatory RNA	Biofilm formation
<i>creB</i>	CreD regulator	Resistance
<i>lecA</i>	LecA lectin	Adhesion and biofilm formation
<i>mexR</i>	MexAB-oprM repressor	Resistance
<i>prtR</i>	Transcriptional regulator PrtR	Type III secretory system
<i>mexT</i>	MexEF-oprM regulator	Resistance
<i>lecB</i>	LecB lectin	Adhesion
<i>ampP</i>	AmpP lactamase	$\beta$ -Lactamase induction
<i>ampG</i>	AmpG lactamase	$\beta$ -Lactamase induction
<i>lasA</i>	LasA protease	Virulence
<i>creD</i>	Inner-membrane protein CreD	Resistance

membranes, showing rupture with subsequent leakage and efflux of cytoplasmic contents.

One of the major drawbacks to treating and preventing *P. aeruginosa* infection is the ability of the organism to develop resistance to an array of antimicrobial agents; this is not unusual for bacteria, which adapt quickly to change or stress by regulating the synthesis of defensive or resistance proteins in order to ensure survival. In *P. aeruginosa*, the lower outer-membrane permeability to antibiotics has been attributed to the narrower structure of the OprD protein compared to the OmpF of *Escherichia coli*,<sup>18</sup> which makes this pathogen recalcitrant to treatment. Therefore, we studied a number of genes that enable *P. aeruginosa* pathogenicity and protection from antibiotics. Resistance in *P. aeruginosa* is enabled by the synergistic effect of the overexpression of efflux pumps and reduced expression of the *oprD* gene. The OprD protein plays a critical role as a channel for basic amino acids, peptides, and carbapenems, and is a major mediator of resistance of *P. aeruginosa* to carbapenems. To eliminate antimicrobial compounds, the organism reduces its expression of *oprD*, in order to prevent entry of antimicrobials, and to develop resistance to antimicrobial agents.<sup>18</sup> It is known that MexT, a possible indirect regulator of *oprD* and a positive regulator of the multidrug efflux pump MexEF-OprN, and MexR, a negative regulator of the MexAB-OprM pump, enable the organism to acquire resistance by the increased expression of *mexT* and reduced expression of *mexR* and *oprD*.<sup>18,34</sup>

Analyses of these genes in *P. aeruginosa* treated with AgCNTs or gentamicin in this study showed that the expression of *oprD* was significantly downregulated in both strains for both treatments. In contrast, the expression of *mexR* was

downregulated in the AgCNT-treated strains but upregulated in the gentamicin-treated strains. On the other hand, *mexT* was also downregulated in the AgCNT-treated mucoid strain, but remained unaffected in the AgCNT-treated nonmucoid strain, while it was upregulated in both gentamicin-treated strains. This may indicate that AgCNTs cause stress and inhibit *P. aeruginosa* by their effect on the expression of the resistance-response genes. A reduced expression of *rpoS* in the presence of AgCNTs in both strains may suggest a decreased ability for survival under stress imposed by AgCNTs, whereas an increased expression in the presence of gentamicin may suggest the opposite. It is known that the general oxidative stress regulator and alternative sigma factor, RpoS<sup>35</sup> regulates the expression of more than 35 genes, many of which are involved in the general stress response. RpoS also plays a role as an intermediary regulator of catalase activity and hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) tolerance. RpoS has been shown to mediate protection against H<sub>2</sub>O<sub>2</sub> killing, as differential sensitivity to H<sub>2</sub>O<sub>2</sub> in a parent and mutant *rpoS* strain was the result of a 60% reduction in catalase activity in the latter.<sup>36</sup> Other stress-related genes include the small regulatory RNA genes *rsmY* and *rsmZ*, the expression of which are essential to enhance population density under environmental stress,<sup>37</sup> and also for biofilm formation.<sup>38</sup> In this study, we observed a significantly reduced expression of the *rsmZ* gene in AgCNT-treated samples and an upregulation in gentamicin-treated samples. A study has shown that upregulation of these regulatory RNAs resulted in more biofilm formation, by increasing expression of the QS gene *lasR*. Another prior study has shown that biofilm production was markedly impaired in *rsmZ*-, *gacA*-, and *rsmY*-mutant strains.<sup>37</sup> Therefore, our findings suggest that AgCNTs may interfere with biofilm formation in *P. aeruginosa*.

Other genes essential for pathogenicity and resistance include *lecB* and *prtR*. *prtR* expression is upregulated in the presence of (ROS), resulting in increased type III secretory system gene expression and increased bacterial tolerance of stress.<sup>39</sup> *lecB* gene expression has been identified to be critical in *P. aeruginosa* pathogenesis, as it plays a role in adhesion of *P. aeruginosa* to the A549 lung epithelial cell line. *lecA* and *lecB* mutation decrease bacteria dissemination,<sup>40</sup> and in this study a significant downregulation of the *lecA* gene was observed for both strains treated with AgCNTs. We also found that the *creD* gene was significantly downregulated – eightfold – in the nonmucoid strain, but remained unchanged in the mucoid strain. The *creD* gene encodes an inner-membrane protein, and is regulated by a dual *creBC* gene system involved in  $\beta$ -lactam resistance/tolerance.<sup>41</sup>

The outer membrane of the mucoid strain is enclosed in alginate, a complex polysaccharide that may mitigate the damaging effects of AgCNTs on the inner-membrane protein.

In contrast to *creD*, *creB* gene expression was significantly downregulated in both strains. The QS-regulated gene *lasA*, which encodes the LasA protease,<sup>42</sup> an exotoxin produced by *P. aeruginosa*, was significantly downregulated in the nonmucoid strain, suggesting an impaired QS system, and subsequent decreased pathogenicity in the nonmucoid strain. By comparison, *lasA* expression was significantly upregulated in the gentamicin-treated *P. aeruginosa*. However, the *ampP* and *ampG* genes, which encode permease<sup>43</sup> and are critical for  $\beta$ -lactamase induction, remained unchanged in both strains. From these experiments, it appears that the antimicrobial activity of AgCNTs may include several mechanisms, such as membrane damage, affecting antibiotic-resistance genes, downregulating virulence genes, and inducing oxidative and general stress. The downregulation of virulence genes may also suggest a decreased pathogenicity of *P. aeruginosa* after AgCNT treatment. The downregulation of these virulence genes in AgCNT-treated samples is in contrast to the upregulation of these genes in the gentamicin-treated samples, which suggests that not only do the mechanisms of action differ between AgCNTs and gentamicin but upregulation of these virulence genes may indicate the ability of *P. aeruginosa* to respond to the presence of gentamicin. For example, upregulation of stress-regulator and efflux-pump genes may indicate the organism's ability to adapt and develop resistance to gentamicin over time and with continuous usage.

We have previously examined the cytotoxicity of AgCNTs.<sup>44</sup> In that study, the toxicity of pegylated and naked AgCNTs were analyzed, together with their antimicrobial effect. Although the pegylated AgCNTs were comparatively less toxic, antimicrobial activity remained unchanged, thereby indicating that the cytotoxicity of AgCNTs can be reduced while maintaining antimicrobial activity and effect. Further, at their MIC, AgCNTs were nontoxic to eukaryotic cells.

In summary, AgCNTs showed antimicrobial activity and bactericidal effects against both the mucoid and nonmucoid strains of *P. aeruginosa* at low concentrations. AgCNTs also induced significant morphological changes and membrane disruption. Based on these results and changes in gene expression, a probable mechanism of AgCNT bactericidal effect involves direct interaction between AgCNTs and cell membrane, whereby the cell membrane is affected and nanoparticle entry induces stress in the bacteria, resulting in growth inhibition and death. These data suggest that AgCNTs may be applicable as an antimicrobial agent against *P. aeruginosa*,

especially for antimicrobial coatings. AgCNTs may also be useful for disinfecting surfaces, water treatment, and purification due to their insolubility. Membrane disintegration and deregulation of virulence-gene expression could contribute to the rapid bactericidal activity of AgCNTs.

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## Disclosure

The authors report no conflicts of interest in this work.

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## Supplementary material

**Table S1** Gene and primers used for quantitative reverse-transcriptase polymerase chain reaction (sequence 5' → 3')

Gene	Forward primer	Reverse primer
<i>mexT</i>	ACCTCATGGGTTGTGACTGTATCC	TAGGATCACTGACAGGCATAGCCA
<i>mexR</i>	GTTTCGTTGCATAGCGTTGTCCTCA	TTGATAACCGGCCATCGAGCTAAA
<i>oprD</i>	TTTCCGCAGGTAGCACTCAGT	CTTCGCTTCGGCCTGATC
<i>lasA</i>	TTCTGTGATCGATTCCGGCTCGGTT	ACCCGGGAAGACAACCTATCAGCTT
<i>lecA</i>	CACCATTGTGTTTCCTGGCGTTCA	AGAAGGCAACGTCGACTCGTTGAT
<i>lecB</i>	AGACAGCGTAAACAATCGAACGAGC	AGGACGCATCGTTCAGCCAATCTA
<i>rpoS</i>	CGGCGAGTTGGTCATCATCAAACA	ATCGATTGCCCTACCTTGACCTGT
<i>rsmZ</i>	GACGGTACTGGGTGTCAAAGG	TGGTAAATTTCTCCCGGTGTA
<i>priR</i>	TCCCTGCACCCATGTGAAATCTCT	ATCGGCAATCTACAGACCCGATGGA
<i>creB</i>	GCATATCCTGATCGTCGAAGATG	GGCCTGCAGGGCGTAGA
<i>creD</i>	CGCCATCGCCCTACTCAT	GGCGATCGCGGATCAG
<i>ampG</i>	GGCCTGACCTATGCGTTGTT	CGACGGGTTGACGTGGAT
<i>ampP</i>	ACCGCCGCCTGGTCAT	CAGGCCGATGGGAATGC

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