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Impact of Sulfidation of Silver Nanoparticles on Established *P. aeruginosa* Biofilm

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Abstract

Silver nanoparticles (Ag-NPs), one of the most common types of nanomaterials in medical fields and consumer products, are known to have antimicrobial effects; these materials also undergo a series of chemical and biological transformations in the environment. Although the pristine form of silver nanoparticles has been studied, less is known about the impacts of the transformed Ag-NPs on biological systems. This knowledge gap hinders the progress of effectively assessing the impacts of Ag-NPs on the environment and human health. In this study, we demonstrate that the most common form of transformed Ag-NPs, sulfidized silver nano-particles (Ag_2S-NPs), show less damage on established *Pseudomonas aeruginosa* GFP (ATCC® 10145 GFP™) biofilm than the pristine form of the nanoparticle. At a dosage of 0.625 mg/L, the total biomass in the biofilm decreased 64% after being exposed to Ag-NPs and 44% after exposure to Ag_2S-NPs. Live biofilms were also interrogated. We observed high reduction in live population for biofilm exposed to Ag-NPs and relatively low reduction by Ag_2S-NPs at exposure concentrations higher than 0.625 mg/L. Compared with Ag-NPs, the lower solubility of Ag_2S-NPs results in less Ag^+ diffusion into established biofilms. Our results suggest that the sulfidation of Ag-NPs reduces their impacts on established biofilms, indicating that the transformed Ag-NPs may have less environmental or human health risks.

Keywords

Silver Nanoparticles, Sulfidized Silver Nano-Particles, Biofilm, *P. aeruginosa*

1. Introduction

Recent developments in the field of nanotechnology have greatly motivated the widespread use of engineered nanomaterials such as silver nanoparticles. Silver nanoparticles (Ag-NPs) are among the most widely used nanomaterials in healthcare and consumer products. Most of these products incorporate Ag-NPs for their superior antimi-

crobial, antifungal and antiviral properties [1] [2] [3] [4]. The antimicrobial properties of Ag-NPs and their ability to reduce the growth of unwanted freely-growing bacteria in laboratory settings are well-documented [5] [6] [7]. The toxicity of these nanoparticles has been attributed to three main mechanisms: the release of biocidal silver ions, generation of ROS (oxidative damages) and direct damage to cell membranes [8] [9] [10].

However, most bacteria grow within attached, sessile cultures called biofilms. Biofilms are densely packed microbial communities surrounded by a self-secreted matrix of extracellular polymeric substance. This complex biofilm structure constitutes a barrier against the toxic effects of materials such Ag-NPs and confers embedded bacteria cells with an inherent resistance to antimicrobial agents [11] [12] [13] [14]. Current understanding of the biocidal effects of Ag-NPs on planktonic bacterial cells is not sufficient to predict their behavior in biofilms. Recent studies have shown that Ag-NPs can accumulate in biofilms and significantly alter their structure, morphology, and biomass [15] [16] [17] [18]. Additional studies are still needed, however, to characterize the interactions between Ag-NPs and biofilm and elucidate the mechanisms governing the impacts of nanoparticles on natural ecosystems. Given the increasing use of Ag-NPs and their inevitable release from man-made products [19] [20] [21], such studies will also benefit environmental risk assessment and exposure studies.

Like other nanoparticles, Ag-NPs undergo natural transformation processes in biological and environmental systems. These transformation processes include dissolution, aggregation, and adsorption or surface reactions and can profoundly affect the behavior of Ag-NPs *in situ*. Thus, the fate, transport and toxicity of transformed Ag-NPs in biofilms should be considered alongside studies that investigate the pristine form of the nanoparticles. A number of studies reveal that the prevalent route of Ag-NPs transformation in biological media is sulfidation [22] [23] [24]. The surface oxidized silver ions react with inorganic sulfide to form Ag_2S, which eventually transforms into Ag_2S-NPs [23]. Although a few studies have addressed the antimicrobial properties of Ag_2S-NPs against planktonic bacteria [25] [26], little is known about their interactions with biofilms.

The objective of this study was to comparatively investigate the antimicrobial properties of pristine and sulfidized silver nanoparticles (Ag-NPs and Ag_2S-NPs respectively) on bacterial biofilms. *Pseudomonas aeruginosa* GFP (ATCC® 10145GFP™) biofilms were exposed to Ag-NPs and Ag_2S-NPs of similar size and identical surface coating under the same experimental conditions. Total biomass, live population, and surface morphology of the biofilms were monitored to determine the impact of sulfidation on the toxicity of Ag-NPs against biofilms. The differences in NPs morphology and their ability to release silver ions were also compared to gain further insight into the mechanism of action of these two nanoparticles.

2. Materials and Methods

2.1. Nanoparticles Preparation and Characterization

The silver nanoparticles used in this study were provided by the Center for the Environmental Implications and Nanotechnology (CEINT) at Duke University. Both Ag-

NPs and Ag_2S-NPs were synthesized using a modified polyol process [27] [28]. The reported purities of both nanoparticles were 99%. Nanoparticles were provided as concentrated stock dispersions in water with concentrations of 1.8 g/L Ag for Ag-NPs and 1.1 g/L Ag for Ag_2S-NPs. Before each experiment, the stock dispersions were sonicated for 10 mins and diluted with ultra-pure milli-Q water to target concentrations. The size and morphology of nanoparticles were analyzed with a transmission electron microscope (TEM). The hydrodynamic diameters and surface charge of nanoparticles in solution were determined by Nanoseries zeta-sizer (Malvern, MA).

2.2. Bacterial Strain and Biofilm Growth Conditions

Pseudomonas aeruginosa GFP (ATCC® 10145GFP™) served as model organism in this study. The fluorescence and high attachment abilities of this model strain enhanced good characterization and analysis. The ability of the strain to emit green fluorescence protein was verified before experiments. Luria-Bertani (LB) broth, supplemented with 300 μg/mL of ampicillin as per manufacturer's instructions, served as culture medium for all experiments. Prior to each experimental assay, *P. aeruginosa* was grown overnight from frozen stocks on LB agar plates at 37˚C. Then a few colonies were grown in suspension at 37˚C for 4 - 5 hours, harvested, and diluted in LB to a final optical density (OD 600) of 0.005, which was equivalent to 10^8 cells/mL. This diluted subculture was then used to initiate biofilm cultures.

Biofilms were grown in a MBEC™ biofilm inoculator (Innovotech Inc. Technologies Canada) as previously described by Harrison *et al.* [29] with minor modifications. This device is commonly used for high throughput determination of the minimum biofilm eradication concentrations (MBECs) in laboratory settings. The device involves specialized MBEC™ polystyrene culture plates with protruding pegs (on the lids of the plates) that support surface-attached bacterial growth (*i.e.*, biofilms).

In this study, each well of the MBEC™ 96-well plates was filled with 200 μL of *P. aeruginosa* suspension in LB at OD 600 of 0.005. The plates were then incubated in an environmental rotary shaker at a speed of 180 rpm and ambient temperature of 37˚C (Benchmark Scientific Incu-shaker) for 72 hours. Fresh LB medium was added at 24-hour intervals to replenish depleted nutrients. This protocol resulted in the formation of mature *P. aeruginosa* biofilms attached to the surfaces of the pegs on the lids of the MBEC™ plates.

Where applicable, optical density and cell concentration measurements were done with a Genesys spectrophotometer (Thermo Fisher Scientific, Waltham, MA) and a Multisizer™ 4 Coulter Counter (Beckman Coulter, Inc., Fullerton, CA) respectively. Biofilm growth was monitored with confocal laser scanning microscope (CLSM) and scanning electron microscopy (SEM) as described below. All experiments were performed under aseptic conditions, and all media and glassware used were autoclaved and sterile.

2.3. Exposure of Established Biofilms to Nanoparticles

After 72 hours of incubation, MBEC™ pegs harboring mature *P. aeruginosa* biofilms were transferred to a new 96 well plate, filled with various concentrations of silver na-

noparticles (in LB broth). Environmentally-relevant test concentrations of 0.3125 mg/L, 0.625 mg/L, 1.25 mg/L, 2.5 mg/L and 5 mg/L were used to challenge established biofilms [30] [31] [32]. Samples without nanoparticles and samples without biofilms both served as controls. Each experimental condition was replicated in four or more wells per assay plate. The plate was then incubated for another 24 hours at 37˚C with constant rotation of 180 rpm. After 24 h of incubation with nanoparticles, the biofilms were gently transferred into 200 μL of sterile phosphate-buffer saline (PBS) solution for 1 min to remove non-adherent biofilm cells. Thereafter, the attached biofilms and/or the residue LB broth in the culture wells were further analyzed as described in the following sections.

2.4. Biofilm Biomass

To determine the biomass in the biofilms, MBEC™ plate pegs were carefully removed and transferred to 1.5 mL centrifuge vials containing 200 μL of PBS. Thereafter, a water-bath sonicator (Quantrex L & R Ultrasonic, NJ, USA) set at 60 Hz for 10 minutes was used to disrupt biofilms into suspension. Recovered biofilm cells in suspension were pipetted into 96-well clear bottom black micro-well plates and examined with a multi-plate reader (Infinite M200 Pro Tecan microplate reader). The total biomass was determined by optical density (OD 600) measurement, and live population was determined by monitoring the green fluorescent protein (GFP) intensity (GFP Excitation: 475 nm; Emission: 570 nm) expressed by live *P. aeruginosa* GFP cells.

2.5. Confocal Laser Scanning Microscopy

Confocal laser scanning microscopy (CLSM) imaging was conducted to visualize biofilm cells *in-situ* to determine the live/dead population distribution. After biofilms were harvested, sample pegs were then stained with propidium iodide for 30 minutes in the dark at room temperature and immediately imaged with CLSM (Zeiss LSM 710 Confocal Microscope). All sample pegs mounted for confocal microscopy were sequentially scanned frame-by-frame with a 63 x water immersion objective in fluorescence mode using FITC (green) and Rhodamine (red) filters. Confocal image visualization, analysis and two-dimensional (2D) projections of acquired Z-stacks were performed using Carl Zeiss Microscopy software systems (ZEN black edition).

2.6. Scanning Electron Microscopy (SEM)

Scanning electron microscopy (SEM) was used to visually examine the surface morphology of biofilms. All biofilm samples were rinsed with sterile PBS and fixed with a solution of 2.5% glutaraldehyde in 0.1 M cacodylate buffer at 25˚C for 2 hours. Thereafter, biofilm samples were air-dried for 7 days before SEM imaging. SEM measurements were performed after samples were sputter-coated with gold nanoparticles to enhance electron conductivity on sample surfaces. SEM images of fixed biofilms were taken with a JOEL JSM-7600F SEM at an accelerating voltage of 20 kV.

2.7. Nanoparticle Dissolution Test in Water and Biofilm Culture

To further examine the effects of biofilm culture on silver ion release from NPs, silver

dissolution experiments were conducted with nanoparticle concentrations of 0.625 mg/L, 1.25 mg/L, 2.5 mg/L and 5 mg/L. These experiments were setup similarly to the aforementioned nanoparticle challenge experiments, with the following exception: residual media in the plate well were collected in lieu of the pegs on the lid. The contents from each well were transferred into 2 mL centrifuge vials individually. Therefore, the silver ions were separated from silver nanoparticles by a centrifuging method by Wirth *et al.* [14]. The samples were centrifuged (Eppendorf ultracentrifuge 5418) for 45 mins at a rotor speed of 10,000 rpm (4˚C). Subsequently, supernatants containing only dissolved silver were gently removed and diluted with 5% nitric acid and tested with Graphite Furnace Atomic Absorption Spectrometry (PerkinElmer Waltham, MA). The dissolved silver concentration in water was also tested for comparison.

3. Results and Discussion

3.1. Nanoparticle Characterization

The Ag-NPs and Ag_2S-NPs were characterized in terms of their morphologies, size distributions and surface charge. As shown in Figure 1, Ag-NPs and Ag_2S-NPs had a particle size distribution from 30 to 50 nm. Particle size distribution and surface charge may sometimes be influenced by organic matter present in LB media. However, the PVP surface coating stabilized the nanoparticles, preventing aggregation, even in the presence of organic material (Table 1). In addition, the zeta potentials of the nanoparticles were negatively charged, with electrophoretic mobilities (μ) of −11.10 ± 0.600 mV and −9.51 ± 0.636 mV for Ag-NPs and Ag_2S-NPs, respectively.

3.2. Impact of of Ag-NPs and Ag_2S-NPs on Bacterial Biofilms

The antimicrobial activities of Ag-NPs against environmental and clinical bacteria have

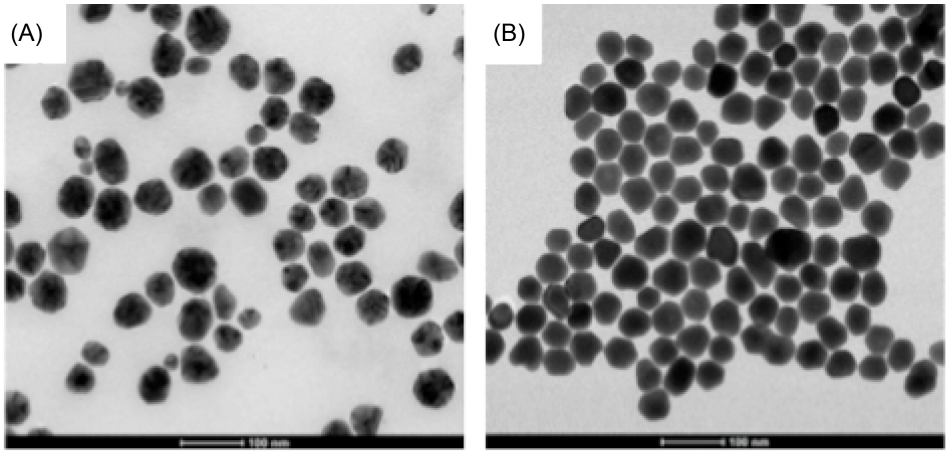

Figure 1. Representative TEM micrographs of (a) Ag-NPs; and (b) Ag_2S-NPs.

Table 1. Diameter and electrophoretic mobility of Ag-NPs and Ag_2S-NPs.

Method	Ag-NPs	Ag_2S-NPs
DLS, Particle diameter	43.32 ± 0.7879 nm	30.67 ± 0.2500 nm
Zetasizer, Electrophoretic mobilities (μ)	−11.10 ± 0.600 mV	−9.51 ± 0.636 mV

been established [33] [34] [35]. A comparative evaluation of the respective activities of Ag-NP and its sulfidized counterpart, Ag_2S-NP, against *P. aeruginosa* biofilms was performed using fluorescence spectrophotometry, scanning electron microscopy (SEM) and confocal laser scanning microscopy (CLSM). Fluorescence spectrophotometry confirmed the ability of both pristine and sulfidized Ag-NPs to disrupt biofilms (Figure 2) and kill bacterial cells (Figure 3). Exposures in the experimental ranges tested (0.3 - 5 mg/L)

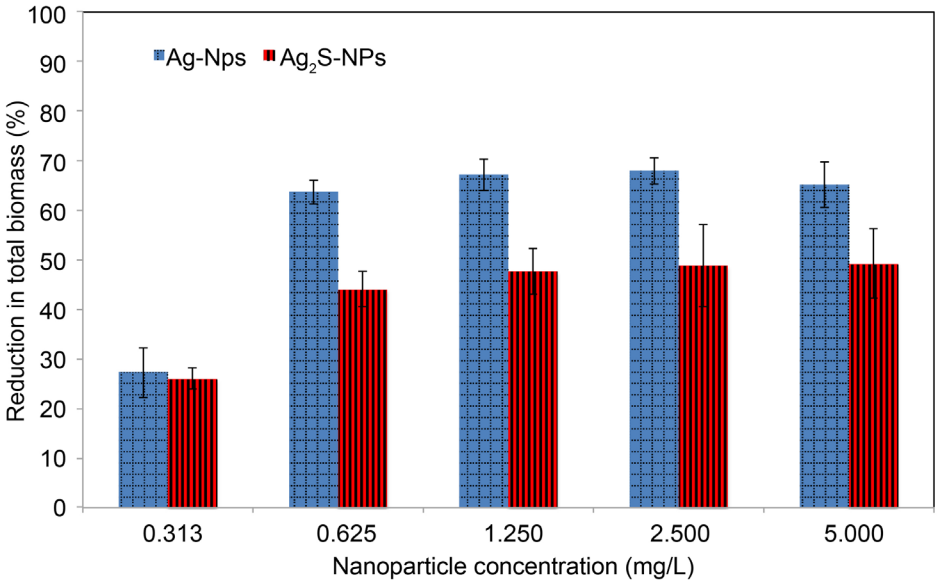

Figure 2. Effects of Ag_2S-NP and Ag-NPs on total biomass of *P. aeruginosa* biofilms. Established *P. aeruginosa* biofilms (72 hours) were exposed to Ag_2S-NPs and Ag-NPs (in LB media) at various concentrations, 0.3125 mg/L, 0.625 mg/L, 1.25 mg/L, 2.5 mg/L and 5 mg/L, respectively. After 24 hours of exposure, the resulting biofilms were carefully harvested and suspended in PBS buffer. The optical density (OD 600) of the respective suspension was determined as the indicator of total biomass in the corresponding biofilm.

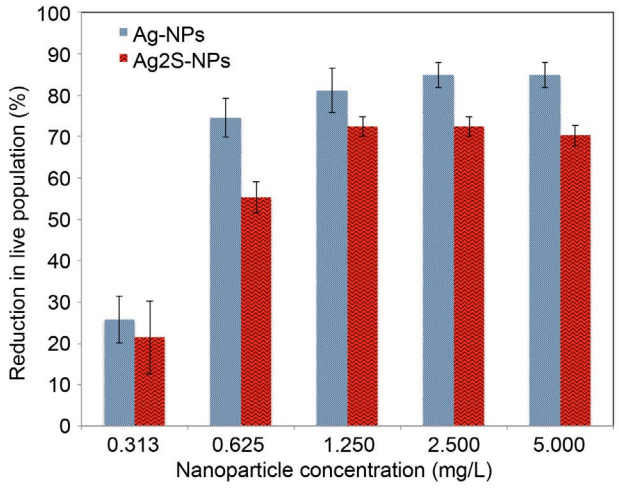

Figure 3. Effects of Ag_2S-NPs and Ag-NPs on total live population biomass of *P. aeruginosa* biofilms. Established *P. aeruginosa* biofilms (72 hours) were exposed to Ag_2S-NPs and Ag-NPs (in LB media) at various concentrations, 0.3125 mg/L, 0.625 mg/L, 1.25 mg/L, 2.5 mg/L and 5 mg/L, respectively. After 24 hours of exposure, the loss of fluoresce intensity from the biofilms were tested.

were not capable of fully eradicating the *P. aeruginosa* biofilms. Biofilm-growing bacteria such as *P. aeruginosa* are capable of withstanding up to 1000 times the concentrations of antimicrobial agents that would inhibit the growth of free-floating bacteria [36]. However, at all exposure concentrations, reduction in total biofilm biomass (determined by absorbance measurements at OD 600; **Figure 2**) corroborated well with the ability of NPs to kill bacterial cells (evaluated by monitoring the fluorescence activity of live bacterial cells; **Figure 3**).

Both NPs revealed a significant increase in antimicrobial activity when their exposure concentrations were doubled from 0.313 to 0.625 mg/L followed by a plateau in activity at higher concentrations (**Figure 2** and **Figure 3**). However, Ag_2S-NPs revealed a significant lower ability to either disrupt established biofilms (**Figure 2**) or kill *P. aeruginosa* cells (**Figure 3**) than Ag-NPs at all concentrations tested. This is consistent with previous findings that the sulfidation of Ag-NPs may reduce nanosilver toxicity [37] [38]. The sole exception to this rule was observed with the least effective exposure concentration (0.313 mg/L) where Ag-NPs and Ag_2S-NPs only resulted in 27.2% and 26.0% reductions in total biofilm biomass (corroborated with 25.8% and 21.5% reductions in live bacteria cells) respectively. This contrasted with 67.9% and 49.2% reductions in total biomass (associated with 84.9% and 70.2% reductions in live cells) for Ag-NPs and Ag_2S-NPs respectively (**Figure 2** and **Figure 3**).

To further assess the nanoparticle antimicrobial properties, pre-established *P. aeruginosa* biofilms were exposed to identical concentrations (0.625 mg/L) of Ag_2S-NPs and Ag-NPs and monitored with CLSM. SEM investigations of unchallenged *P. aeruginosa* cultures (**Figure 4(A)** and **Figure 4(D)**) validated the ability of the MBEC™ assays to

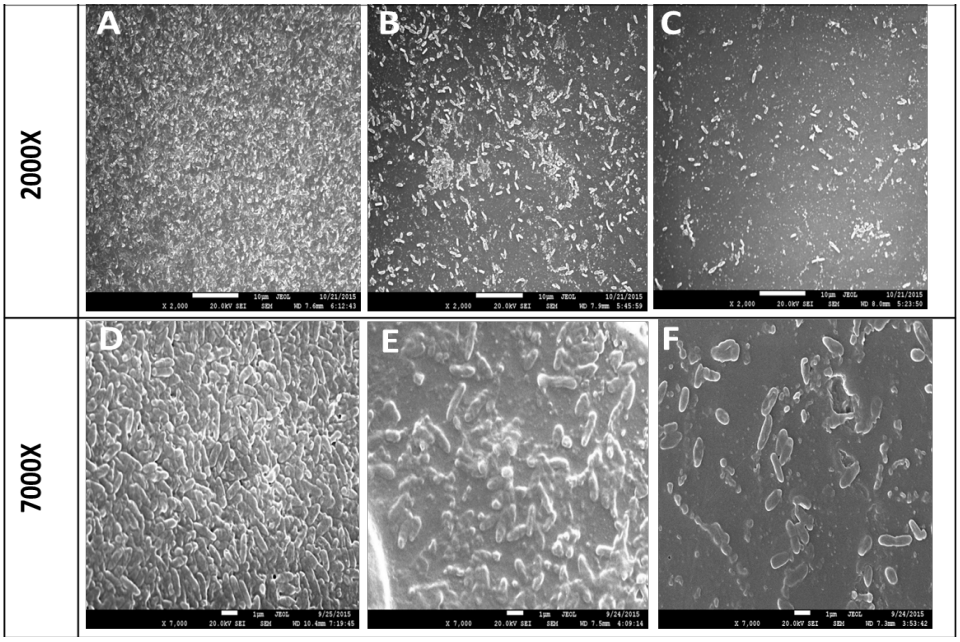

Figure 4. Micrographs (A), (B) & (C) are SEM images with magnification of 2000x, while (D), (E) & (F) are with 7000x. (A) & (D) represent unchallenged *P. aeruginosa* biofilms; (B) & (E) are biofilms challenged with Ag_2S NPs; C & F are biofilms treated with Ag-NPs. Established *P. aeruginosa* biofilms (72 hours) were exposed to 0.625 mg/L nanoparticles (in LB media) for 24 hours. The resulting biofilms were carefully preserved and sputter-coated for SEM imaging.

develop and sustain fully established biofilms on the surfaces of MBEC™ pegs. Again, Ag_2S-NPs were shown (**Figure 4(B)** and **Figure 4(E)**) to be less efficient at disrupting pre-established *P. aeruginosa* biofilms than Ag-NPs (**Figure 4(C)** and **Figure 4(F)**), corroborating the results achieved during quantification of total microbial biomass (**Figure 2**). Similar trends were observed with CLSM analysis. Corroborating previous live/dead assay findings (**Figure 3**), confocal images of biofilms challenged with NPs demonstrated the enhanced ability of Ag-NPs to kill *P. aeruginosa* cells as compared to Ag_2S-NPs (**Figure 5**). Taken together, these data emphasize the fact that sulfidation of Ag-NPs significantly impacts its antimicrobial properties, namely here its ability to kill biofilm-embedded cells and disrupt established *P. aeruginosa* biofilms. Since sulfidation of is one of the transformation processes that occur naturally in the environment, these data further suggest that the rate and extent of transformation of nanosilver should always be monitored and considered as a major factor when evaluating the potential impact of nanosilver on biological systems [26].

3.3. Effects of Nanoparticle Transport from Media to Biofilms

The bioavailability of nanoparticles is greatly affected by the transport and diffusion of NPs into the biofilms. In this section, we examined the transport of Ag-NPs and Ag_2S NPs into biofilms. Firstly, SEM imaging of nanosilver challenged biofilms confirms the transport activity of both NPs. Significant amount of Ag-NPs and Ag_2S NPs were observed on the biofilms. As shown in **Figure 6**, the nanoparticles appear to form loose clusters on biofilms, and the majority of the nanoparticles remain unaggregated, most likely due to the hydrophilic PVP coating [38].

Silver nanoparticles suspensions are a mixture of various forms: dissolved NPs and

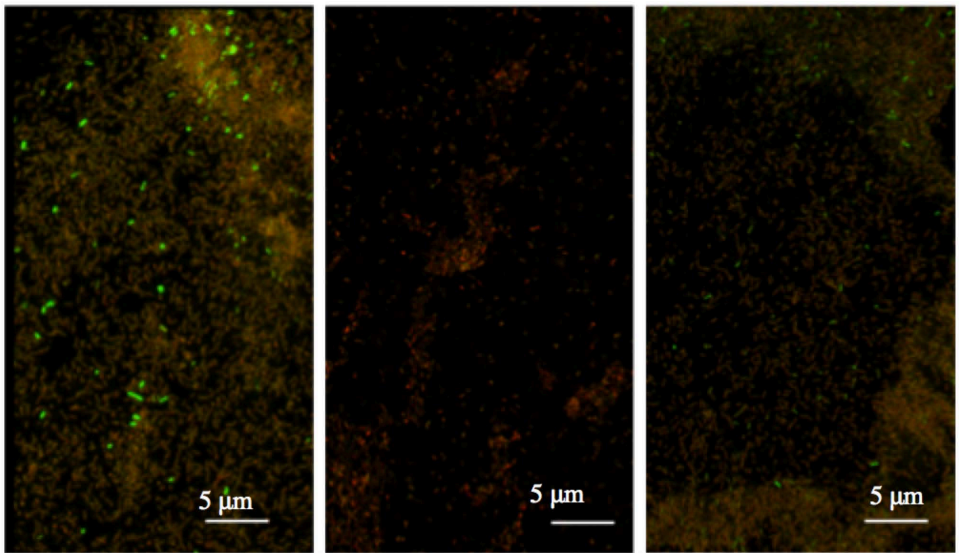

Figure 5. Confocal laser scanning microscopy images of (A) Unchallenged *P. aeruginosa* biofilm, (B) *P. aeruginosa* biofilm challenged by Ag-NPs, and (C) biofilms after challenged by Ag_2S-NPs. Established *P. aeruginosa* biofilms (72 hours) were exposed to 0.625 mg/L nanoparticles (in LB media) for 24 hours. The resulting biofilms were stained with PI for 30 mins and imaged immediately.

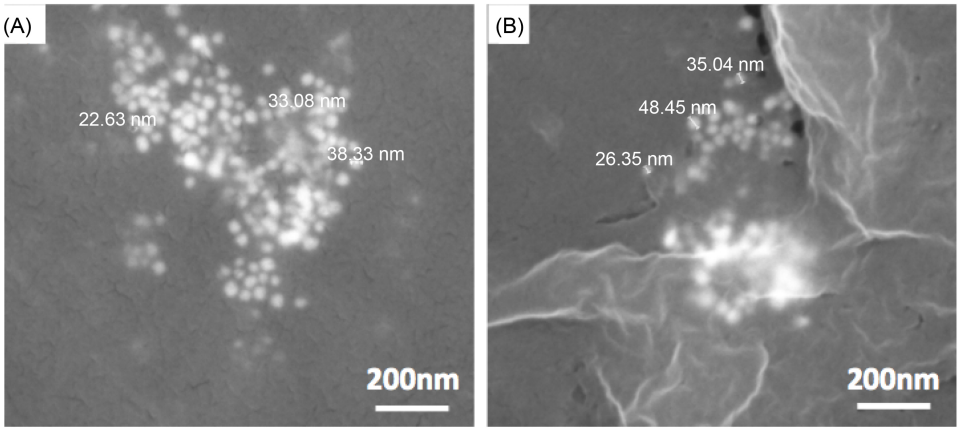

Figure 6. SEM images of the morphology of (A) Ag-NPs and (B) Ag_2S NPs in biofilm. Established *P. aeruginosa* biofilms (72 hours) were exposed to 0.625 mg/L nanoparticles (in LB media) for 24 hours. The resulting biofilms were carefully preserved and sputter-coated for SEM imaging.

dissolved silver ions. A difference in diffusion rate and transport into biofilms, as well as effective bioavailability for antimicrobial challenge, may exist between Ag-NPs and Ag_2S-NPs due to the differences in the transport abilities of dissolved NPs and diffusion of silver ions. Research has shown that silver nanoparticles can effectively transport from culture media to biofilms and that silver ions diffuse much faster than the dissolved NP forms [15] [39]. Additionally, the sulfidation process changes the dissolution rate of silver nanoparticles in water and reduces the release of silver ions. However, the presence of organic matters in culture solution may also alter the toxicity threshold [40] [41].

Therefore, we investigated the dissolution of Ag-NPs and Ag_2S-NPs in culture in the presence of *P. aeruginosa* established biofilms. After an exposure period of 24 hours, the residual silver ion concentrations in the culture were tested. As shown in **Figure 7**, concentrations of dissolved silver were significantly higher in suspensions of Ag-NPs than those of Ag_2S-NPs across the test concentrations after exposure to biofilms. This suggests a higher rate of silver ions transport into *P. aeruginosa* biofilms. Together, the higher solubility of Ag-NPs and higher amount of transported silver ions into biofilms would correlate with the higher impacts on biomass and live populations in the challenged biofilms by Ag-NPs than Ag_2S-NPs. However, the difference in biofilm inhibition effects between Ag-NPs and Ag_2S-NPs were not proportional to the difference in solubility, this may result from the difference in the transport behaviors of the two NPs. Overall, the bioavailabity of silver nanoparticles is greatly reduced by sulfidation.

4. Concluding Remarks

Our results suggest that sulfidation of Ag-NPs to Ag_2S-NPs resulted in lower damage to *P. aeruginosa* biofilms. Solubility of Ag-NPs is higher than that of Ag_2S-NPs in culture media, resulting in greater transport of Ag^+ into biofilms. Sulfidation of Ag-NPs changed their transport behavior and antimicrobial impacts on biofilms. Based on the information presented in this study, we can make predictions that sulfidation of Ag-NPs may reduce their bioavailability and antimicrobial abilities in different applications.

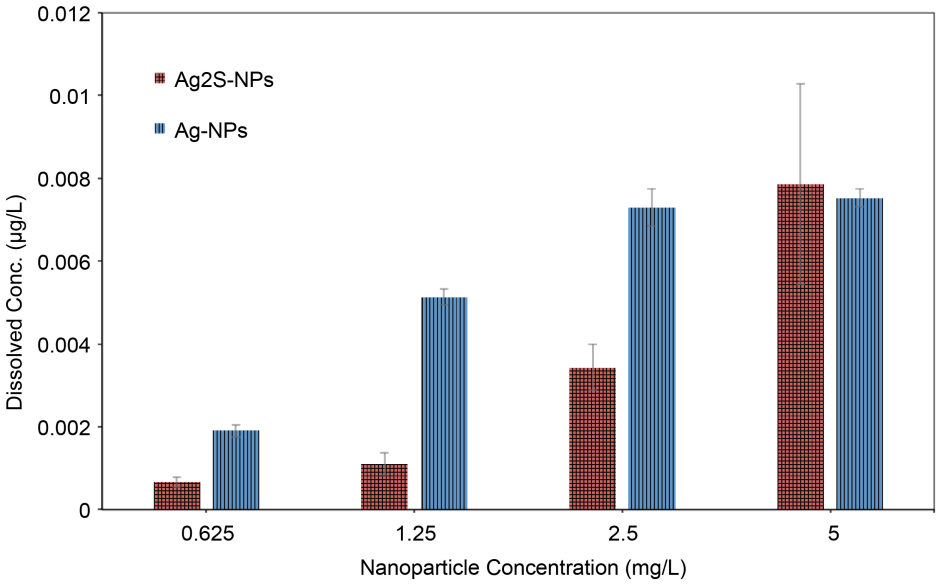

Figure 7. Concentration of silver ions dissolved from Ag-NPs and Ag_2S NPs after 24-h biofilm exposure. Ag-NPs and Ag_2S NPs were dissolved in LB media (at concentrations of 0.625 mg/L, 1.25 mg/L, 2.5 mg/L and 5 mg/L) and exposed to established *P. aeruginosa* biofilms for 24 hours. The concentrations of residual silver ions in the media were tested by AAS.

However, this process may also result in reduced environmental or human health risks could also be reduced when released into the environment.

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