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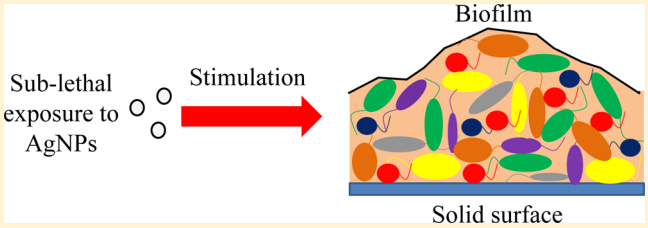
Sublethal Concentrations of Silver Nanoparticles Stimulate Biofilm Development

Yu Yang and Pedro J. J. Alvarez*

Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States

Supporting Information

ABSTRACT: Although silver nanoparticles (AgNPs) are used as antimicrobial agents in a wide variety of commercial products, sublethal exposure can counterproductively promote the development of biofilms. We observed by fluorescence microscopy denser biofilm growth with mixed cultures from a wastewater treatment plant after exposure to 21.6 μg/L 10 nm AgNPs. To further understand biofilm promotion mechanisms, experiments were conducted with a pure culture of *Pseudomonas aeruginosa* PAO1. Sublethal exposure of PAO1 to AgNPs (10.8 and 21.6 μg/L) also enhanced biofilm development and upregulated quorum sensing, lipopolysaccharide biosynthesis, and antibiotic resistance (efflux pump) genes. An increase in the sugar and protein contents of the PAO1 biofilm matrix (by 55 ± 3 and 114 ± 32%, respectively, relative to unexposed controls) corroborated the transcriptional upregulation of PAO1 biofilm-related genes. Enhanced biofilm development by exposure to low AgNP concentrations might accelerate biofouling and biocorrosion and harbor pathogens that pose a risk to public health.



INTRODUCTION

Silver nanoparticles (AgNPs) are widely used as antimicrobial agents in consumer products for domestic, environmental, medical, and industrial applications.[1−3] In 2014, 438 of 1816 nanotechnology-enhanced commercial products contained AgNPs.[4] Release of AgNPs from nanoenabled products has been observed,[5−8] and the potential impacts of such releases on microbial ecosystem services such as nutrient cycling and wastewater treatment have been recognized.[9−14] However, most of these studies have been conducted with relatively high (bactericidal) AgNP concentrations, and the effects of sublethal (low concentrations) exposure are poorly understood. This is a critical knowledge gap because low (rather than high) AgNP concentrations are expected to predominate following dilution and dispersion in the environment or engineered systems.[11,15]

Most studies of the microbial response to AgNPs have been conducted with planktonic cells,[14,16−18] even though bacteria in the environment commonly attach to surfaces and form biofilms.[19] Whereas biofilms can beneficially participate in biogeochemical cycles, waste degradation, and clean energy generation,[20−22] they are also implicated in biofouling, biocorrosion, pathogenic infection, and disease outbreaks.[23−26] Thus, prevention of biofilm formation is one objective of incorporating AgNPs on material surfaces.[15,27,28]

In this study, we address the effect of sublethal exposure to AgNPs on biofilm formation by a mixed culture from a wastewater treatment plant (WWTP). To gain mechanistic insight, additional experiments were conducted with the model bacterium *Pseudomonas aeruginosa* PAO1, which is ubiquitous in the environment, is known to be associated with biofouling,[29] and has been widely studied as a potential opportunistic human pathogen.[30] PAO1 is also a convenient bacterium for studying quorum sensing (QS),[31,32] which is a cell-to-cell signaling mechanism that is critical for initiating biofilm formation as well as for the regulation of virulence.[33−36] We compare the effects of AgNPs and Ag^+ ions (added as $AgNO_3$), because released Ag^+ ions from AgNPs are the critical effectors of the antimicrobial activity of silver.[37,38] Protein and sugar contents in the biofilm extracellular polymeric substances (EPS) as well as the expression of genes associated with biofilm formation, such as QS, lipopolysaccharide (LPS) biosynthesis, and antibiotic resistance (ABR), are also considered.

MATERIALS AND METHODS

Bacterial Strain and AgNPs. *P. aeruginosa* PAO1 (ATCC 15629) was purchased from the American Type Culture Collection (Manassas, VA) and was grown aerobically at 37 °C in Davis minimal nutrient broth from BD (Sparks, MD). Polyvinylpyrrolidone-coated AgNPs (PVP-AgNPs, 10 nm nominal size, 1 mg/mL) were purchased from NanoComposix Inc. (San Diego, CA), and their size and ζ potential in Davis broth were measured by a Zen 3600 Zetasizer Nano (Malvern Instruments). AgNP concentrations were calculated on a silver mass basis. Details about the broth components, biofilm growth condition, quantification of PVP coating, characterization of AgNPs, and other reagents are provided in the Supporting Information.

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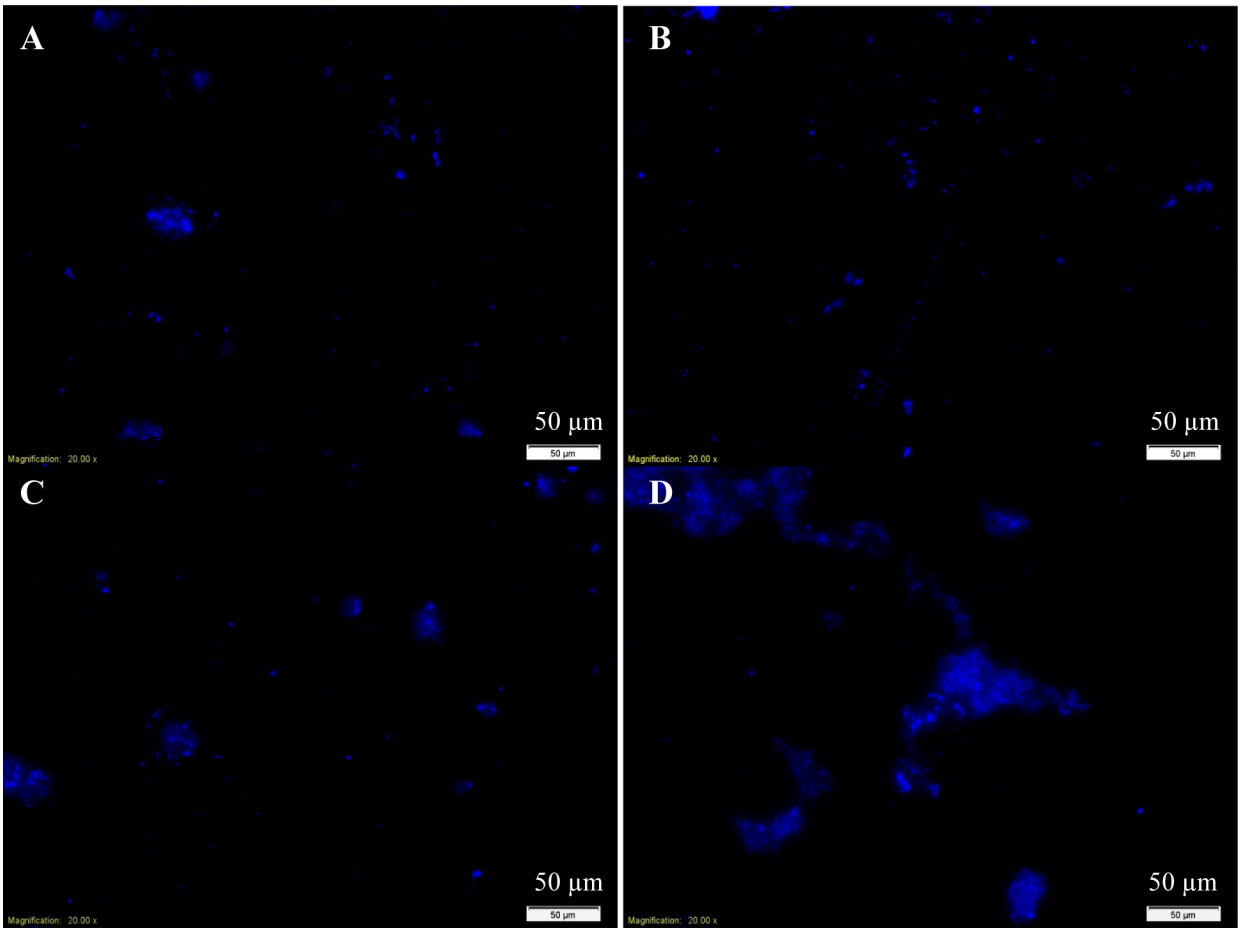

Figure 1. Images of biofilm in control (A) and treatments with PVP (41.7 μg/L) (B), 21.6 μg/L Ag^+ (C), and 21.6 μg/L PVP-AgNP (as total silver) (D). The samples were stained with propidium iodide (blue).

Assessment of Biofilm Development by Fluorescence Microscopy. Effluent from the West University Place wastewater treatment plant (WWTP) (Table S1 of the Supporting Information) was used "as is" for the mixed culture biofilm development test. The effluent was amended with glucose [0.2% (w/v)] and 21.6 μg/L silver as either PVP-AgNPs or $AgNO_3$. *P. aeruginosa* PAO1 in Davis minimal nutrient broth (A_{600} = 0.003−0.005) was exposed to PVP-AgNPs or Ag^+ (21.6 μg/L) for the pure culture biofilm development test. This sublethal AgNP concentration was selected on the basis of preliminary dose−response assays with PAO1 (Figure S1 of the Supporting Information). Controls received no AgNPs, and the PVP coating control contained 45.7 μg/L PVP. This concentration was chosen because the PVP density on PVP-AgNPs is 2.21 ± 0.23 mg/mg of Ag, and 21.6 μg/L PVP-AgNPs could result in (at most) 45.7 μg/L PVP. Uncoated glass Petri dishes containing the effluent and an uncoated glass slide were prepared in triplicate and incubated on a rotary shaker at 37 °C and 50−60 rpm for 24 h (WWTP effluent) or 48 h (*P. aeruginosa* PAO1). The longer incubation time was used for PAO1 because of the slower growth in the minimal medium used to minimize PVP−AgNP aggregation. The biofilm on the glass slide was gently rinsed with distilled water and then fixed at 80 °C for 0.5 h in an isothermal oven (Fisher Scientific, Pittsburgh, PA). The biofilm was stained with propidium iodide (30 μM) for 15 min at 37 °C, and the excessive dye was washed off with phosphate-buffered saline (PBS). The biofilm on each glass slide was observed, and cells were counted with an Olympus IX71 inverted fluorescence microscope (Olympus, Center Valley, PA). At least 10 fields of view (444 μm × 333 μm) were recorded for each slide.

Investigation of the Transcriptional Mechanism in a *P. aeruginosa* Biofilm. *P. aeruginosa*, exposed to sublethal concentrations of Ag^+ (21.6 μg/L) or PVP-AgNPs (21.6 and 108 μg/L on a silver mass basis), was grown at 37 °C in 12-well plates (Corning Inc., Corning, NY) filled with Davis minimal nutrient broth (A_{600} = 0.003−0.005). The plates were placed on a rotary shaker at 37 °C and 50−60 rpm. Controls, receiving no AgNPs, were treated in the same way. After exposure for 48 h, the biofilm was rinsed with PBS to remove unbound cells and removed from the plate with cell scrapers.[39] Biofilm RNA was extracted using the RNeasy Mini Kit according to the manufacturer's protocol, and concentrations were determined using a Nanodrop ND-1000 instrument (Nanodrop products Inc., Wilmington, NE). cDNA was synthesized overnight at 42 °C by reverse transcription polymerase chain reaction (PCR) using random primers, RNaseOUT, dNTPs, and Superscript II reverse transcriptase.[10] Quantitative real-time polymerase chain reaction (q-rt-PCR) was performed in 15 μL of reaction mixture composed of 2 ng of cDNA, SYBR Green Master Mix (7.5 μL), each primer at 0.3 μM, and water, to quantify the expression of target chromosomal genes involved in QS (i.e., *lasI/R* and *rhlI/R*), lipopolysaccharide (LPS) biosynthesis (i.e., *pelA*, *pslA*, and *sagS*), and antibiotic resistance (i.e., *mexA* and *ndvB*).[40,41] The $2^{-\Delta\Delta CT}$ method was used to quantify differential gene expression with recombinase A gene *recA* as

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the reference gene,[42] and the results were analyzed with SDS 1.3.1.[43] All treatments and q-rt-PCR analyses for each sample were run in triplicate. Additional experimental details and primer sequences are described in Table S2 of the Supporting Information.

Quantification of Protein and Carbohydrate in the PAO1 Biofilm Matrix. Following a 48 h exposure to 21.6 μg/L Ag^+ (added as $AgNO_3$) or PVP-AgNPs, the biofilm matrix of PAO1 was extracted following a previously described method[39,44,45] (please refer to the Supporting Information for details). The protein content was determined with a BCA kit using bovine serum as a standard (Sigma-Aldrich, St. Louis, MO), based on the kit manual. The total carbohydrate content was measured according to the method of Dubois et al.[46] The total protein and total carbohydrate contents were expressed as a function of the biofilm dry weight (milligrams per gram of biofilm dry weight). For each treatment (including no-treatment controls), plates were prepared in triplicate and incubated under identical conditions.

■ RESULTS AND DISCUSSION

PVP-AgNPs Enhanced Biofilm Formation by Bacteria in WWTP Effluent. Sublethal exposure to PVP-AgNPs (21.6 μg/L) enhanced biofilm formation by a mixed culture from WWTP effluent, while Ag^+ did not at the tested equivalent concentration (Figure 1). The biofilm coverage fractions were 0.9 ± 0.2% for control, 0.9 ± 0.3% for PVP, 1.1 ± 0.3% for Ag^+, and 5.2 ± 2.1% for PVP-AgNPs (Table S3 of the Supporting Information). Thus, biofilm coverage increased by 2.1−6.3-fold after sublethal 24 h exposure to PVP-AgNPs. An increased level of biofilm development was also observed by fluorescence microscopy for *P. aeruginosa* PAO1 exposed to 21.6 μg/L PVP-AgNPs relative to unexposed controls or treatments with an equivalent Ag^+ concentration ($p < 0.05$) (Figure S2 and Table S3 of the Supporting Information). The negligible effect of PVP coating on biofilm development rules out its possible role in the promotion in biofilm formation. Moreover, PVP coating has been reported to hinder biofilm formation by *P. aeruginosa* on silicone tubes.[47] The stronger effect of AgNPs than Ag^+ at an equivalent concentration is likely due to the presence of inorganic ligands (e.g., chloride and phosphate) and organic matter [e.g., soluble microbial products (SMPs) and EPS], which can reduce the bioavailability and effect of Ag^+ to a greater extent than AgNPs.[48,49]

AgNPs are used on a variety of commercial products such as toys and medical devices to mitigate bacterial colonization and biofilm formation.[50,51] The AgNP coating (PVP, up to 5%) is not toxic to *Pseudomonas* spp.,[52] suggesting that PVP coating does not directly contribute to antibacterial activity of PVP-AgNPs. Our counterintuitive observation that sublethal exposure to AgNPs could promote biofilm formation calls for long-term studies to assess whether this phenomenon would occur when treated surfaces reach low AgNP concentrations after leaching and dissolution. Another potential concern is enhanced biofilm formation by sublethal microbial exposure to AgNPs released to engineered systems (e.g., water and wastewater infrastructure), which could accelerate biofouling and biocorrosion and harbor pathogenic bacteria.

Low concentrations of AgNPs might promote biofilm formation as a defense mechanism against toxicity. Cells in the biofilms might benefit from protection against antibacterial agents.[53] Environmental factors and chemical stressors exert similar stimulatory effects. For example, subinhibitory concentrations of tetracycline upregulated expression of polysaccharide intercellular adhesin genes in *Staphylococcus epidermidis*, and other stress (e.g., osmolarity and high temperatures) also induced biofilm formation.[54,55] Other examples include promotion of *Escherichia coli* biofilm formation by dissolved Ni^{2+}[53] and *P. aeruginosa* biofilm formation by mucin.[56] However, the stimulatory mechanisms remain elusive, which motivated us to investigate the transcriptomic response of biofilm-forming *P. aeruginosa* PAO1 exposed to sublethal AgNP concentrations.

Upregulation of QS, LPS Biosynthesis, and ABR by AgNPs. Low levels of PVP-AgNPs (21.6 or 108 μg/L) induced expression of PAO1 genes associated with biofilm formation (QS and LPS biosynthesis) (Figure 2). PAO1 contains two QS

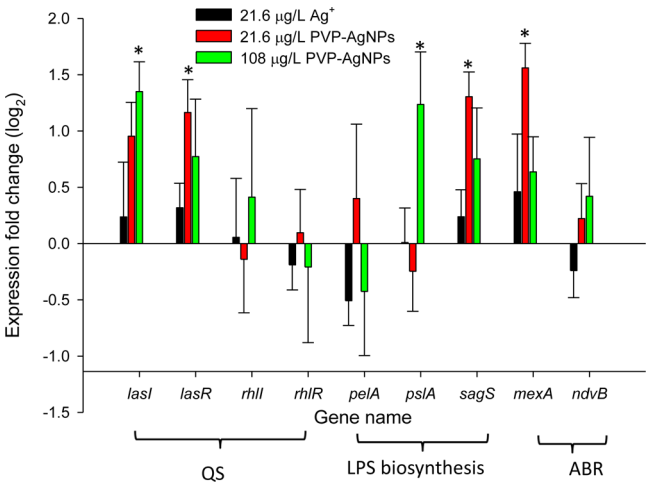

Figure 2. Transcriptomic analysis for *P. aeruginosa* exposed to sublethal concentrations of Ag^+ or PVP-AgNPs. Asterisks indicate significant upregulation compared to unexposed controls ($p < 0.05$). Error bars represent ±one standard deviation from the mean of triplicate measurements.

systems, *las* and *rhl* systems, and either system consists of one transcriptional activator (LasR or RhlR) and an autoinducer synthase (LasI or RhlI).[57] The *las* system dominates and controls the *rhl* system, although these two systems are linked to each other.[57] For the *las* system, 21.6 and 108 μg/L PVP-AgNPs induced *lasR* (2.1−3.1-fold) and *lasI* (1.8−2.7-fold), respectively, while the *rhl* system was not affected by PVP-AgNPs. Furthermore, 21.6 μg/L PVP-AgNPs induced expression of surface attachment and growth sensor hybrid *sagS* (2.1−2.9-fold), and 108 μg/L upregulated expression of probable glycosyl transferase *pslA* (1.7−3.3-fold). LPS contributes to biofilm attachment and structure maintenance and also functions as an important virulence factor.[58] This stimulation of QS and LPS synthesis corroborates the observation that sublethal concentrations of AgNPs can promote biofilm development and increase the risk of biofouling and pathogenesis.

Upregulation of antibiotic resistance genes (ARGs) by AgNPs suggests potential enhancement of antibiotic tolerance.[59] Expression of *mexA*, encoding multidrug efflux membrane fusion protein, was induced by 21.6 μg/L PVP-AgNPs (2.5−3.4-fold) (Figure 2). This efflux pump system seems to contribute to both antibiotic resistance and silver resistance, and coregulation between heavy metal and antibiotic resistance in *P. aeruginosa* has been reported.[60]

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AgNPs Increased the Level of Production of Biofilm Matrix Components. An increase in the level of production of sugar and protein components of the biofilm matrix corroborated the observed transcriptional upregulation. After exposure to 21.6 μg/L PVP-AgNPs, the protein and sugar contents in the PAO1 biofilm matrix increased by 114 ± 32 and 55 ± 3%, respectively (Figure 3). The enhanced EPS secretion might exacerbate biofouling and biocorrosion, because EPS are involved in cell adhesion, aggregation, water retention, barrier protection, sorption of substrates, nutrient supply, electron exchange, and enzyme and energy storage.[61]

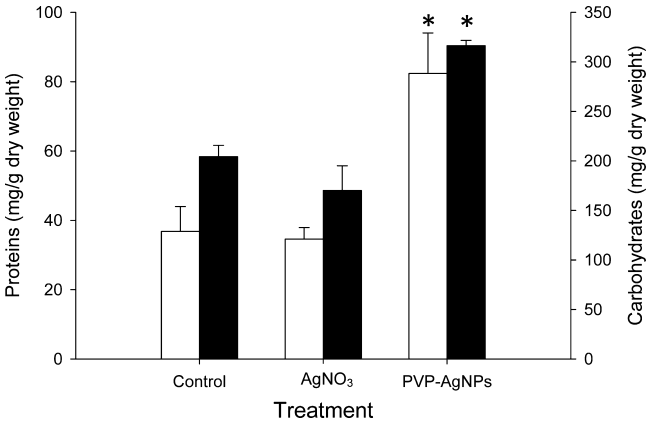

Figure 3. Quantification of proteins (white bars) and carbohydrates (black bars) in a biofilm matrix exposed to $AgNO_3$ or PVP-AgNPs. Asterisks indicate significant increases compared to control samples ($p < 0.05$). Error bars represent ±one standard deviation from the mean of triplicate measurements.

Consistent with the mixed culture biofilm results, no significant effect was observed on either biofilm gene transcription (Figure 2) or EPS production (Figure 3) following exposure to an equivalent concentration of Ag^+ ions. Whereas released Ag^+ has been demonstrated to be the critical effector of the antibacterial activity of AgNPs,[37] the bioavailability of Ag^+ is often decreased to a much greater extent than that of AgNPs because of scavenging by a wide variety of inorganic ligands and organic matter,[48] including SMPs and EPS.[49,62] Thus, we postulate that the increased level of EPS and SMP production may scavenge free Ag^+ ions and mitigate their influence on biofilm development.[63−65]

Overall, this work demonstrates an overlooked potential unintended consequence associated with bacterial exposure to sublethal AgNP concentrations: upregulation of QS and EPS production genes and the associated stimulation of biofilm formation. This implies that incidental or accidental releases of low concentrations of AgNPs might accelerate biofouling and biocorrosion. Furthermore, considering that biofilms are more difficult to eradicate than planktonic bacteria,[66,67] the observed upregulation of antibiotic resistance genes suggests the potential for exacerbating bacterial virulence and pathogenesis, which underscores the need for judicious use of AgNPs and control of their incidental releases.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Detailed description of AgNP dissolution, gene expression quantification, biofilm EPS extraction, biofilm biomass measurements, and characterization of AgNPs. The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.estlett.5b00159.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: alvarez@rice.edu. Phone: (713) 348-5903. Fax: (713) 348-5268.

Notes

The authors declare no competing financial interest.

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