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Interaction of silver nanoparticles with pure nitrifying bacteria

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HIGHLIGHTS

- *N. europaea* and electrolytes contributed to AgNPs aggregation in the medium.
- The inhibitory effect of AgNPs on *N. europaea* was size- and coating-dependent.
- AgNPs caused the damage of cell wall of *N. europaea* and made the nucleoids disintegrated and condensed next to cell membrane.
- AgNPs inhibited activity of *N. europaea* via repressing the expression of selected functional proteins.

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ABSTRACT

In this study, *Nitrosomonas europaea* ATCC 19718 was exposed to silver nanoparticles (AgNPs) of different particle size (7 ± 3 and 40 ± 14 nm) and different coatings (polyvinyl alcohol and adenosine triphosphate disodium). For all different AgNPs used in the study, large aggregates were gradually formed after addition of AgNPs into the media containing *N. europaea*. The scanning electron microscopy and energy dispersive X-ray spectroscopy of the microstructures suggested that bacterial cells and electrolytes had significant effects on AgNP aggregation. Size- and coating-dependent inhibition of ammonia oxidation by AgNPs was observed, and our analysis suggested that the inhibition was not only due to the released dissolved silver, but also the dispersity of AgNPs in the culture media. Electron microscopy images showed AgNPs could cause the damage of cell wall of *N. europaea* and make the nucleoids disintegrated and condensed next to cell membrane. Surface-enhanced Raman scattering signals also implied the damage of cell membrane caused by AgNPs. Further protein expression analysis revealed that AgNPs would inhibit important protein functions, including biosynthesis, gene expression, energy production and nitrification to further cause toxicity to *N. europaea*. Our findings explain the susceptibility of *N. europaea* to inhibition by AgNPs and the possible interaction between each other. Future research is needed to characterize these effects in more complex cultures and media such as activated sludge and wastewater.

1. Introduction

The use of silver nanoparticles (AgNPs) in our daily life increases rapidly since the first AgNPs-containing product (Algaedyn) in 1954 was registered in the US as an algaecide based on the patent by Moudry (Nowack et al., 2011). To date, AgNPs have been widely used in textiles, food storage containers, numerous household products, cosmetics, and biomedical applications (Farkas et al., 2011; Rejeski et al., 2011; Walser et al., 2011). The AgNPs-containing consumer products have accounted for 55.4% (313 consumer products) of nanobased consumer products in 2011 (Rejeski et al., 2011). Thus, the release of AgNPs to the environment cannot be avoided due to human activities and environmental conditions (e.g., washing AgNPs-containing products and raining on AgNPs-containing wall) (Farkas et al., 2011; Walser et al., 2011), which may bring about a potential risk to microorganisms in the natural and engineered systems because of their broad-spectrum antimicrobial properties (Choi et al., 2010).

The AgNPs, released from the consumer products, most possibly enter sewer systems with the domestic sewage and then reach wastewater treatment plants (WWTPs) (Kim et al., 2010). Some studies have confirmed that spiked AgNPs can decrease the microbial activity and change the microbial population structure in the lab-scale bioreactor (Liang et al., 2010; Yang et al., 2012). Nitrifying bacteria, a group of ubiquitous and important autotrophic microorganisms in the WWTPs, convert ammonia to nitrate in two steps called nitrification (Roh et al., 2009). The first step of nitrification, from ammonia to nitrite, is carried out by ammonia-oxidizing bacteria (AOB), and the second step, from nitrite to nitrate, is carried out by nitrite-oxidizing bacteria. The previous studies have shown that they are more sensitive to AgNPs than heterotrophic bacteria and low concentrations ($\leqslant 1\ mg\ L^{-1}$) of AgNPs can effectively

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inhibit their activity (Choi and Hu, 2008; Liang et al., 2010). The shock loading of 1 mg L^{-1} AgNPs to activated sludge inhibited 41% of nitrification and led to shift or loss of nitrifying bacteria in the reactor (Liang et al., 2010). Therefore, for the risk of AgNPs to WWTPs, it should be paid more attention to the effect on nitrifying bacteria.

As mentioned in recent publications, researchers hypothesized that the toxicity of AgNPs to microorganisms was mainly caused by the dissolved silver ions from AgNPs, and AgNPs themselves had limited contribution to the toxicity effects (Radniecki et al., 2011; Xiu et al., 2011). However, other researchers demonstrated that the elimination of Ag^+ by Ag^+ ligands such as cysteine or sulfide, could partially remove AgNPs toxicity (Xiu et al., 2011). Researchers also suggested that the toxicity of AgNPs are related to particle size (Carlson et al., 2008; Liu et al., 2010), shape (Pal et al., 2007), surface coatings (Amato et al., 2011), aggregation (Cheng et al., 2011), generation of reactive oxygen species (ROS) (Choi and Hu, 2008), and environmental conditions (Badawy et al., 2010). Although considerable progress has been made in elucidating factors affecting AgNPs toxicity, there is less information regarding the toxicity mechanism of AgNPs to test microorganisms, especially nitrifying bacteria. Therefore, in this study, we used three different AgNP suspensions (7 ± 3 nm, 7 ± 3 nm, and 40 ± 14 nm, coated with adenosine triphosphate disodium (Na_2-ATP), polyvinyl alcohol (PVA) and Na_2ATP, respectively) to study their toxicity to pure nitrifying bacteria. In addition, this work also characterized the interactions of AgNPs with the pure AOB, *Nitrosomonas europaea*, by electron microscopy, surface-enhanced Raman scattering (SERS) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) to elucidate the possible toxicity mechanism of AgNPs to AOB.

2. Materials and methods

2.1. Preparation and characterization of AgNP stock suspensions

Two different types of AgNPs with similar particle size were synthesized but were stabilized by different coatings. The PVA-coated AgNPs (herein referred to as PVA_7) were synthesized according to the method described by Choi et al. (2010). In short, 1 mL of 14 mM sodium borohydride was added into a 18 mL solution of 0.06 wt% PVA, and 1 mL of 14 mM silver nitrate was then injected slowly at one drop per second at room temperature, and the mixture was stirred by a magnetic stirrer with a speed of 700 rpm for 5 min in the dark. Na_2ATP-coated AgNP suspension (herein referred to as ATP_7) were synthesized according to the method described by Shi et al. (2012). 0.0318 g Na_2ATP and 1 mL of 25 mM $AgNO_3$ were respectively brought into 48 mL ultra-pure water with stirring at a speed of 400 rpm, followed by injection of 1 mL of 29 mM $NaBH_4$ and mixing continuously for 12 h in the dark. The third AgNP suspension (herein referred to as ATP_{40}) was prepared by adding purchased commercial AgNP powders (Nanostructured & Amorphous Materials Inc., USA; 35 nm and spherical) into the ultra-pure water, followed by addition of Na_2ATP as the stabilizer and dispersion by ultrasonication in the ice bath. These AgNP suspensions were store in the dark at 4 °C before use.

The morphology and particle size of these AgNPs were determined by transmission electron microscopy (TEM, Hitachi H-7650, Japan) at acceleration voltage of 80 kV and analyzed by the free software of Image J (http://imagej.nih.gov/ij/). TEM samples were prepared by placing a drop of the AgNP suspension on a standard copper grid and drying at room temperature overnight. The characteristic surface plasmon absorption band of AgNPs was checked with scanning from 300 to 700 nm using a Thermo Evolution 300 UV–Vis spectrophotometer (USA). The release of silver ions from AgNPs in the suspensions was examined by using Amicon centrifugal ultrafilter devices (Millipore 3 kDa, USA) for 30 min at 5000 rpm (Multifuge X1R, Thermo Fisher Scientific) according to the previous study (Liu and Hurt, 2010). Total silver was digested with HNO_3 in a 95 °C water bath, and dissolved silver concentrations were measured by inductively coupled plasma mass spectrometry (ICP-MS, Agilent 7500Cx, USA).

2.2. Growth conditions of nitrifying bacteria and AgNPs toxicity assays

N. europaea ATCC 19718 was purchased from Nite Biological Resource Center. *N. europaea* was grown in a defined mineral salt medium containing 0.5 g KH_2PO_4, 11.92 g HEPES, 0.5 g $NaHCO_3$, 100 mg $MgSO_4{\cdot}7H_2O$, 5 mg $CaCl_2{\cdot}2H_2O$ and 75 mg Fe-EDTA per liter and 19 mM $(NH_4)_2SO_4$ under 150 rpm in the dark at 30 °C. The initial pH of the culture medium was maintained between 7.8 and 8.0. The cells were harvested at late-exponential growth phase (3 d after inoculation) by centrifugation at 10000 g for 30 min at 4 °C using a Hitachi centrifuge (CR22GII, Japan), washed once with phosphate buffer solution (50 mM KH_2PO_4 and 2 mM $MgSO_4{\cdot}7H_2O$, pH 7.8), and then resuspended in the mineral salt medium and stored on ice for subsequent experiments (Roh et al., 2009). The cell concentrations were measured at OD_{600} using a Thermo Evolution 300 UV–Vis spectrophotometer.

To assess the toxicity of AgNPs on *N. europaea*, aliquots of previously prepared cell stock solution were added in 250 mL flasks with culture medium prewarmed at 30 °C and containing a series of different concentrations AgNPs. The initial OD_{600} of cell concentration in the flasks was controlled at 0.05. Subsequently, the flasks containing cells and AgNPs were incubated under the same conditions as cell growth. Nitrite generation rate was used as the indicator of the activity of *N. europaea*, and nitrite concentration was measured every 60 min for 3 h using a Dionex ion chromatograph (ICS-3000, USA). All the experiments were conducted in triplicate. The inhibition was calculated by analyzing nitrite generation rate (NGR) as described in Eq. (1) (Wang and Gunsch, 2011)

$$\text{Inhibition } \% = \frac{(\text{NGR}_{\text{control}} - \text{NGR}_{\text{sample}})100\%}{\text{NGR}_{\text{control}}} \quad (1)$$

The remaining concentration of nitrite, sodium borohydride, Na_2ATP and PVA present in the toxicity assay medium had no inhibition on NGR (data not shown). Growth of *Nitrobacter winogradskyii* NBRC 14297 and its toxicity assays are described in Supplementary material (SM).

2.3. Characterization of aggregation of AgNPs and morphology of N. europaea

Microstructures of the visible aggregates and morphology changes of *N. europaea* during the toxicity assays were characterized via scanning electron microscopy (SEM, Hitachi S-4800, Japan) with energy dispersive X-ray (EDX) spectroscopy (Genesis XM2) and TEM with EDX Spectroscopy (Genesis XM2). Details of the methods are described in SM.

2.4. SERS assays

A confocal microscope Raman spectrometer (HORIBA Jobin Yvon S.A.S. LabRAM Aramis, France) with a 632.8 nm diode laser source was used to analyze the biomolecular changes of the cellular surface in exposure to AgNPs. The spectral signals of 300–1800 cm^{-1} range were enhanced with 60 nm gold nanoparticles (AuNPs) and recorded using a 10 s exposure time and 10 mW laser (Hodges et al., 2011). The system was calibrated using the 520.5 cm^{-1} Raman band as a baseline from a silicon wafer. The bacteria exposed to a series of concentrations (0, 1, 5 and