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Bactericidal activity of silver nanoparticles in environmentally relevant freshwater matrices: Influences of organic matter and chelating agent

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ABSTRACT

Toxicity of nanomaterials, especially silver nanoparticles (AgNPs), is attracting increasing interests in both research and applications as the emerging of nanoscience and nanotechnology. The objective of this study was to elucidate the influences of various natural organic matter (NOM) and chelating agent, ethylenediaminetetraacetic acid (EDTA), on AgNPs stability (aggregation, dissolution) and bactericidal activity in environmentally relevant freshwater matrices. Bacterial viability, in terms of half maximal inhibitory concentration (IC_{50}) value, was adopted to judge the toxicity of AgNPs against bacteria. Our results showed that three model NOM, humic acid, bovine serum albumin and alginic acid, at concentration comparable to that of natural freshwaters did not substantially affect the bactericidal activity of AgNPs against *Bacillus subtilis* and *Escherichia coli*. Nevertheless, the combined addition of Ca^{2+} and humic acid induced severe aggregation of AgNPs, resulting in a reduced nanotoxicity. The chelating agent, EDTA, exhibited a concentration-dependent effect on AgNPs bactericidal activity: at low concentration (1 mg/L), EDTA decreased toxicity of AgNPs likely by converting the relatively high toxic dissolved Ag species into a series of low toxic Ag–EDTA complex; at high concentrations (5 and 20 mg/L), EDTA mainly acted as a synergist and increased the toxicity of AgNPs probably by reacting with divalent cations (such as Ca^{2+} and Mg^{2+}) on bacterial cell membranes. Our findings have significant implications for understanding the influencing factors of AgNPs toxicity under natural freshwater conditions, which may also benefit the studies of nanotoxicity mechanisms as well as environmental impact assessments of nanomaterials.

Introduction

Nanomaterials are defined as materials with at least one dimension on nanoscale, usually ranging from 1 to 100 nm in size [1–3]. Different from the traditional bulk materials of same composition, nanomaterials exhibit unique properties stemming from their nanoscale dimensions, which enables their widespread applications in almost all industrial sectors including materials, healthcare, transport, energy, water remediation, and information and communications technologies [4,5]. According to some estimates, nanotechnology is likely to develop into a $1 trillion industry by 2015 [6].

While the frontier of nanotechnology is expanding rapidly, new challenges in understanding, predicting and controlling potential adverse effects of nanomaterials also arise. For example, silver nanoparticles (AgNPs) are the most widely used nanomaterial among all commercial nano-products [7,8]. They can be released into the environment through variety routes during their production, usage, and disposal, which inevitably leads to their accumulation, transformation and degradation in the atmosphere, water, soils, or organisms [9,10]. AgNPs could be dangerous to the environment, especially in aquatic environments, due to the relatively high toxicity of silver against some aquatic organisms and microbial communities [11,12]. Hence, understanding the potential risks of AgNPs imposed to the environment is as important as studying their novel physicochemical properties used in promising applications.

Previous studies suggested that toxicity of AgNPs in different matrices may differ greatly [13,14]. While most currently documented ones were conducted in bio-relevant medium that is rich of various nutrients and with a high concentration of salts.

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Little is known about the transport, fate, and toxicity of AgNPs in natural environments, especially in natural freshwaters [14]. Natural freshwaters are enriched with natural organic matter (NOM) with varied structures and compositions, which may adsorb on the surface of AgNPs and produce a core-shell structure [15]. Stability and toxicity of AgNPs could be affected during this process. In addition, relatively subtle changes in water chemistry conditions can significantly alter interfacial morphology, hydrophobicity, and charge of AgNPs, which dictate their stability, reactivity, bioavailability and potential toxicity in aquatic environments [16,17]. Therefore, studies on stability and bactericidal activity of AgNPs with the presence of different NOM in environmentally relevant freshwater matrices is important to elucidate the fate, reactivity and impacts of AgNPs on microorganisms in ecosystems.

Chelating agents may also enter natural water system through the effluents of a variety of industrial applications [18]. Their concentrations can be comparable to those of NOM [19]. They are a series of polydentate ligands that may change the water chemistry conditions [20] and form a variety of complexes with different kinds of metals. Among them, EDTA has been one of the most widely used chelating agents with high extraction efficiency [21]. EDTA may significantly affect the size, shape, surface charge, bioavailability and toxicity of AgNPs during the formation of complexes [22]. However, to the best of our knowledge, no systematic research has been conducted on learning the effects of chelating agent on AgNPs toxicity in environmentally relevant freshwater matrices.

The objective of this study was to illustrate the influences of NOM and chelating agent on the stability and bactericidal activity of AgNPs in environmentally relevant freshwater matrices.

Materials and methods

Chemicals and solution chemistry

Humic acid (HA), bovine serum albumin (BSA) and alginic acid (AA) were used as model NOM to represent humic substances, protein and polysaccharides in natural streams. HA was Suwannee River Humic Acid Standard II obtained from International Humic Substances Society (IHSS), St. Paul, MN. Both BSA (lyophilized powder, $\geq$98%) and AA (alginic acid sodium salt from brown algae) were obtained from Sigma–Aldrich, St. Louis, MO. Ethylenediamine-tetraacetic acid (EDTA, 99.4–100.6%, Sigma–Aldrich; St. Louis, MO) was used to observe the effects of chelating agent on bactericidal activity of AgNPs. Commercial silver nanoparticles (10 nm, 40 nm and 100 nm AgNPs, 20 mg/L in 0.059% sodium citrate aqueous buffer) used in all experiments were purchased from Sigma–Aldrich, St. Louis, MO.

Environmentally relevant freshwater matrices were prepared by using Milli-Q water referring to the water quality parameters reported for the fresh river water in Wheeling, West Virginia [23] and the White River in Muncie, Indiana [24]. The total ionic strength in each solution was maintained at a constant level of 5.6 mM by replacing specific cation or anion with ionic strength equivalent, NaCl, unless specified. In order to simulate realistic situation, 5 mg/L [16,23–25] was chosen as the concentration of three model NOM (HA, BSA and AA) and chelating agent (EDTA) in test solutions, unless otherwise specified. The pH value of each solution was adjusted to 7.0 by using 0.1 M NaOH or HCl. All solutions were either autoclaved or disinfected using 0.2 μm syringe filters with Supor membrane (Pall Corporation, Ann Arbor, MI). All chemicals and reagents were either ACS reagent grade or analytical grade unless specified. Compositions, pH, and conductivity of each solution are shown in Table S1, Supplementary information.

Preparation and characterization of AgNPs suspensions

AgNPs (20 mg/L in 0.059% sodium citrate aqueous buffer) with different particle sizes (nominal diameters of 10 nm, 40 nm and 100 nm) were tested to investigate the influence of particle size on their toxicity. The AgNPs stock solutions were prepared by simply diluting the products to a silver concentration of 10 mg/L. In order to make sure that AgNPs were uniformly dispersed in stock solutions, 15 min ultrasonication (Ultrasonic Bath S450H, 50/60 Hz, 2000 W, Fisher Scientific Pte. Ltd.; SG) was employed before experiments. Hydrodynamic diameters and ξ-potentials of AgNPs in respective water matrix were determined using Zetasizer (Nano ZS, Malvern Instruments; Worcestershire, UK).

To determine the concentration of dissolved silver, AgNPs suspensions (same conditions as those used in nanotoxicity studies) were filtered through a 3 kDa ultrafiltration centrifugal filter units (Amicon Ultra-4 3K, Millipore; Billerica, MA) by centrifuging for 40 min at 4000 × *g*. The filtrate was digested in 7% HNO_3 according to a modified U.S. EPA method 3050B [26] and measured by using Inductively Coupled Plasma Mass Spectrometer (ICP-MS, ELAN DRC-e, Perkin Elmer; Waltham, MA).

Preparation of bacterial cultures

Gram-positive bacteria *Bacillus subtilis* (ATCC 6633) and Gram-negative bacteria *Escherichia coli* (ATCC 8739) were used as model organisms to evaluate the toxicity of AgNPs against bacteria in various water chemistry conditions. Both genera are widely existed in environment [14]. Planktonic bacterial cultures were prepared in nutrient broth (BD DIFCO 234000) with shaking (150 rpm) at their respective optimum growth temperature, i.e., 30 °C for *B. subtilis* and 37 °C for *E. coli*. Bacterial cells were harvested at mid-exponential growth phase (24 h for *B. subtilis* and 12 h for *E. coli*, respectively) and then were washed twice with 0.9% NaCl and specific freshwater matrices sequentially, and then resuspended in specific freshwater matrices to achieve a cell density of 1.0×10^8 cells/mL for bacterial viability tests.

Bacterial viability assay by high-throughput screening analysis

Live/dead Baclight bacterial viability kit (Molecular Probes, Eugene, OR) was used to determine bacterial viability by high-throughput screening analysis [14,27–29]. 100 μL of AgNPs suspensions of different concentrations (from 0.01 μg/L to 10 mg/L) and 100 μL of bacterial suspensions were added and completely mixed into specific wells of a 96-well F-bottom polystyrene microplate (Greiner Bio-One; Monroe, NC). Each combination was tested in 2–4 independent batches with at least 9 replicates, therefore, 18–36 replicates were used to determine one testing condition. All plates with bacteria were incubated for 24 h under respective optimum growth temperature. After 24 h exposure, stain solutions with a mixture of SYTO9, green-fluorescent nucleic acid stain that labels all bacteria in a population, and propidium iodide, a red-fluorescent nucleic acid stain that only affects the bacteria with membrane damage, were added into each well.

The green/red fluorescence ratio detected by Synergy 2 Multi-Mode Microplate Reader (BioTek Singapore, SG) provided the percentage of live bacterial cells in each well (excitation at 485 nm and emission at 635 and 500 nm) according to calibration curves obtained by blending different known ratios of live-to-dead bacteria (3–4 independent batches with at least 18 replicates). Silver-free controls contained only bacteria and stain mixture. Percentages of live bacteria cells calculated by calibration curves were normalized by the silver-free controls and analyzed by fitting the biostatistics software Prism 5 "Dose-response Inhibition,