Does the bacterial media culture chemistry affect the stability of nanoparticles in nanotoxicity assays?

M. Millour, K. Doiron, K. Lemarchand, J.-P. Gagné
Institut des sciences de la mer de Rimouski, Université du Québec à Rimouski, Rimouski, Québec, Canada

Introduction

New classes of silver nanoparticles (AgNPs) with antimicrobial activities are under rapid development for incorporation into consumer products. Important applications are in food packaging materials, odor-resistant textiles, paints, cosmetics, medical devices, water disinfectants, etc. This increasing use of AgNPs raised the issue of the release of nanosilver from a variety of sources, under different forms, into aquatic environments. Recent studies have confirmed the release of AgNPs from socks and nanotextiles during washing or from surfaces coated by nanosilver paints during rain-water runoff events. The environmental effects of the AgNPs discharged into aquatic environments are largely unknown. However, laboratory ecotoxicological studies on fishes, crustaceans, algae and bacteria have shown toxic effects on test organisms exposed to AgNPs. Due to the antimicrobial properties of AgNPs, it is important to understand which factors control their toxicity toward environmental microorganisms since they play important roles in major biogeochemical cycles, in the production of natural organic matter and in the recycling of nutrients. Bacterial sensitivity to silver nanoparticles and ionic silver have been demonstrated on reference bacterial strains of the genera Bacillus, Escherichia, Pseudomonas and Vibrio as well as on bacterial communities. Today, there is no consensus on the mechanisms of antimicrobial action of AgNPs. Proposed processes include: i) free silver ion dissolution from AgNPs followed by cellular uptake and disturbance of intracellular reactions; ii) cellular uptake of AgNPs followed by reactive oxygen species (ROS) generation and release of silver ions impacting DNA replication and ATP production; and iii) direct interaction of AgNPs with cell surface altering protein function and membrane permeability. These processes were demonstrated to be dependent on nanoparticle’s size and specific properties (shape, chemical composition, surface charge, coating, etc.). Moreover, the chemical composition of exposure media (pH, ionic strength, ionic composition, quality and quantity of natural organic matter) is also a relevant factor that would affect the toxicity of AgNPs. The solution chemistry could play an important role in the aggregation and in the fate of AgNPs. If aggregation occurs, the size of AgNPs in solution will change, modifying the sedimentation and the ability of AgNPs to interact with proteins at the cellular surface or to cross-cellular membranes. Thus, aggregation can modulate the toxicity of nanoparticles. Considering that the size of AgNPs can be a significant factor controlling the toxicity of silver nanoparticles, it is important to understand how the exposition media (e.g., culture broths) modify the size of the nanoparticles used in toxicity assays. The purpose of this study is to assess the stability of AgNPs suspensions with time in different exposure solutions characteristic of natural waters or of media currently used in microbial ecotoxicology studies. We aim to show the importance to control the aging of AgNPs colloidal suspensions during toxicity assays.

Materials and Methods

All experiments were conducted with citrate coated silver nanoparticles obtained by the reduction of AgNO₃ with sodium citrate. Suspension of AgNPs was purified to remove excess of AgNO₃ and citrate and to concentrate AgNPs. AgNPs synthetized had a hydrodynamic diameter of 9±2 nm. Salts used in this study were NaCl, CaCl₂, MgCl₂. Artificial seawater (ASW) was made by mixing 400 mM of NaCl, 41.4 mM of MgSO₄, 7 H₂O and 1.8 mM of NaHCO₃. Salt solutions and ASW were prepared and regulated at pH 7 and at 25%. This salinity is characteristic of estuarine environments. These solutions were filtered on 0.2-µm pore-sized polycarbonate membrane. Culture media tested were Guillard’s F/2, as reference bacterial strain of the genera Bacillus, Escherichia, Pseudomonas and Vibrio as well as on bacterial communities. All experiments were conducted with citrate coated silver nanoparticles obtained by the reduction of AgNO₃ with sodium citrate. Suspension of AgNPs was purified to remove excess of AgNO₃ and citrate and to concentrate AgNPs. AgNPs synthetized had a hydrodynamic diameter of 9±2 nm. Salts used in this study were NaCl, CaCl₂, MgCl₂. Artificial seawater (ASW) was made by mixing 400 mM of NaCl, 41.4 mM of MgSO₄, 7 H₂O and 1.8 mM of NaHCO₃. Salt solutions and ASW were prepared and regulated at pH 7 and at 25%. This salinity is characteristic of estuarine environments. These solutions were filtered on 0.2-µm pore-sized polycarbonate membrane. Culture media tested were Guillard’s F/2, generally used for marine microalgal culture, and Luria Broth (LB), commonly used for bacterial growth. F/2 media was prepared by diluting trace element (nitrate, phosphate, metal and vitamins) in ASW. LB media was composed of 10 g of tryptone, 5 g of yeast extract and 10 g of NaCl for 1 L of nanopure water at pH 7.2. The evolution of hydrodynamic diameters of the AgNPs suspensions at concentrations of 93 µg Ag.L⁻¹ was measured by time resolved dynamic light scattering (TR-DLS) using Zetasizer Nano ZS (Malvern Instruments Ltd., Malvern, Worcestershire, UK) instrument during 17 h at 25°C. Parameters used for the TR-DLS was one measure each 10 min with an angle of 173°. One measure was obtained with 10 scans of 15 s each. The concentration used (93 µg Ag.L⁻¹) in the experiments was in the lower range of concentrations used to test the toxicity of AgNPs to microorganisms.

Results and Discussion

Figure 1 shows the evolution in hydrodynamic diameter (Dh) for AgNPs over 17 h in nanopure water, NaCl, CaCl₂, or MgCl₂ solution. In nanopure water, the Dh of AgNPs remained stable at 9±2 nm during 17 h (Figure 1A). In presence of NaCl at 25%, the Dh of AgNPs (9 nm) evolved rapidly (Figure 1B) to reach 550±280 nm after 100 min. When CaCl₂ was added to nanopure water at 25%, the Dh of AgNPs increased rapidly during the first 50 min (Figure 1C) suggesting that the rate of AgNPs aggregation is modulated by the nature of cations in solution. After 50 min, the Dh did not evolve anymore and stabilized at 424±69 nm. In presence of MgCl₂, added to nanopure water at 25%, the behavior of AgNPs was the same as in the CaCl₂ solution (Figure 1D). After the rapid increase (50 min), the Dh remained at 548±105 nm. The rapid, but different, increase of Dh observed in NaCl, CaCl₂ and MgCl₂ solutions reflected the aggregation of AgNPs in presence of various cations and showed that divalent cations were more effective in accelerating aggregation than monovalent cations.

Figure 2 presents the time evolution of AgNPs hydrodynamic diameter in ASW, F/2 and LB media. In ASW, from 0 min to 200 min, the Dh increased rapidly from 9 nm to near 400 nm.
as shown in Figure 2A. After 200 min the Dh reached 422±255 nm. In F/2 medium (Figure 2B), the behavior of AgNPs was similar to that observed in ASW. A rapid increase in Dh was observed during the first 200 min afterward the Dh remained at 635±253 nm. In LB broth, the Dh of AgNPs increased rapidly during the first 250 min. Later on, the diameter stabilized around 511±365 nm. In complex solutions (Figure 2) slower changes in aggregation rates were observed compared to those observed in simple solutions (Figure 1) during the first 100 min. This involves that for a complex aqueous solution, the rate of aggregation is modified by the presence of cations but also by other components, such as organic matter (vitamins in F/2 medium and tryptone and yeast extract in LB medium) present in the culture media.

Stability or aggregation of AgNPs could be rationalized, in first approximation, by the Derjarguin, Landau, Verwey and Overbeek (DLVO) theory. Stability is controlled by attractive and repulsive interactions. Attractive interactions are van der Waals forces and repulsive interactions are governed by the electrical double layer. When repulsive interactions are greater than attractive interactions, the conditions are unfavorable for aggregation, but when repulsive interactions are equal or lower than attractive forces, the aggregation is promoted. The transition between these two steps is characterized by the critical coagulation concentration (CCC). For AgNPs coated with citrate, the CCC was generally close to 50 mM for NaCl and near 2 mM for divalent cations present in CaCl₂ or MgCl₂ solution. In this study, the pH solution was near 7 and the salt concentrations were greater than the CCC, thus aggregation occurs.

In nanopure water, the citrate moieties of AgNPs were negatively charged. This promotes the electrostatic repulsions between AgNPs, thus avoiding the aggregation of AgNPs. Moreover, the presence of salts has increased the ionic strength of the solution decreasing the thickness of the electrical double layer around the silver nanoparticles. This has enhanced the close encounter of AgNPs and therefore the aggregation. However, the presence of salts also affected the charges at the surface of AgNPs. Citrate moieties were negatively charged and can interact with cations. Interactions between citrate and cations decreased the negative charges of AgNPs and lowered the electrostatic repulsion between particles. These phenomena enhanced the aggregation of AgNPs and can explain the behavior of AgNPs in nanopure water and salt solutions. Another process that could contribute to the aggregation was the presence of multivalent cations. Those cations could form a bridge between two adjacent AgNPs facilitating the aggregation of nanoparticles. This modification was caused by the reaction of Ag⁺, released from AgNPs, with anions like Cl⁻ or HCO₃⁻. The salts formed during the reaction were insoluble and could interact with the surface of AgNPs to create a new coating. With this new coating, the toxicity of nanoparticles could be different than the toxicity of the native nanoparticles.

In F/2 medium, the trace element concentration could be too low to affect AgNPs aggrega-
Aggregation of AgNPs could extend the lag phase or decrease growth rate of cultured microorganisms since they need to adapt to the evolution of AgNPs size and particle numbers in solution. During the rapid increase of Dh of AgNPs, the culture media are in constant evolution. This could increase the lag phase as observed in some studies.\(^6\)\(^7\) During aggregation, the toxicity mechanism could change. The small aggregates of AgNPs could penetrate in the cell and produce ROS inside the cell. When the aggregates become larger they cannot penetrate the cell but can interact with their surface and alter membrane permeability and protein functions. The decrease of particle concentration can also modify the response of organisms exposed to AgNPs. Depending of the aggregation state of the AgNPs suspension, toxicity of AgNPs could be different. This study demonstrates that characterizing the behavior of AgNPs in the toxicity assay media is essential to better understand the real effects of AgNPs toward microorganisms.

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