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Toxicity of silver ions and differently coated silver nanoparticles in Allium cepa roots

Petra Cvjetko[a], Anita Milošić[a], Ana-Marija Domijan[b], Ivana Vinković Vrček[c], Sonja Tolić[d], Petra Peharec Štefanić[a], Ilse Letofsky-Papst[e], Mirta Tkalec[a], Biljana Balen[a,*]

[a] Department of Biology, Faculty of Science, University of Zagreb, Horvatovac 102a, 10000 Zagreb, Croatia
[b] Department of Pharmaceutical Botany, Faculty of Pharmacy and Biochemistry, University of Zagreb, Ul. Ante Kovačića 1, 10000 Zagreb, Croatia
[c] Institute for Medical Research and Occupational Health, Ksaverska cesta 2, 10000 Zagreb, Croatia
[d] Department of Ecology, Institute of Public Health "Dr. Andrija Štampar", Mirogojska cesta 16, 10000 Zagreb, Croatia
[e] Institute of Electron Microscopy and Nanoanalysis (FELMI), Graz University of Technology, Graz Centre for Electron Microscopy (ZFE), Austrian Cooperative Research (ACR), Steyrergasse 17, 8010 Graz, Austria

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ABSTRACT

Silver nanoparticles (AgNPs) are the dominating nanomaterial in consumer products due to their well-known antibacterial and antifungal properties. To enhance their properties, different surface coatings may be used, which affect physico-chemical properties of AgNPs. Due to their wide application, there has been concern about possible environmental and health consequences. Since plants play a significant role in accumulation and biodistribution of many environmentally released substances, they are also very likely to be influenced by AgNPs. In this study we investigated the toxicity of $AgNO_3$ and three types of laboratory-synthesized AgNPs with different surface coatings [citrate, polyvinylpyrrolidone (PVP) and cetyltrimethylammonium bromide (CTAB)] on *Allium cepa* roots. Ionic form of Ag was confirmed to be more toxic than any of the AgNPs applied. All tested AgNPs caused oxidative stress and exhibited toxicity only when applied in higher concentrations. The highest toxicity was recorded for AgNPs-CTAB, which resulted with increased Ag uptake in the roots, consequently leading to strong reduction of the root growth and oxidative damage. The weakest impact was found for AgNPs-citrate, much bigger, negatively charged NPs, which also aggregated to larger particles. Therefore, we can conclude that the toxicity of AgNPs is directly correlated with their size, overall surface charge and/or surface coating.

1. Introduction

Interest in nanomaterials with controlled structures and functionality is growing rapidly. Silver nanoparticles (AgNPs) are of particular interest because of their well-known antibacterial and antifungal properties. They can be found in medical devices, textiles, food packaging, and healthcare and household products (Ahamed et al., 2010). According to the Woodrow Wilson Inventory 38 (http://www.nanotechproject.org, November 2013), AgNPs are the dominating nanomaterial in consumer products. With their increasing usage, concerns about their impact on environmental and human health raise. AgNPs are known to induce toxicity in prokaryotic (Suresh et al., 2010) and eukaryotic (Ahamed et al., 2010) organisms. Their (cyto)toxicity has been attributed to several possible mechanisms, including disruption of cell-membrane integrity (Suresh et al., 2010), protein or DNA binding and damage (Arora et al., 2009) and reactive oxygen species (ROS) generation (Mirzajani et al., 2013). However, several contradicting reports show that it is still not clear to which degree the toxicity of AgNPs results from AgNPs *per se* and how much toxicity is related to the released Ag^+ (Navarro et al., 2008). Different surface coatings are used in the synthesis of AgNPs to enhance their stability (Tejamaya et al., 2012) and they influence the physical/chemical properties of the NPs including size and shape, surface charge, differential binding and aggregation potential, consequently determining cytotoxicity and cellular interactions of AgNPs (Suresh et al., 2010, 2012).

Since plants play a significant role in accumulation and biodistribution of many environmentally released substances, they are also very likely to be influenced by AgNPs, serving as a potential pathway for AgNP-transport and bioaccumulation into food chains (Rico et al., 2011). Plants can be exposed to AgNPs indirectly, via AgNP-containing products for human usage that are being released to the environment

* Corresponding author.
E-mail address: bbalen@biol.pmf.hr (B. Balen).

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(Luoma, 2008), and directly by the application of the commercially available products implemented in agriculture (Sekhon, 2014). Research studies aiming to elucidate mechanism of AgNPs toxicity on plants are still scarce. Published results indicate that the impact of AgNPs on higher plants depends on the species and age of plants, the size and concentration of the particles, the experimental conditions and the duration and method of exposure (Yin et al., 2012; Vannini et al., 2013, 2014). Bioavailability of AgNPs can be influenced by composition of nutrient media, aquatic solutions or soils as well as surface coatings, but only a few studies with plants have considered these aspects so far (Lee et al., 2012; Yin et al., 2012). Although accumulation of Ag in AgNPs treated plants has been revealed (Lee et al., 2012; Dimkpa et al., 2013), uptake mechanisms and distribution of AgNPs in plant cells are still insufficiently explored.

The aim of this research was to reveal the effect of surface coating of AgNPs on toxicity effects to plant tissue in order to contribute to the environmental hazard assessment of AgNPs as well as to the basic knowledge about the mechanisms of their toxicity. As the model organism we used *Allium cepa*, an economically interesting plant, and also a frequently used model plant organism in abiotic stress research (Kumari et al., 2009; Geremias et al., 2011). Laboratory-synthesized AgNPs with three different surface coatings were used to determine the effect of surface properties of AgNPs on their induction of oxidative stress, damage to lipids, proteins and DNA molecule, parameters that are frequently used in investigations of metal-induced toxicity (Balen et al., 2011; Tkalec et al., 2014), but have only recently been explored in AgNP-toxicity studies performed on plants (Mirzajani et al., 2013; Yasur and Rani, 2013; Dimkpa et al., 2013).

2. Materials and methods

2.1. Synthesis, characterization and stability evaluation of AgNPs

The syntheses of AgNPs with different surface functionalization were conducted using three structurally diverse surface coatings. Citrate-coated AgNPs (AgNP-citrate) were prepared by reduction of $AgNO_3$ with sodium citrate as reported by Milić et al. (2015). The AgNPs coated with cetyltrimethylammonium bromide (AgNP-CTAB), or polyvinylpyrrolidone (AgNP-PVP) were synthesized by reducing $AgNO_3$ with $NaBH_4$ as previously described (Vinković Vrček et al., 2015). In order to obtain purified and stable AgNPs, the freshly prepared nanoparticle suspensions were washed twice with ultrapure water (UW) immediately after synthesis using centrifugation at 15,790 × *g* for 20 min. The washed AgNPs were resuspended in UW by sonification, and stored in the dark at 4 °C until use.

Total silver concentrations in AgNPs colloidal suspensions were determined in acidified solutions (10% HNO_3) using an Agilent Technologies 7500cx inductively coupled plasma mass spectrometer (ICP-MS) (Agilent, Waldbronn, Germany). The formation of nanosized Ag particles was verified by the presence of a surface plasmon resonance (SPR) peak measured using a UV–Vis spectrophotometer (CARY 300, Varian Inc., Australia). The size and charge of AgNPs were measured using Zetasizer Nano ZS (Malvern, UK) equipped with green laser (532 nm). Intensity of scattered light was detected at the angle of 173°. All measurements were conducted at 25 °C. The data processing was done by the Zetasizer software 6.32 (Malvern instruments). Results are reported as an average value of 10 measurements and the size distributions are reported as volume distributions. The charge of AgNPs was evaluated by measuring electrophoretic mobility of AgNPs. Results are reported as an average value of 5 measurements. In addition, synthesized and purified AgNPs were visualized using a monochromated TF20 (FEI TecnaiG2) transmission electron microscope (TEM), Schottky cathode, operated at 200 kV, equipped for energy-dispersive X-ray spectroscopy (EDX) with a SiLi detector and an ultrathin window. TEM samples were prepared by depositing a drop of the sample suspension on a Formvar®/Carbon copper grid. Samples were air-dried at room temperature.

The possible silver dissolution in UW was determined by centrifugal ultrafiltration (Millipore Amicon Ultra-4 3K) through a membrane with a 3 kDa molecular weight limit. Suspensions were centrifuged for 30 min at 15,000 × *g*. The silver concentration in the filtrate as determined by ICP-MS was related to the Ag concentration before ultrafiltration to calculate dissolved Ag^+.

2.2. Plant material and treatment

Onion (*Allium cepa* L.) bulbs were purchased from Sjemenarna d.o.o (Zagreb, Croatia). Bulbs were scrapped and the yellowish-brown scales and their bottom plates were removed so that the apices of the root primordial were exposed and their dry scales peeled off. Bulbs were afterwards placed on the top of the test tubes (one bulb per each test tube) filled with distilled H_2O and placed in the dark. After 2 days the bulbs with 2–3 cm long roots were washed in running tap water for 5–10 min and then subjected to treatment with aqueous solutions of AgNP-citrate, AgNP-PVP and AgNP-CTAB as well as of $AgNO_3$. Aqueous solutions of all AgNPs were made by dispersing the NPs stock solutions in ultrapure ion free Milli-Q (Millipore, 18.2 MΩ-cm resistivity) water without solvents in order to obtain concentrations of 25, 50, 75 and 100 μM. $AgNO_3$ treatments were applied in the same concentrations. The treatments lasted for 72 h in the absence of light at 25 °C. Roots of control bulbs were immersed in ultrapure Milli-Q water. After 72 h, the onion bulbs were taken out and roots were thoroughly rinsed with water. For these experiments the 6 onion bulbs were taken for each exposure treatment, including control. For each parameter analysis all roots from the each of the bulbs were taken together.

2.3. Root growth, mitotic index and dry matter content

The length of the three longest roots was measured from each control and treated bulb (6 bulbs per treatment), after which the average length was calculated for each treatment.

Mitotic index (MI) analysis was performed as previously described by Kumari et al. (2009). The MI was calculated as the ratio between the number of mitotic cells and the total number of cells scored (3,000) and expressed as percentage.

Root dry matter content was expressed as a ratio between root dry mass and root fresh mass.

2.4. Silver uptake measurements

At harvest, roots were washed with ultrapure Milli-Q water to remove AgNPs adhered to tissue. Roots were oven-dried at 80 °C for 24 h until a constant weight was recorded. Dried samples were prepared for analysis, according to the modified US EPA method 3052. Tissue was microwave digested, firstly in 10 mL of concentrated HNO_3 at 130 °C for 10 min, then at 180 °C for another 15 min. The second step was digestion in 1 mL of H_2O_2 at 85 °C for 5 min and then at 130 °C for 4 min. After cooling, the samples were diluted with 1% (v/v) HNO_3 up to the total volume of 50 mL. The samples were analyzed using ELAN DRC-e ICP-MS (Perkin Elmer, USA) to determine total Ag concentration. Silver concentration was calculated according to the calibration curve obtained with a set of standards of known concentrations. Detection limits and limit of quantification (LOQ) were 0.2 and 1 mg kg^{-1} respectively. Spike recovery tests were 92.4% for $AgNO_3$-treated plant tissue and between 90.7 and 95.4% for AgNPs-treated roots.

2.5. TEM analysis of plant material

Roots were carefully washed with distilled water, dried with filter paper and fixed with 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.2), postfixed with 1% OsO_4 in the same buffer and embedded in