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Properties of silver nanoparticles influencing their uptake in and toxicity to the earthworm *Lumbricus rubellus* following exposure in soil☆

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ABSTRACT

Physicochemical properties of nanoparticles influence their environmental fate and toxicity, and studies investigating this are vital for a holistic approach towards a comprehensive and adequate environmental risk assessment. In this study, we investigated the effects of size, surface coating (charge) of silver nanoparticles (AgNPs) – a most commonly-used nanoparticle-type, on the bioaccumulation in, and toxicity (survival, growth, cocoon production) to the earthworm *Lumbricus rubellus*. AgNPs were synthesized in three sizes: 20, 35 and 50 nm. Surface-coating with bovine serum albumin (AgNP_BSA), chitosan (AgNP_Chit), or polyvinylpyrrolidone (AgNP_PVP) produced negative, positive and neutral particles respectively. In a 28-day sub-chronic reproduction toxicity test, earthworms were exposed to these AgNPs in soil (0–250 mg Ag/kg soil DW). Earthworms were also exposed to $AgNO_3$ at concentrations below known EC_{50}. Total Ag tissue concentration indicated uptake by earthworms was generally highest for the AgNP_BSA especially at the lower exposure concentration ranges, and seems to reach a plateau level between 50 and 100 mg Ag/kg soil DW. Reproduction was impaired at high concentrations of all AgNPs tested, with AgNP_BSA particles being the most toxic. The EC_{50} for the 20 nm AgNP_BSA was 66.8 mg Ag/kg soil, with exposure to <60 mg Ag/kg soil already showing a decrease in the cocoon production. Thus, based on reproductive toxicity, the particles ranked: AgNP_BSA (negative) > AgNP_PVP (neutral) > Chitosan (positive). Size had an influence on uptake and toxicity of the AgNP_PVP, but not for AgNP_BSA nor AgNP_Chit. This study provides essential information on the role of physicochemical properties of AgNPs in influencing uptake by a terrestrial organism *L. rubellus* under environmentally relevant conditions. It also provides evidence of the influence of surface coating (charge) and the limited effect of size in the range of 20–50 nm, in driving uptake and toxicity of the AgNPs tested.

1. Introduction

The anticipated increase in the production and use of nanotechnology in the design and manufacture of numerous consumer products (Ahamed et al., 2010; Haider and Kang, 2015; Vance et al., 2015), is likely to result in an increase in the environmental release of nanoparticles (NPs), potentially causing harmful impacts (Benn et al., 2010; Geranio et al., 2009; Reidy et al., 2013; Wijnhoven et al., 2009, 2010). At the nanoscale (1–100 nm), the small size

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and increased surface area of NPs result in novel properties, which can be enhanced by their stabilization or functionalization using biocompatible molecules. Essentially, the type of surface coating and process used in stabilizing NPs during synthesis determine their surface charges, solubility and/or hydrophobicity (Abou El-Nour et al., 2010; Bastús et al., 2014; Evanoff and Chumanov, 2005; Shenashen et al., 2014). This in turn, influences the behaviour and environmental fate of NPs, as well as their effects on organisms (Chanana and Liz-Marzán Luis, 2012; Kreuter, 2004; Roohani-Esfahani et al., 2010; Shoults-Wilson et al., 2011a). Considering the barrage of nano-based products entering the global market annually and the necessary regulatory requirements for assessing the health and environmental risks of these engineered NPs, studies elucidating the synthesis, fate and outcome of NPs exposures are essential and are increasing (Baalousha et al., 2016; Izak-Nau et al., 2015; Reidy et al., 2013; Sharma et al., 2015; Topuz and van Gestel, 2015; Yu et al., 2013).

Currently, silver nanoparticles (AgNPs) are among the nanomaterials most frequently used in products on the European market (Vance et al., 2015) owing to the well demonstrated antimicrobial properties of silver (Franci et al., 2015; Hwang et al., 2012; Lara et al., 2010; Sondi and Salopek-Sondi, 2004). In recent times, our understanding of the fate and effect of various NPs has been improved from investigations utilizing both *in vivo* and *in vitro* models (Foldbjerg et al., 2012; van der Ploeg et al., 2014b; Yu et al., 2013). In addition to the exposure matrix-associated factors, the importance of physicochemical properties of the NPs including size and size dispersion (both mono- and polydispersity), shape, zeta potential, surface coating (charge) and agglomeration and dissolution rates (Reidy et al., 2013) in influencing their fate and toxicity has been highlighted. However, available information on this issue varies widely and is often inconsistent (Makama et al., 2015; Yu et al., 2013). Some studies have implicated size (Powers et al., 2011), charge (Suresh et al., 2012), or surface coating and dissolved ions (Tan et al., 2012; Yang et al., 2012) to be of eminent importance. In another study however, no significant impact of the influence of AgNPs surface coating (PVP or oleate) on toxicity to *Eisenia fetida* was observed (Shoults-Wilson et al., 2011a). Also, the debate on the involvement of particulate Ag in the toxicity of AgNPs has remained. With the development of techniques that can characterize NPs in biological matrices (Makama et al., 2015; Peters et al., 2014; van der Zande et al., 2012), it has become more evident that both particulate and ionic Ag are involved.

Certainly, a better understanding of the properties that influence both fate and effects of AgNPs in organisms will facilitate appropriate risk assessment, which in turn will assist the regulation of nanomaterials. This is especially applicable for soil organisms where available data are limited. In a previous study investigating the effect of AgNPs (NM-300 K) on *Lumbricus rubellus* populations during a 28-day exposure experiment, reproduction was especially impaired with number of cocoons laid dropping to 18% (van der Ploeg et al., 2014a). van der Ploeg et al. (2014a) also exposed coelomocytes from *L. rubellus* to the AgNPs (NM-300 K), resulting in reduced cell viability of these immune cells. In a recent study (Makama et al. *submitted*), we investigated the influence of size (20, 35 and 50 nm) and surface coating (BSA, chitosan and PVP) of AgNPs on toxicity to mammalian macrophages and found that reduced overall viability was observed to a similar extent irrespective of AgNPs coating type or size. On specific mechanisms of toxicity (TNF-α and ROS) however, we found that the AgNPs differed significantly. Also, negatively charged BSA-coated AgNPs were the most potent in inducing cellular effects. To validate these *in vitro* observations, we used an *in vivo* model in this present study. Here, we systematically investigated the influence of physicochemical properties of AgNPs on their uptake in and toxicity to a model soil organism common in Europe, the red earthworm *L. rubellus*. To achieve this, AgNPs were synthesized that differed in size and surface charge, two important properties influencing uptake and effects of engineered NPs (Reidy et al., 2013). The outcome of the current study will provide a valuable insight into how AgNP properties determine their fate and effects in soil organisms.

2. Materials and methods

2.1. Experimental design

During a 28-day exposure period, earthworms at a density of 5 individuals per experimental unit and in triplicates (n = 3), were exposed to the different AgNPs at nominal exposure concentrations of 0, 15.6, 31.3, 62.5, 125 and 250 mg Ag/kg soil dry weight (DW). To compare the effects of the AgNPs to those of ionic silver (Ag^{+}), two concentrations of $AgNO_3$ solution (1.5 and 15 mg Ag/kg soil DW) were also included. Soil for the control groups were spiked with only the dispersing and moisturizing media, without AgNPs nor $AgNO_3$. Upon termination of exposure, whole earthworms were collected and their tissues analysed for both ionic and particulate Ag content. Additionally, population dynamic parameters like cocoon production, mortality (survival) and growth rates were assessed. The AgNPs used in this study were synthesized at the Catalonia Institute of Nanoscience and Nanotechnology (ICN2), Barcelona, Spain by methods earlier reported (Bastús et al., 2014) with modifications (Makama et al., *submitted*), necessitating only a brief description here.

Results were processed with Microsoft Excel (2013), and data are presented as mean ± standard deviations. Where appropriate, the experimental data generated were subjected to one-way analysis of variance (ANOVA) with the aid of GraphPad Prism 5.04 for Windows (GraphPad Software, San Diego California USA, www.graphpad.com), and logistic regression was done using GenStat 17th ed. (17.1.0.14713; VSN International, Hemel Hempstead, UK, GenStat.co.uk). A p value of <0.05 is considered to be significant.

2.2. Reagents and instruments

Chemicals, enzymes and reagents were of analytical grade. All glassware used in this study were first acid-washed by soaking in a 21% HNO_3 solution overnight, then rinsed 3 times in milliQ water (Millipore, resistivity 18.2 MΩ/cm) and allowed to dry under a fume hood. Unless where otherwise stated, all chemicals, enzymes and reagents were purchased from Sigma-Aldrich® (Zwijndrecht, The Netherlands).

2.3. AgNPs synthesis and pre-exposure characterization

The details of the synthesis and characterization of these AgNPs have been provided previously (Makama et al. *submitted*), with additional information included in the online Supporting Information (SI) accompanying this manuscript. Colloidal, dispersed AgNPs of three different sizes (20, 35 and 50 nm) were prepared separately, following a kinetically controlled seeded-growth method previously reported (Bastús et al., 2014) with slight modifications (Makama et al. *submitted*). The approach is based on the reduction of silver nitrate ($AgNO_3$) at 100 °C by tannic acid (TA) and trisodium citrate hexahydrate (SC). In order to generate negative, positive and neutral NPs, the AgNPs were subsequently surface-coated with bovine serum albumin (AgNP_BSA), chitosan (AgNP_Chit) or polyvinylpyrrolidone (AgNP_PVP), respectively.

All nine AgNPs were characterized in re-suspension media (i. e. soil extract), moisturizing media (milliQ water), or both, using a combination of different techniques in order to enhance a more

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