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Geno- and Ecotoxicity Evaluation of Silver Nanoparticles in Freshwater Crustacean *Daphnia magna*

Sun-young Park, Jinhee Choi†

University of Seoul/Faculty of Environmental engineering, College of University of Seoul, Jeonnong-dong 2-ga, Dongdaemun-gu, Seoul 130-743, Korea

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Abstract

Genotoxic- and ecotoxic assessments of silver nanoparticles (AgNPs) were conducted on the freshwater crustacean *Daphnia magna*. AgNPs may have genotoxic effects on *D. magna*, given that the DNA strand breaks increased when exposed to this nanoparticle. Increased mortality was concomitantly observed with DNA damage in the AgNPs-exposed *D. magna*, which suggests AgNPs-induced DNA damage might provoke higher-level consequences. The results of the comparative toxicities of AgNPs and Ag ions suggest that AgNPs are slightly more toxic than Ag ions. Overall, these results suggest that AgNPs may be genotoxic toward *D. magna*, which may contribute to the knowledge relating to the aquatic toxicity of AgNPs on aquatic ecosystems, for which little data are available.

Keywords : *Daphnia magna*, DNA damage, Ecotoxicity, Genotoxicity, Silver nanoparticles

1. Introduction

Silver nanoparticles (AgNPs) have a wide range of current and potential future applications, including spectrally selective coatings for solar energy absorption, chemical catalysts, surface-enhanced Raman scattering for imaging and; in particular, antimicrobial sterilization, which has made them one of the most commonly used nanomaterials[1-5]. Widely used NPs, such as AgNPs, will most likely enter the environment, and may produce a physiological response in certain organisms, possibly altering their fitness, and might ultimately change their populations or community densities. Research and literature regarding the ecotoxicity of NPs is still emerging, and gaps still exist in our knowledge of this area.

Despite the dramatic increase in the use of such nanomaterials, little information is available on their potential harmful effects on the environment. Most current literature on the toxicity of nanoparticles; however, comes from mammalian studies that have focused on respiratory exposure, or from *in vitro* assays using mammalian cells[6-11]. Ecotoxicological studies on nanoparticles are more limited, with only a few reports on the acute toxic effects of nanoparticles on aquatic organisms[12-16]. Few ecotoxicity studies on aquatic organisms have been performed that include genotoxic endpoints. However, the presence of genotoxic and potentially carcinogenic compounds in aquatic environments is of major concern with respect to the health of aquatic media biota[17-19]. The potential genotoxic effects of emerging nanomaterials, such as AgNPs, on aquatic systems should be identified to allow for their safe use.

Genotoxic assessments of AgNPs were conducted on aquatic sentinel species, the freshwater crustacean *Daphnia magna*. The small-sized freshwater crustacean, *D. magna*, holds an important position in the aquatic food chain, respond to many pollutants, are easy to culture and have short life cycles; thus, are considered suitable species for aquatic biomonitoring[20, 21]. Conventional ecotoxicity tests were also conducted on the Daphnia systems, as they may provide insights to the potential toxic effects of AgNPs on aquatic environments. Given the importance of *D. magna* in aquatic ecosystems, information concerning the geno- and ecotoxicity of widely used nanomaterials on these species could be valuable in relation to aquatic nanoecotoxicology. To compare the toxicity of AgNPs to that of Ag ions, the toxicity of Ag ions was also investigated in *D. magna* using the same toxic endpoints as used in the AgNPs toxicity assay.

2. Experimental Methods

2.1. Organism Culture, AgNPs, and Ag Ion Preparation and Exposure to *D. magna*

Using an original strain provided by the Korea Institute of Toxicology (Daejeon, Korea), *D. magna* were obtained in our

†Corresponding author
E-mail: jinhchoi@uos.ac.kr
Tel: +82-2-2210-5622, Fax: +82-2-2244-2245

laboratory from adults reared using M4-media, as described previously[22, 23]. AgNPs (size <100 nm, Sigma-Aldrich Chemical, St. Louis, MO, USA) were homogenously dispersed in deionized water by sonication for 13 hours (Branson-5210 sonicator, Branson Inc., Danbury, CT, USA), stirring for 7 days, and filtering through a cellulose membrane (pore size 100 nm, Advantec, Toyo Toshi Kaisha, Tokyo, Japan) to remove NP aggregations. To compare the toxicities of AgNPs and Ag ions, aqueous $AgNO_3$ (AG002, Next Chimica, Centurion, Republic of South Africa), in deionized water, was used, with the final concentrations of AgNPs and $AgNO_3$ estimated using a Multitype Inductively Coupled Plasma Emission Spectrometer (ICPE-9000, Shimadzu, Tokyo, Japan). The concentrations for AgNPs and $AgNO_3$ had equivalent Ag masses. From stock solutions (4 mg/L), experimental concentrations of AgNPs and AgNO3 were prepared in M4-media. Neonates, aged less than 24 hours, were used for Comet assays and ecotoxicity tests.

2.2. Characterization of AgNPs

Energy filtering transmission electron microscopy (TEM) was used to examine the particle shape and size of the AgNPs. Twenty μL of the particle suspension were dried onto a 400 mesh carbon-coated copper grid and imaged with a LIBRA 120 TEM (Carl Zeiss, Oberkochen, Baden-Württemberg, Germany) at 80-120 kV. The size distribution of the AgNPs was evaluated using a photal dynamic light scattering (DLS) spectrometer, DLS-7000 (Otsuka Electronics Co., Inc., Osaka, Japan).

2.3. Mortality, Growth, Reproduction Assays

For the mortality test, 10 individuals, less than 24 hours, were exposed to AgNPs and Ag ions for 24 hours, with live and dead individuals then counted[23]. For the growth test, 20 individuals, less than 24 hours, were incubated with AgNPs and Ag ions for 96 hours, with the fresh weights measured immediately after exposure. The body dry weight was evaluated after drying *Daphnia* at 105°C for 24 hours. *Daphnia* reproduction tests were conducted according to the OECD guidelines[24]. Ten individuals, less than 24 hours, were exposed to various concentrations of the test chemicals, and then observed and fed daily for 21 days. Three replicates were prepared for each concentration, with jars filled with 100 mL of test media. Each jar was provided with Chlorella as the food source, at a concentration of 5×10^5 cells/mL daily. Test animals were transferred to new medium every 2 days. Neonates were removed from the jar daily, and the numbers of neonates counted.

2.4. Comet Assay

To prepare *Daphnia*, a total of 150 neonates were collected from the control and experimental tanks after 24 hours exposure to nanoparticles, and pooled for the Comet assay. Organisms were placed in 1 mL of phosphate-buffered saline (PBS), containing 20 mM ethylene diamine tetra acetic acid (EDTA) and 10% dimethyl sulfoxide (DMSO), and disintegrated mechanically by mincing. An alkaline comet assay was performed based on the method of Singh et al.[25], with adaptation for *Daphnia*, as described previously[22]. Briefly, about 50 cells per slide (3 slides per treatment) were analyzed using a fluorescence microscope (Nikon, Kanagawa, Japan), equipped with an excitation filter with a BP 546/12 nm and 590 nm barrier filter at 400× magnification. DNA damage was expressed as the olive tail moment (OTM) using an image analysis computerized method (Komet 5.5, Kinetic Imaging Limited, Nottingham, UK).

2.5. Data Analysis

The genotoxic- and ecotoxic assays results were tested for significance using an analysis of variance (ANOVA) test, employing the Dunnett's multiple comparison test. All statistical tests were performed using SPSS version 12.0 (SPSS Inc., Chicago, IL, USA).

3. Results and Discussion

The AgNPs used for the toxicity assays were characterized using TEM and DLS methods (Fig. 1). The TEM provided information on the size and shape of the nanoparticles, and showed sizes mainly <50 nm (Fig. 1a); however, it could not provide information on whether the nanoparticles existed in single or aggregated forms in the test medium, as the nanoparticles form

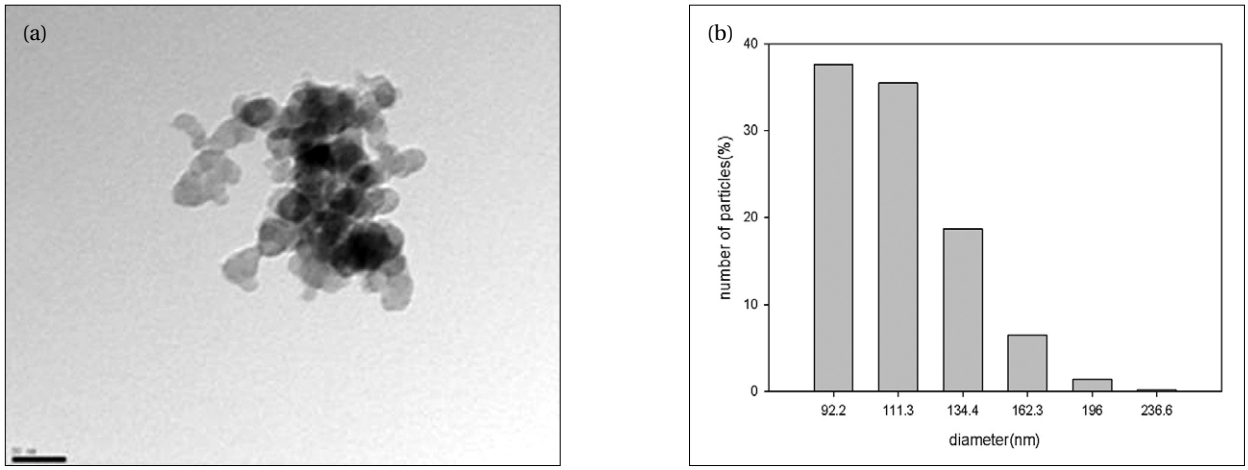

Fig. 1. Images of AgNPs in the test media using transmission electron microscopy (a) and dynamic light scattering spectrometers (b).

Table 1. Acute toxicity of silver nano particles estimated in *Daphnia magna*

Concentration (μg/L)	**0**	**0.1**	**1**	**2**	**4**	**8**	**50**
Mortality (%)	0±0	5±5.0	20±0	100±0	100±0	100±0	100±0

Twenty-four hours exposure to various concentrations, number=2, mean±SE.

aggregates when dried on the microscopic observation slide. The results of the DLS suggested that the AgNPs did not exist as single particles, but tended to aggregate in the test medium, as the main nanoparticle sizes distributed in the test medium were about 100 nm (Fig. 1b). In relation to nanotoxicity, it is often expected that the smaller the size, the stronger the toxicity exerted[26]. However, the relationship between the physico-chemical properties of nanoparticle and their toxicities seems to be much more complicated than just being related to their size and surface area (i.e. shape, charge, concentration, etc.); there is still much on going debate[8, 27, 28]. Many studies have failed to show any clear relationship between toxicity and the size of nanoparticles [8, 29]. The TEM images of the nanoparticles from the test medium showed the size of the nanoparticles tested. However, the line of evidence provided from the present study is rather limited; therefore, to identify key properties of nanoparticles with respect to causing ecotoxicity, toxic responses of a broad range of physico-chemical properties to various classes of nanoparticles may be investigated in various environmental relevant species.

To find the range of sublethal concentrations for geno-and ecotoxicity tests, an acute toxicity test was performed on *D. magna* exposed to AgNPs, using mortality as an endpoint (Table 1). A broad range of AgNPs concentrations was tested (data not shown from 1 to 4 mg/L). Beyond exposure to 2 μg/L of AgNPs, complete mortality was observed. A steep concentration-response relationship was observed (i.e. 0% mortality at 1 μg/L and 100% mortality at 2 μg/L of AgNPs exposure). Based on the acute toxicity test, 0.5, 1, and 1.5 μg/L of AgNPs were selected as concentrations for geno- and ecotoxicity tests. To compare the toxicity of AgNPs to that of Ag ions, the toxicity of Ag ions was also investigated in *D. magna* using the same concentrations and toxic endpoints as used in the AgNPs toxicity assay.

DNA damage, particularly DNA strand breaks, was measured using the Comet assay to evaluate whether AgNPs induced any genetic toxicity in *D. magna* (Table 2). AgNPs and Ag ions may exert genotoxic effects on *D. magna*, given that DNA strand breaks (OTMs) increased in *D. magna* exposed to AgNPs and Ag ions. A statistically significant increase in OTMs was observed in *D. magna* exposed to 1 and 1.5 μg/L of AgNPs and Ag ions. However, the degree of increase in OTM was more consequential in AgNPs exposed *D. magna* than in those exposed to Ag ions. Moreover, exposure concentration dependant DNA damage was observed in AgNPs exposed *D. magna*. Even though genotoxicity tests with the Comet assay are widely used in aquatic environmental monitoring, most Comet assays have been performed on *in vitro* systems of aquatic species, mostly using fish-driven cell lines[19, 30]. The measurement of genotoxic effects of emerging nanomaterials, using *in vivo* genotoxicity biomarker in aquatic invertebrates, could be a useful tool for monitoring aquatic toxicity due to nanoparticles. AgNPs may influence the genetic constitution of populations by directly damaging DNA molecules within the individual cell nucleus, but the ecological relevance of changes in single cells within some tissues of certain individual organisms is extremely difficult to assess.

Therefore, conventional ecotoxicity tests, using mortality, growth and reproduction as endpoints, were subsequently conducted to validate the ecotoxicological relevance of the response of DNA to damage in *D. magna* exposed to AgNPs and Ag ions (Table 2). The response of *Daphnia* to AgNPs in terms of their mortality, growth and reproduction may explain the higher biological-level consequences of the observed DNA damage. Aquatic toxicity tests may provide insights to the relative sensitivity of *D. magna* to AgNPs, which may also provide information on the impact of nanoparticles on water systems, as these species hold important positions in aquatic ecosystems[24, 31-33]. A significant increase in mortality was observed in *D. magna* exposed to 1.5 μg/L of AgNPs; whereas, no significant

Table 2. Growth, reproduction parameters investigated in *Daphnia magna* exposed to silver nanoparticles and $AgNO_3$, and DNA damage (as OTM obtained by comet assay) measured in *D. magna* exposed to silver nanoparticles and $AgNO_3$

	Concentrations	**DNA damage**	**Growth**		**Reproduction**
	(μg/L)	OTM (control=1) weight (mg/individual)	Body fresh (mg/individual)	Ash free weight	No. of neonates born
Control	0	1.000±0.214	0.301±0.010	0.0048±0.000	52.52±3.44
AgNPs	0.5	2.488±0.914	0.335±0.017	0.0055±0.000	49.91±1.82
	1	4.411±1.431	0.310±0.025	0.0053±0.000	49.20±6.07
	1.5	7.833±2.774a	0.284±0.016	0.0045±0.000	48.58±1.04
$AgNO_3$	0.5	1.866±0.387	0.409±0.054	0.0068±0.000	44.14±1.65
	1	3.009±1.209 a	0.301±0.128	0.0061±0.000	41.87±2.85
	1.5	2.543±0.501	0.512±0.027	0.0065±0.000	40.92±4.35

The results were expressed as OTM obtained using the Comet assay (number=3, mean±SE, $^a p<0.05$).
OTM: olive tail moment.

alteration was observed in growth and reproduction. It seemed Ag ion exposure leads to a slight increase in mortality, but decrease in reproduction potential; however, those alterations were not statistically significant. An increase in DNA strand breaks occurred concomitantly with an increase in mortality in *D. magna* exposed to 1.5 μg/L of AgNPs, which suggests DNA alteration induced by AgNPs might provoke higher level consequences. As mortality is the most obvious sign of progression of serious toxicity at the organism level, the impairment of survival due to AgNPs exposure may be considered a consequence of a serious progression of sub-organism level toxicities, such as the increased DNA damage in *Daphnia*. The relationships between the responses of the genotoxic biomarker and the physiological /individual/population effects are complicated due to the compensatory mechanisms regulating the physiological/ individual fitness and population dynamics in a natural system. As the mere presence of genotoxic compounds, which are potentially carcinogenic, is of major concern in human and ecosystem health, the sensitive and rapid detection of the genotoxic properties of aquatic systems themselves is considered important, although does not necessarily include alteration at a higher level of biological organization. Especially for the nanomaterials concerned, despite the dramatic increase in the use of nanomaterials and; hence, their ubiquitous distribution in aquatic environments, little information is available on their potential genotoxicity on aquatic organisms. Considering the potential of *D. magna* as a bioindicator species, and the importance of the genotoxicity of nanoparticles in ecotoxicity monitoring, the measurement of the DNA damage in these species after exposure to nanoparticles could provide useful information for freshwater monitoring. There have been discussions regarding the comparative toxicity of AgNPs and Ag ions[34, 35], with the latter's bactericidal action having been studied previously[36, 37]. Our previous ecotoxicity study using *Caenorhabditis elegans*, comparing the toxicity of AgNPs and Ag ions, suggested that AgNPs were slightly more toxic than Ag ions in terms of their effect on reproduction potential, and it also appeared that different mechanisms exerted the toxicity of AgNPs and with Ag ions[38]. Results of the geno- and ecotoxicities (Table 2) in *D. magna* exposed to AgNPs and Ag ions also suggest that AgNPs are slightly more toxic than Ag ions. However, as it appeared that the biocidal effects of AgNPs might be partially due to Ag ion generation, further studies on this aspect of toxicity are required.

In this study, the geno- and ecotoxicities of AgNPs on *D. magna* were evaluated. The results suggested that AgNPs may have genotoxic potential toward *Daphnia*, and AgNPs-induced DNA damage might provoke higher-level consequences, which could comprise a contribution to the knowledge on the aquatic toxicity of AgNPs on aquatic ecosystems, for which little data are available. However, further studies on the mechanism behind AgNPs-induced DNA damage and mortality are needed to better explain the ecotoxicity of AgNPs in *D. magna*.

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