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Effect of agglomeration of silver nanoparticle on nanotoxicity depression

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Abstract−Silver nanoparticles (AgNPs) are used commercially in a variety of applications, including textiles, cosmetics, spray cleaning agents, and metal products. AgNP itself, however, is classified as an environmental hazard by Environmental Protection Agency (EPA, USA) Nanotechnology White Paper, due to its toxic, persistent and bioaccumulative characteristics when exposed to the environment. We investigated the cumulative mortality and abnormalities in Japanese medaka (*Oryziaslatipes*) embryos after exposure to AgNPs. Free AgNPs in solution have a high activity with respect to biological interactions regarding blocking blood flow and distribution of AgNPs into the cells from head to tail of hatched *O. latipes*. Interestingly, the agglomeration of AgNPs (loss of nanosized characteristics) played an important role in the environmental toxicity. The present study demonstrated that when the AgNPs were exposed in the ecosystem and then formed agglomerates, nanotoxicity was reduced.

Key words: Nanomaterials, Silver Nanoparticles, Nano-hazard, Bioaccumulation, Agglomeration

INTRODUCTION

A number of studies on the possible hazards of nanomaterials have recently appeared, because the novel size-dependent properties of nanomaterials have the potential to impact humans and the ecosystem [1]. It has been reported that engineered nanomaterials can cause various effects in systems ranging from human cells to bacteria, which include oxidative stress [2], catalysis of cell necrosis [3], or protein aggregation [4]. Actually, cases have already reported in which nanomaterials have demonstrated significant effects on humans and the environment [5].

It is important to understand the fate and behavior of nanoparticles for understanding effects on ecological systems (or receptors). When engineered nanomaterials, containing chemically modified surfaces to promote stabilization, were exposed in the environment, specific behaviors such as agglomeration, aggregation, sedimentation, or adsorption were observed [6,7]. Nanoparticles readily agglomerate or aggregate, *via* the effect of Ostwald ripening in the aqueous phase or via electrostatic force in the gas phase, and consequently lose their 'nano' properties. This failure to naturally sustain such properties has made it difficult to test the toxicity of nanomaterials in organisms [8-10]. However, considering studies on environmental exposure, fewer studies of the relation between agglomeration and nanoparticle toxicity in the environment have been reported.

The goal of the present study was to examine the relationship between the agglomeration of nanoparticles (specifically, AgNPs) and their toxicity. Bare AgNPs, without a chemical stabilizer to sustain nano-size suspension, were used to induce agglomeration in an aqueous phase and exclude adverse effects contributed by chemical additives. The toxicity of the AgNPs was tested using Japanese medaka (*Oryziaslatipes*) embryos. The role of released metal ions in AgNPs toxicity needs to be taken into account in an AgNPs toxicological study. We selected the percentage of silver in form of Ag^+ present in suspended AgNP, which Ag^+ ratio was maintained to be stable for several days, as the toxic metric. Different impacts on the development of *O. latipes* embryos (accumulative mortality and abnormality) were selected as toxicological endpoints such as mortality, heart rate, histopathology, abnormality.

EXPERIMENTAL

1. Chemicals

For bare AgNPs, silver powder (Sigma-Aldrich) was purchased and suspended in Milli Q water through physical processes, such as an ultra-sonication, stirring, and nano-filtration (100 nm isopore, Adventech). Details of preparation and characterization of bare AgNPs are shown in our previous report [11]. The prepared AgNPs suspensions contained about 50% Ag^+ ions. The amount of Ag^+ ions was adjusted by a potentiostatic method (below 50% Ag^+), operated at −0.3 mV and by adding $AgNO_3$ solution (above 50% Ag^+). We maintained ionic ratios (2.1, 8, 15, 24, 41, 50, 76, and 100% Ag^+ to total Ag) at constant levels over time (during 10 days). The agglomeration of AgNPs was observed by a dynamic light scattering method (DLS; Otsuka, ELS-8000). The present study focused on relationships between agglomeration and different ionic ratios with the similar size distribution of suspended AgNPs. The AgNPs suspension was mixed with dechlorinated tap water (test medium) and the AgNPs then underwent agglomeration within 10 minutes.

2. Embryo Exposure

The evaluation of toxicity was based on several fish exposures: early-life-stage toxicity test [12], short-term toxicity on fish embryos and sac-fry stages [13], and fish embryo toxicity [14]. The eggs of Japanese medaka were collected from females before the early blastula

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stage. Each egg was exposed to an AgNPs suspension in a 96-well microtiter plate. The eggs were incubated for 16 h and subjected to a 16 : 8 h (light:dark) photoperiod at an illumination of 500 lux and 25±1 °C.

Developmental abnormality of *O. latipes* was determined at 25 mg/L AgNPs varying Ag^+ ratio (5, 10, 20, 45, 75, and 100%). The toxicity test was performed while the sac-fry were still being nourished from the yolk-sac. Mortality was evaluated in fish embryos exposed to i) the concentration of AgNPs (50, 100, and 150 mg/L) and ii) Ag^+ ratio (2.9, 5, 14, 23, 40, 52, 76, and 100%) at 25 μg/L of silver nanoparticle. Twelve individual embryos were treated per exposure concentration, with three independent replicates of a microtiter plate. The protocol was semi-static and the media was changed every 48 hrs. The percentage of surviving eggs was calculated using Abbott's formula, which was corrected for the mortality in the controls. Heart rate of embryos was determined at 3, 5, and 7 day. During embryo exposure, the number of surviving eggs was then counted and any morphological changes were determined from images taken with a digital camera (Olympus, E1) at 48, 72, and 120 h. After exposure, the morphological changes of embryo egg (membrane) and hatched sac fry (tissue) were investigated by transmission electron microscopy (TEM, JEM 1010, JEOL). For this purpose, primary fixation and post fixation for the *O. latipes* (egg membrane and tissue of hatched sac fry) were performed with 2% glutaraldehyde and 1% osmium tetroxide, respectively. The *O. latipes* were then stained with 0.5% uranyl acetate and dehydrated sequentially with 30, 50, 70, 80, 90, and 100% ethyl alcohol. The *O. latipes* were infiltrated and polymerized with Spurr's resin. The polymerized the *O. latipes* was then sectioned with an ultramicrotome (MT-X, RMC).

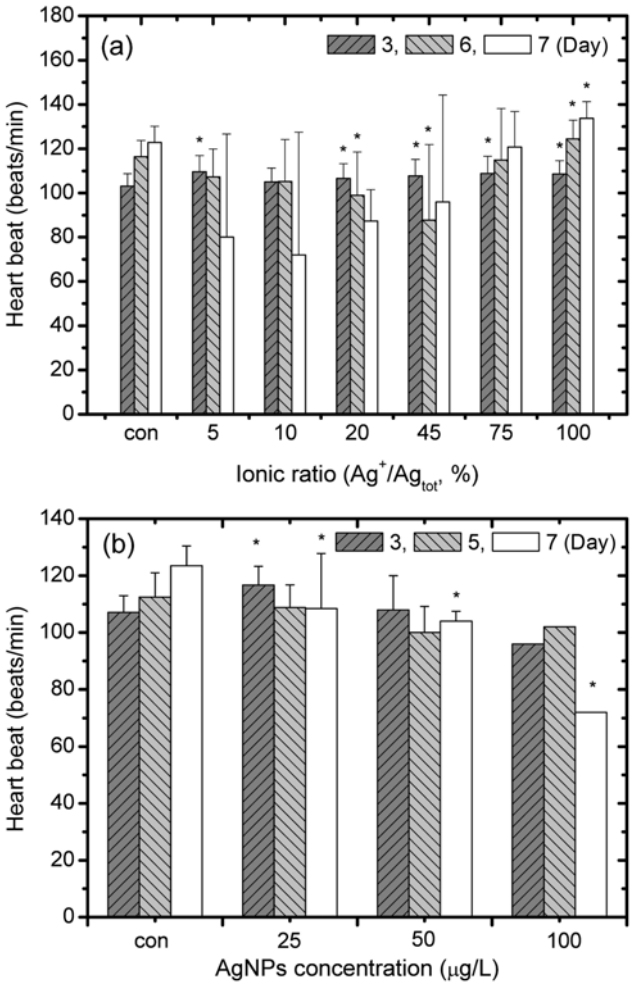

Fig. 1. Abnormality (heart rate) of embryo depending on (a) concentration of the AgNPs (at 45% ionic ratio) and (b) ionic ratio (at 25 μg/L total AgNPs) (con=control).

RESULTS AND DISCUSSION

The heart rate of embryos (control) increased normally with development time. The heart rate of the treated embryos decreased with time in the range of 5-45% ion ratio (Fig. 1(a)); at >45% ion ratio, it appeared to increase similar to controls. At 45% ionic ratio, the heart rate of the treated embryos was dependent on the AgNPs concentration (Fig. 1(b)). Importantly, the heart rate (at 7 days) decreased with the AgNPs concentration (25-100 mg/L) and reached an average of 72 heart beats/min (100 mg/L) as compared to an average of 125 heart beat/min (control).

O. latipes embryos treated with the AgNPs (45% ion ratio) had internal bleeding (Fig. 2; 1a), developmental arrest (Fig. 2; 1b, 2a, 2b), precipitation (Fig. 2; 2a, 3b), curved spine of kyphosis (Fig. 2; 3b), and static circulation (Fig. 2; 3a), which were commonly observed. Otherwise, other abnormalities such as malformation of tail and head, pericardial edema, and optic size were not observed.

The *O. latipes* chorion was observed to consist of three layers: an ultrathin layer (UL, outermost layer, ≈200 nm thick), an outer layer (OL, 2 μm thick), and an inner layer (IL, 15-20 μm thick) (Fig. 3). The AgNPs were able to readily attach to the surface of the ul-

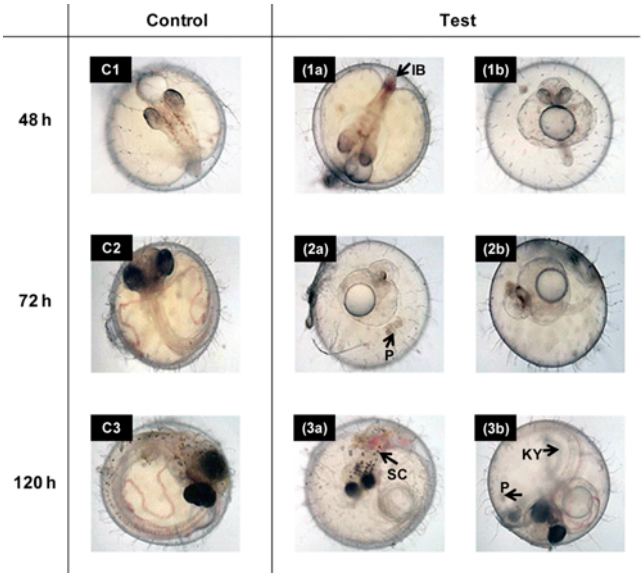

Fig. 2. Images of *Oryziaslatipes* embryos exposed to an AgNP suspension with exposure times of 48 h (Fig. 2; C1, 1a, 1b), 72 h (Fig. 2; C2, 2a, 2b), and 120 h (Fig. 2; C3, 3a, 3b). The embryos showed various abnormalities such as internal bleeding (IB; Fig. 2; 1a), developmental arrest (Fig. 2; 1b, 2a, 2b), precipitation (P; Fig. 2; 2a, 3b), static circulation (SC; Fig. 2; 3a), or curved spine of kyphosis (KY; Fig. 2; 3b) (Fig. 2; C1-C3: control; the eggs size: 0.8-1.0 mm).

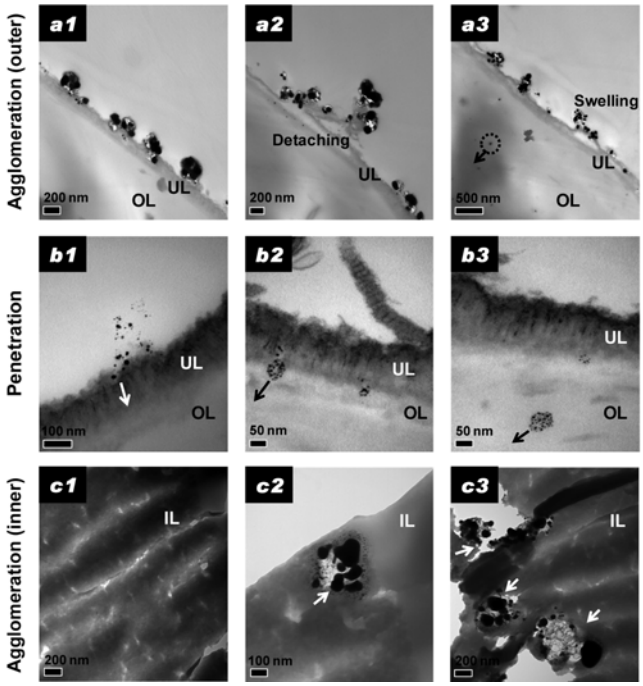

Fig. 3. TEM images of the egg membrane of *Oryziaslatipes*: an ultrathin layer (UL, outermost layer, 200 nm thick), outer layer (OL, 2 μm thick), and inner layer (IL, 15-20 μm thick, 9-10 lamellae). The AgNPs were adsorbed to the surface of the UL (Fig. 3; a1) and some caused the pore plug (PP) material to be detached (Fig. 3; a2) or swollen (Fig. 3; a3). Some AgNPs were adsorbed to the surface (Fig. 3; b1). Otherwise, some were easily absorbed into the UL (Fig. 3; b2), and penetrated the OL (Fig. 3; b3). The normal IL has a lamellar layered structure (Fig. 3; c1). The AgNPs became agglomerated (Fig. 3; c2, c3) and the lamellar layers were broken down (Fig. 3; c3).

trathin layer (UL) of the membrane (Fig. 3; a1-a3). Some of the AgNPs became agglomerated on the surface (Fig. 3; a1) and caused the pore-plugging material to swell and detach from the UL structure (Fig. 3; a2-a3). However, the very small dimensions of the AgNPs allowed them to easily penetrate the UL (Fig. 3; b1). Once agglomerated in the OL (Fig. 3; b2), transport towards the IL (Fig. 3; b3) was evident. In the IL, called lamellar layer (9-10 lamellae) (Fig. 3; c1), the AgNPs became aggregated (Fig. 3; c2) and potentially caused destruction of the lamellar layer (Fig. 3; c3).

Hatched *O. latipes* contained the AgNPs in their body from head to tail (Fig. 4), even though it was exposed in the development step of embryo. The AgNPs were distributed in the cytoplasm (Fig. 4; a4-a5) near the gill, tail muscle (Fig. 4; c2), blood vessel (Fig. 4; c4), dermal layer (Fig. 4; a1) and epithelium layer (Fig. 4; a2, b2-b4, c3). The magnification of TEM images showed that the AgNPs (30-150 nm) were agglomerated or aggregated (Fig. 4; a1-a3, b1-b4, c1-c2) or deposited as a single particle (Fig. e4; a4-a5, b5, c3-c5).

The AgNPs were suspended in Milli-Q water, in the form of spherical nanoparticles (not shown here), which have a dominant Ag(111) crystal face [11]. The average size of the AgNPs was determined to be approximately 63 nm by TEM analysis. The AgNPs are known to function as a non-corrosive metal in the environment, but they still have the potential to release ions.

The initial agglomerations varied with Ag^+ ionic ratio (Fig. 5). The agglomeration rate constants [11] increased from 4.6×10^{-20} to 1.1×10^{-19} m^2/s in the range of 8-50% ion ratio (Fig. 5). However, at higher ion ratios (50-95%), the agglomeration rate constants decreased slightly. The higher ionic sample was relatively preferred for agglomeration than the lower ionic sample in tested medium.

The cumulative mortality by AgNPs exposure level (50-150 mg/L) with ion ratio (3-100%) was measured by exposure to *O. latipes* embryos during a 7 day period (Fig. 5). The cumulative mortality of the *O. latipes* embryos was dependent on the AgNPs dosage and exposure time. A solution of Ag containing 23% of Ag^+ and 77% of AgNPs had the most severe effect on the ecological system. This may be because Ag^+ and the AgNPs play a complementary role to each other in the toxicity mechanism (Fig. 5). The cumulative mortality increased with changes in the range of ion ratios of 3-23%. However, interestingly, the toxic effect on *O. latipes* embryos at higher ion ratios (40-100%) was lower than that for a ratio of 23% and decreased with increasing ion ratio.

An AgNPs suspension containing 45% Ag^+ ions was observed for their abnormal effects on chorion, in an attempt to determine how the AgNPs specifically interact with *O. latipes*. The chorion of the egg functions to block contaminants from outside the egg. The findings indicate that AgNPs penetrate into the egg with great difficulty, unlike fish, which readily take up nanoparticles (even though their agglomerate) through the gills. AgNPs agglomerates were attached on the adhesive surface of chorion (UL). Unagglomerated AgNPs invaded the UL of chorion. These AgNPs were easily found in the outer layer (OL). The pore canals play an important role in transferring nutrients from the outer environment and are also attacked by viruses and toxic contaminants. Because the pore canal of medaka are filled with the ZI-1, 2 and ZI-3 proteins and plugged with osmophilic material from the environment after fertilization, most AgNPs are internalized through the process of agglomeration/aggregation of the AgNPs in the lamella (IL), rather than through the canal pores.

Embryo exposures to AgNPs showed distribution of agglomerated or single particle through the body (mainly epithelial and dermal layer) of hatched *O. latipes* (Fig. 2). During early embryonic stages, the AgNPs might enter the cells through the skin. The wide distribution from head to tail likely resulted from translocation of the internalized AgNPs. Nanoparticles were shown to selectively translocate into the brain via the olfactory bulb in fish, suggesting an ability to bypass some of the mechanical barriers. It was noteworthy that malfunctions related with blood flow such as internal bleeding, static circulation, and decrease of heart rate with exposure time were observed in the development steps of embryos (Fig. 2). Otherwise, the development of the embryos was unaffected by Ag^+ ion treatment. Results point out the low suspension stability of bare AgNPs. If AgNPs surface are functionalized, the results may be quite different. In the present study, the AgNPs were not stabilized with any chemicals and their toxicity was depressed by agglomeration. These reasons might explain differences between embryo abnormalities observed in this study (edama in heart/yolk sac/heart, eye abnormality, or tail flexture) from those of other researchers.

However, the toxicity of AgNPs did not increase linearly with ion ratio, but reached a maximum at an ion ratio of 23%; otherwise

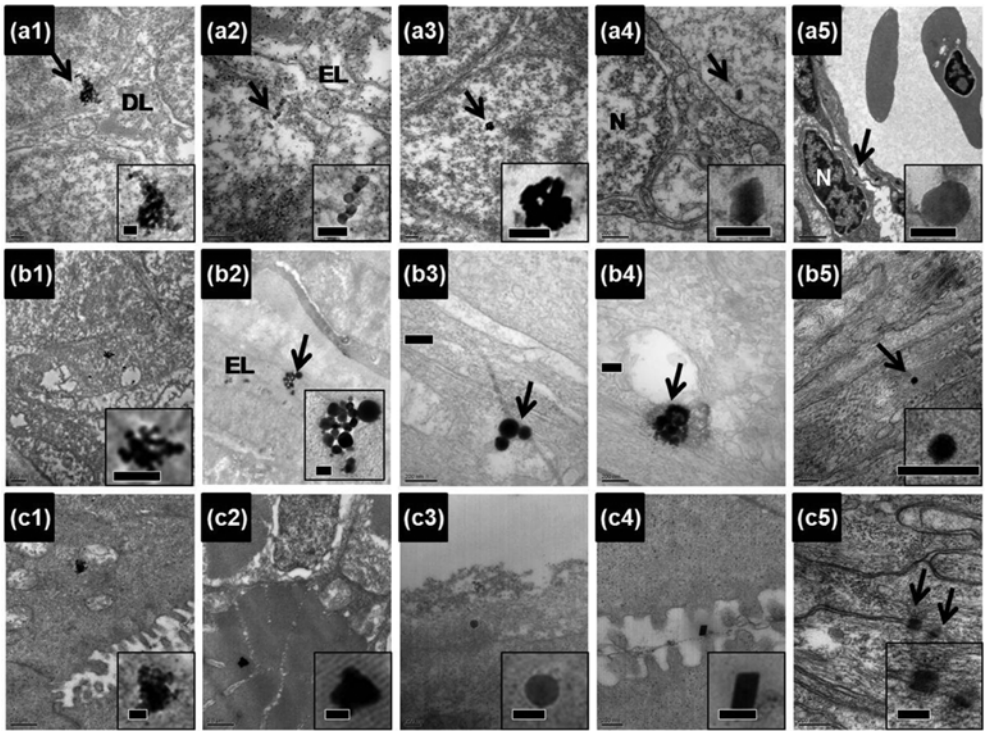

Fig. 4. TEM images of Sac fry tissue after the AgNPs (25 μg/L) exposure in the step of embryo development; (a) head; (b) main body; (c) tail part: nucleus (N), epithelium layer (EL), dermis layer (DL) (bar scale: 100 nm).

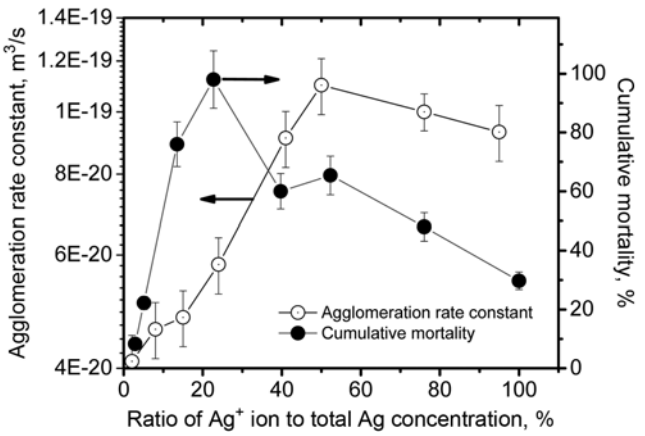

Fig. 5. Agglomeration rate constants and cumulative mortality as a function of ionic ratio. The AgNPs with Ag^+ ions agglomerated readily with salt in dechlorinated tap water and this agglomeration involved a decrease in the cumulative mortality of *O. latipes*.

the agglomeration rate reached a maximum at an Ag^+ ion ratio of 50%, as shown in Fig. 5. Importantly, the difference between these maximization conditions clearly shows that it is possible to reduce the toxicity of AgNPs by creating a high agglomeration rate constant (more than 1×10^{-19} m^3/s). We refer to this phenomenon as 'toxicity depression by agglomeration' of nanoparticles. This was because the agglomeration of AgNPs would cause a decrease of diffusion and penetration into the egg membrane. The toxicity depression was mainly dependent on the agglomeration rate, while the effect of the size of the agglomerate on the toxicity depression was relatively small.

Some studies have reported that there is no difference in toxicity between nanoparticles and bulk particles, because they form similar sized aggregates [15,16]. While, the toxicity depression found in the present study was observed in a mixture of bare AgNPs (including Ag^+ ion coexisting status) and solutions containing salt (e.g., tap water). When nanoparticles are exposed in the ecosystem and then form agglomerates, nanotoxicity can be reduced. The present study shows that this is possible. This result is consistent with agglomeration phenomena discussed in nanotoxicological studies [17].

CONCLUSION

AgNP suspensions were undergone to accelerate agglomeration in an aqueous phase. Both the agglomeration rate and toxicity of AgNPs solution were related to the Ag^+ ionic ratio in the solution. Ag^+ ions not only increase the toxicity, but also accelerate suspension instability of AgNPs. Despite the toxicity depression by agglomeration, the safety of AgNPs should be considered with care. The AgNPs were able to penetrate through the chorion and distribute widely in the body structures of hatched *O. latipes*. Thus, the agglomeration phenomena of AgNPs suspensions provide important information on both toxicity depression and the actual toxicity mechanism.

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