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Original Article

Does physical production of nanoparticles reduce their ecotoxicity? A case of lower toxicity of AgNPs produced by laser ablation to zebrafish (*Danio rerio*)

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Abstract: Use of nano-materials has increased in various aspects of human life. However, possible outbreak of nano-materials toxicity in humans and other organisms is one of the future challenges. Different chemical precursors which are used in chemical approaches for production of nano-materials may have secondary and sometimes toxic effects in living organisms. These secondary effects may be reduced in physical approaches due to not use of chemicals. To test this hypothesis, acute toxic effects of two types of silver nanoparticles (AgNPs) which were produced by physical (top-down) and chemical (bottom-up) methods on survival rate of Zebrafish (*Danio rerio*) were compared. According to the results, AgNPs produced by physical method were 38 times less toxic than ones generated by chemical method and therefore, the hypothesis was approved. The estimated 96 hr LC_{50} values of AgNPs produced by physical and chemical methods for zebrafish were 0.540 ± 0.032 and 0.014 ± 0.001 mg/L, respectively. According to these values and regarding the rules of European Union, both types of AgNPs are considered as highly toxic chemicals to aquatic organisms. Generally, AgNPs seems to have toxic effects on aquatic organisms regardless of the method used for their production, and so, their accidental or intentional entrance into the aquatic ecosystems should be inhibited.

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Introduction

Nanotechnology is a highly promising technology that has extensive applications in many areas of science and industry. A nano-material is a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or agglomerate and where, 50% or more of the particles in the number size distribution and one or more external dimensions is in the size range of 1-100 nm (European commission, 2011). In specific cases and where warranted by concerns for the environment, health, safety or competitiveness, the number size distribution threshold of 50% may be replaced by a threshold between 1 and 50%.

The use of nano-materials is growing and number of nano-products has increased from 54 in 2005 to 1628 in 2013 (Woodrow Wilson Database, 2014). The silver, titanium, carbon, silicon, zinc and gold are respectively the more frequent nano-materials used in consumer products (Woodrow Wilson Database, 2014). With rapid development of nanotechnology and the extension of nano-material applications in human life, concerns are rising on probable threats caused by releasing them to environment. As aquatic ecosystems are one of the final destinations of the man-made chemicals, the study of these materials effects on the aquatic organisms (aquatic toxicology) is of great importance (Hosseini et al., 2013;

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Mansouri Chorehi et al., 2013; Roy et al., 2013; Banaee et al., 2014; Ghelichpour, 2014). In this respect, "Aquatic nanotoxicology" can be considered as a suitable indicator for prediction of possible impacts of nano-materials release into aquatic ecosystems. Hence, this study was conducted to evaluate the toxicity of silver nanoparticles (AgNPs) on adult zebrafish (*Danio rerio*). This species is a valuable model for investigating the impacts of chemical contaminants (Hill et al., 2005) and suitable vertebrate for nanotoxicology studies and the prediction of safety and hazard level of nano-materials on human is much simpler and less expensive compared with rodents (Kim and Tanguay, 2013).

Nano-materials can be manufactured by bottom-up and top-down approaches. In bottom-up approaches, molecules are joined together during special processes to generate larger structures with nano dimensions, whereas in top-down approaches, large dimension materials are changed into nano dimension structures by physical methods (Rodgers, 2006). In top-down approach, a large volume of silver metal is first ablated by mechanical method and then the manufactured AgNPs are fixed by adding colloids protectants (Gaffet et al., 1996; Amulyavichus et al., 1998). Bottom-up approaches include chemical reduction of silver ions, electrochemical methods and sonochemical processes (Prabhu and Poulose, 2012). Different chemical precursors which are used in bottom-up approaches for reducing silver ion to AgNPs, may cause secondary effects and sometimes have toxic impacts on organisms. In contrast, no use of chemicals in top-down approaches may reduce these secondary effects. Therefore, to test this hypothesis in this study, acute toxicity effects of two types of AgNPs produced by physical and chemical methods were investigated and compared based on the survival rate of zebrafish.

Materials and Methods

Nanoparticles and characterization: Two types of colloidal silver nanoparticles (AgNPs) were used in the present study. The first type of colloidal AgNPs produced by physical method (top-down approach), manufactured by Particular GmbH (Germany) through laser ablation of silver metal in water (Bärsch et al., 2009). According to the information provided by the manufacturer, this product is a ligand-free colloidal AgNPs with concentration of 500 mg/L in pure distilled water. For enhancing the resistance and preventing the nanoparticles from settling, polyvinylpyrrolidone (PVP) has been added as 0.01% wt. The second type of colloidal AgNPs produced by chemical method (bottom-up approach), manufactured by Nano Nasb Pars Company (Tehran, Iran) through photochemical reduction of silver nitrate solution in presence of hydrazine solution and linear alkyl benzene sulfonate (Rahman Nia, 2009). According to the information provided by the manufacturer, this product is a citrate capped colloidal AgNPs with concentration of 4000 mg/L. The detailed specifications of this colloid have been analyzed and reported previously (Asghari et al., 2012; Johari et al., 2013).

Also, prior to using the above products in the present study, dynamic light scattering (DLS), was performed using a Zetasizer (Malvern Instruments, Nano ZS), to determine the hydrodynamic diameter of particles in colloids.

Experimental Animals and Conditions: A population of 1000 zebrafish with average weight of 1580 ± 282 mg was used in this research. For adaptation to experimental conditions, fish were kept for one week in a 1000 L tank with aeration and under 12L: 12D photoperiod. During this period, mean water temperature was 23 ± 1°C and fish were daily fed to 1% of body weight with aquarium fish food (BioMar, Germany). The water used during adaptation and toxicology experiments was de-chlorinated tap water. The properties of water including ammonia, chloride, calcium and pH were 0.00 mg/L, 0.00 mg/L, 100 mg/L and 8.5, respectively. Also during the experiments, mean water temperature was 24 ± 1°C and dissolved oxygen was always more than 8 mg/L.

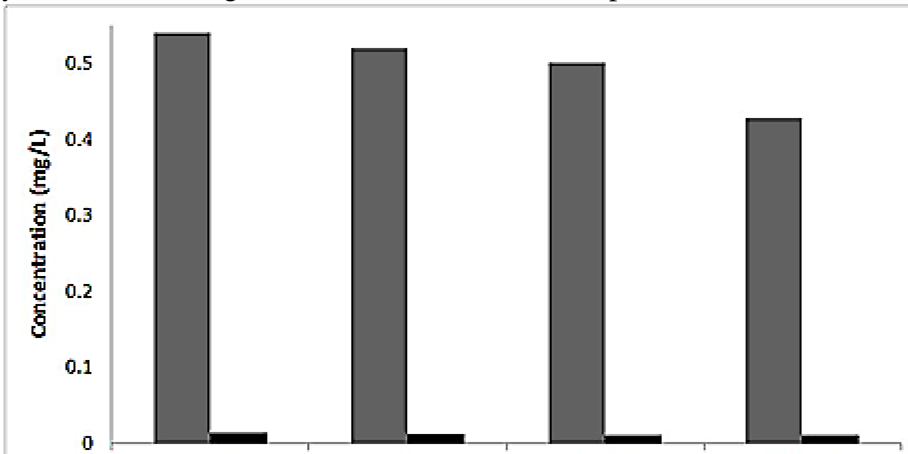

Figure 1. Magnitudes of 48 hours LC_{50}, MATC, LOEC, and NOEC of silver nanoparticles (AgNPs) produced by top-down approach (laser ablation method) compared to bottom-up approach (chemical reduction) in zebrafish.

Ecotoxicity Tests: Toxicology experiments were conducted according to Organisation for Economic Co-operation and Development (OECD) guidelines for the Testing of Chemicals (OECD, 1992) in aquariums containing ten liters of aerated water. According to the OECD guidelines, feeding of fish was stopped 24 hours before and during the experiments. First, several range finding tests were conducted to determine the range of lethal concentration of each chemical. For this purpose, 200 zebrafish were exposed to concentrations of 5, 3, 1, 0.5, 0.3 0.1, 0.05, 0.03, 0.01, and 0 (control) mg/L of each type of the AgNPs for 96 hours. Each concentration was in two replicates and five fish were exposed to chemicals in each replicate. The mortalities were recorded at 24, 48, 72 and 96 hours post-exposure. Based on the data acquired from the preliminary experiments, lethal range of chemically produced AgNPs was 0.01-0.03 mg/L, while this range was 0.50-1.00 mg/L for AgNPs produced by physical method.

For conducting the main experiments, a number of 600 fish were exposed to ten concentrations of AgNPs produced by physical method (including 0.98, 0.90, 0.82, 0.74, 0.66, 0.60, 0.58, 0.56, 0.54 and 0.52 mg/L) and nine concentrations of chemically produced AgNPs (including 0.030, 0.028, 0.025, 0.023, 0.020, 0.018, 0.015, 0.013 and 0.010 mg/L) along with control groups (aerated water without nanoparticles) for 96 hours with three replicates (10 fish per replicate). The mortalities were recorded at 24, 48, 72 and 96 hours post-exposure. The results obtained from the mortalities of fish during the main experiments were assessed using the EPA Probit analysis program (version 1.5) to calculate the lethal concentrations (LCs).

Results

Based on the results of dynamic light scattering, the hydrodynamic diameter of the physically produced AgNPs ranged from 5-20 nm in colloid, where particle sizes of 9.244 nm were dominant and Polydispersity Index (PDI) of these particles was 0.396. In the same way, the hydrodynamic diameter of the chemically produced AgNPs in colloid was in the range of 5 to 220 nm, where particle sizes of 63.45 nm were dominant and PDI of these particles was 0.544 (PDI <20, showing that particles are monodisperse, and their diameter distribution is uniform in the colloid).

The number of mortalities in the control groups was always less than 5%. In the case of fish exposed to higher concentrations of both types of AgNPs, increase of gill mucus secretion and unbalanced swimming were observable that were finally resulted in the death of fish.

Based on the results, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) of AgNPs produced by chemical method were 0.01 and 0.0125 mg/L, respectively. Furthermore, according to the Probit analysis, maximum acceptable toxicant concentration (MATC) of this type of AgNPs was calculated 0.011 ± 0.0015 mg/L. Median lethal concentration (LC_{50}) of chemically produced AgNPs colloids was estimated 0.014 ± 0.001 mg/L during 96 hours of exposure (Fig. 1).

In the case of physically produced AgNPs, the NOEC, LOEC and MATC were 0.50, 0.52, 0.427 ± 0.0597, respectively. LC_{50} of physically produced AgNPs colloids in zebrafish was estimated 0.540 ± 0.032 mg/L during 96 hours of exposure (Fig.1).

Discussion

The aim of this research was to test this hypothesis that chemically produced AgNPs are more toxic than AgNPs produced by physical method due to different chemical precursors which are used in bottom-up approaches for reducing silver ions to AgNPs, and may cause secondary effects and sometimes have toxic impacts on organisms. Based on the results, no observed effect concentration, lowest observed effect concentration, maximum acceptable toxicant concentration and median lethal concentration of AgNPs produced by chemical method were respectively 50.00, 41.60, 38.81 and 38.57 times less than ones produced by physical method, therefore our hypothesis is accepted.

In the acute aquatic toxicity tests, the median lethal concentration is usually the most important end point. With regard to the results, 96 h LC_{50} of physically and chemically produced AgNPs colloids in zebrafish were estimated to be 0.540 ± 0.032 and 0.014 ± 0.001, respectively. As both of these values are less than 1 mg/L, and based on the rules of the European Union (European commission, 2008) and Recommendation No. 67/548/EEC of this union (European commission, 1999), both types of AgNPs colloids are classified as "highly toxic chemicals" to this fish species and therefore if these nano-materials were released into the aquatic ecosystems, they cause unfavorable effects on aquatic organisms. Similar results on the acute toxicity of AgNPs in *Oncorhynchus mykiss* (Johari et al., 2013) and *Daphnia magna* (Asghari et al., 2012) have been reported. Also sublethal toxicity of AgNPs has been shown by other works (Sharifian et al., 2013).

Based on the findings of this research and also the results of other published studies, it seems that AgNPs regardless of the method used for their production (chemical or physical), have toxic effects on aquatic organisms, therefore more attention should be paid to preventing from their accidental or intentional entrance into aquatic ecosystems.

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