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Toxicity comparison of silver nanoparticles synthesized by physical and chemical methods to tadpole (*Rana ridibunda*)

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ABSTRACT

One of the possible threats in increasing use of nanomaterials is the emergence of toxicity in humans and other animals which is discussed in nanotoxicology. In addition to toxic effects of nanomaterials themselves, different chemical precursors which are usually used in bottom-up approaches for production of nanomaterials may have secondary toxic effects in living organisms. In contrast, less use of chemicals in top-down approaches may reduce these secondary effects. To test this hypothesis, toxic effects of two types of silver nanoparticles (AgNPs) produced by physical (top-down) and chemical (bottom-up) methods were investigated and compared on the tadpole, *Rana ridibunda* survival. The estimated 48 h LC_{50} values of AgNPs produced by chemical and physical methods for tadpoles were 0.055 ± 0.004 and 0.296 ± 0.085 $mg.L^{-1}$, respectively. No observed effect concentration, lowest observed effect concentration, maximum acceptable toxicant concentration and median lethal concentration of AgNPs produced by chemical method were respectively 3.42, 4.50, 4.84 and 5.38 times less than those produced by physical method. Therefore, approving the mentioned hypothesis, it was revealed that AgNPs produced by chemical method are more toxic than those generated by physical method. However, it seems totally that AgNPs regardless of the method used for their production, have toxic effects on aquatic organisms and so, inhibiting their accidental or intentional entrance into the aquatic ecosystems should be more considered.

Key words: Amphibian, Aquatic Nanotoxicology, *Rana ridibunda*, Silver Nanoparticles, Tadpole.

INTRODUCTION

Nanotechnology is of promising technologies that has found extensive applications in many scientific and industrial fields. Based on the statistics obtained from thirty countries of the world, the number of consuming products by human being in which the nanomaterials are used, has increased from 54 in 2005 to 1317 in 2011 and it is predicted that this number would be rapidly increasing in future years too (Woodrow Wilson Database, 2011). According to these statistics, the more frequent nanomaterials used in consumer products are silver, carbon, titanium, silicon, zinc and gold, respectively. Increasing development of nanotechnology and the extension of the applications of nanomaterials in human life has caused many concerns on possible dangers of the release of these materials into the environment. Aquatic ecosystems are one of the final destinations of the released nanomaterials in the environment. These materials may have harmful effects on the aquatic organisms and so, the study of these effects is of great importance. Nanotoxicology is the assessment of toxic effects of nanomaterials on living organisms and hence, aquatic nanotoxicology investigates the toxic effects of nanomaterials on aquatic organisms including aquatic bacteria, unicellular and multicellular algae, zooplanktons, mollusks, crustaceans, amphibians, fish, and etc.

Despite numerous published studies which focused on the toxicity of different chemicals on aquatic organisms (Imanpour Namin *et al*., 2011; Khodabakhsh *et al*., 2014; Ramzanpour *et al*., 2014; Shirdel and Kalbassi, 2014), the field of aquatic nanotoxicology is relatively new field (Kalbassi *et al*., 2011; Salari Joo *et al*., 2012, 2013; Hosseini *et al*., 2014; Johari, 2014; Johari *et al*., 2015; Tavana *et al*., 2014; Sohn *et al*., 2015a, b). Among the various aquatics, amphibians are good candidates for aquatic nanotoxicology since they have the potential of being bioindicators and also are of great importance for investigation of the pollution transference between terrestrial and aquatic ecosystems (Sparling *et al*., 2000) although much less attention have been paid to them in ecotoxicology studies compared other vertebrates.

Rowe & Freda (2000) stated that some species of amphibians can avoid contaminated breeding sites but according to Weir *et al.* (2010), some unexpected events like runoff or leaching may contaminate the sites and so, water dependent early-life stages of amphibians which are sensitive to contaminants face contamination.

Amphibians play both the role of prey and top predators in trophic chains based on their habitat and life stage (Murphy *et al*., 2000). This ability makes them even more important in ecotoxicology studies because their roles are very important in accumulation and transfer of toxic substances. Sensitivity of amphibians to contamination is more in larval than in adult stage.

Furthermore, since nanoparticles aggregate in sediment surface and amphibians' larvae feed on them, their role as good bioindicators is more focused. In this study the marsh frog (*Pelophylax ridibundus* or *Rana ridibunda*) tadpole was selected to evaluate the toxicity of AgNPs.

This species occurs in the largest part of Europe, Asiatic Russia, south to western Iran and Afghanistan, as well as isolated populations in Saudi Arabia. This species is classified as least concern according to IUCN criteria (Kuzmin *et al*., 2009). In general, there are two methods for production of nanomaterials. In bottom-up approaches, molecules are joined together during very special processes to generate larger structures with nano dimensions; Vice versa in top-down approaches, large dimension materials are changed to nano dimension structures by physical methods (Rodgers, 2006). AgNPs may be also manufactured by both physical and chemical methods. 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 & 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. To test this hypothesis, acute toxic effects of two types of AgNPs produced by physical and chemical methods were investigated and compared on the survival of marsh frog tadpoles.

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), were 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 $mg.L^{-1}$ 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), were 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 $mg.L^{-1}$. 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 marsh frog (*Pelophylax ridibundus* or *Rana ridibunda*) tadpoles which were obtained from single spawning was used in the present study. According to the identification key of Gosner, at the beginning of toxicology experiments tadpoles were at the developmental stage of 25 (Gosner, 1960). The water used during toxicology experiments was drinking water which was vigorously aerated at least for two weeks for dechlorination. In investigation of aquatic toxicity of AgNPs, it is very important to pay attention that because of possible reaction of silver with sulfate, sodium thiosulfate cannot be used for dechlorination. Some properties of aerated water were measured with chemical water test kit (Sera Company, Italy) and according to its results, ammonia, chloride and calcium were 0.00, 0.00 and 100 $mg.L^{-1}$, respectively and pH was 8.5. Toxicology experiments were conducted in glass beakers containing one liter of aerated water and under 12L: 12D photoperiod.

Ecotoxicological Experiments

At first, several range finding tests were performed to determining the range of lethal concentration of AgNPs produced by physical and chemical methods.

Briefly 200 tadpoles were exposed to an extensive range of concentrations of each type of the AgNPs and also the control group (the water without nanoparticles). Each concentration was in two replicates and 5 tadpole juveniles were exposed to nanoparticles in each replicate.

The mortalities were recorded at 24 and 48 hours post-exposure. No reactions to the touching of the caudal peduncle with a glass rod confirmed the mortality of tadpoles. Based on the data acquired from the preliminary experiments, lethal range of chemically produced AgNPs was 0.035 - 0.075 $mg.L^{-1}$ while this range was 0.12- 0.60 $mg.L^{-1}$ for AgNPs produced by physical method.

For conducting the main experiments, a number of 420 tadpoles were exposed in triplicate (10 tadpoles for each replicate) to seven concentrations of AgNPs produced by physical method (including 0.60, 0.48, 0.42, 0.36, 0.24, 0.18 and 0.12 $mg.L^{-1}$) as well as seven concentrations of chemically produced AgNPs (including 0.075, 0.070, 0.060, 0.050, 0.045, 0.040 and 0.035 $mg.L^{-1}$). Control groups (aerated water without nanoparticles) consisting of 30 tadpoles in 3 replicates were also included. The mortalities (as described before) were recorded at 24 and 48 hours post-exposure.

The results obtained from the mortalities of tadpoles during the main experiments were assessed using the EPA Probit analysis program (version 1.5) in order to calculate the lethal concentrations.

RESULTS

Based on the results of dynamic light scattering, the hydrodynamic diameter of the physically produced AgNPs ranging 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).

During the ecotoxicological experiments, mean water temperature was 27 ± 1°C and dissolved oxygen was always more than 8 mg.L^{-1}. Based on the obtained results in this research, no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) of AgNPs produced by chemical method were 0.035 and 0.040 mg.L^{-1}, respectively in tadpoles. Furthermore, according to the analysis of mortalities data by Probit software, maximum acceptable toxicant concentration (MATC) of these types of AgNPs was calculated 0.039 ± 0.0045 mg.L^{-1}.

Median lethal concentration (LC50) of chemically produced AgNPs colloids in tadpoles was estimated 0.055 ± 0.004 mg.L^{-1} during 48 hours of exposure (Fig. 1).

In the case of physically produced colloidal silver nanoparticles, the NOEC, LOEC, MATC, and LC50 were 0.12, 0.18, 0.189 ± 0.092, and 0.296 ± 0.085 mg.L^{-1} respectively within a period of 48 hours (Fig. 1).

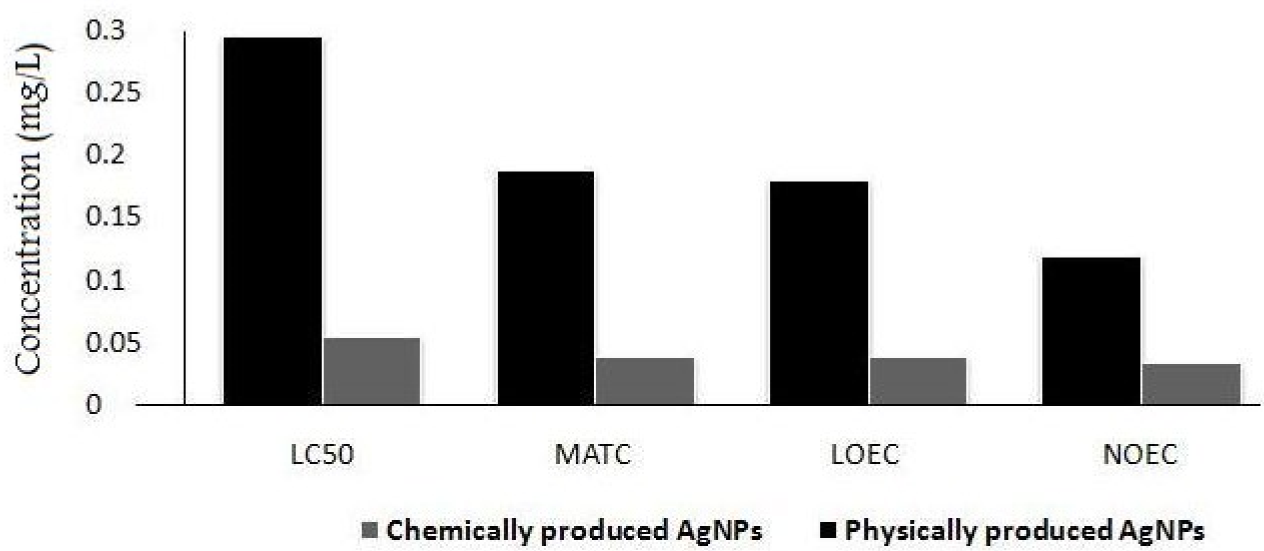

Fig. 1. Magnitudes of 48 hours LC_{50}, MATC, LOEC, and NOEC of silver nanoparticles (AgNPs) produced by top-down approach (laser ablation method) compare to bottom-up approach (chemical reduction).

DISCUSSION AND CONCLUSION

With regard to increasing production and presentation of nanomaterials and consumable products containing these materials, aquatic nanotoxicology can be considered as a very suitable index for predicting the possible impacts of the release of nanomaterials into the aquatic ecosystems. In this case, the results of acute and chronic toxicology studies are each of great importance.

To the end of August 2013, based on our investigations, a number of 690 scientific papers have been published in valid journals on the toxicology of different types of nanomaterials in aquatic organisms. After precise investigation of these papers, it was found that only in very few of them, toxic impacts of nanomaterials have been investigated in frogs. For example and briefly, in *Xenopus laevis,* ecotoxicological potential of double-walled carbon nanotubes (DWNTs) in larvae (Mouchet *et al*., 2008), the effects of ZnO, TiO_2, Fe_2O_3 and CuO nanomaterials (20 – 100 nm) during 96 h of exposure on embryos (Nations *et al*., 2011), teratogenic potential of commercially available CuO, TiO_2 and ZnO nanoparticles (Bacchetta *et al*., 2012) and also, the effect of increasing nano-TiO_2 concentration on growth, survival and developmental stages (Zhang *et al*., 2012) have been investigated. In addition Yslas *et al.* (2012) investigated the impacts of polyaniline nanofibers exposure on growth and morphology of *Rhinella arenarum* embryos at early life stages. Also, Salvaterra *et* *al.* (2013) evaluated sublethal effects of a short-term exposure (96h) to titanium silicate nanoparticles ($TiSiO_4$-NP) on *Pelophylax perezi* tadpoles.

As it was explained in the introduction part, the objective of this research was to test this hypothesis that chemically produced AgNPs are more toxic compared to the AgNPs produced by physical method. Based on the results of the present study, no observed effect concentration, lowest observed effect concentration, maximum acceptable toxicant concentration and median lethal concentration of AgNPs produced by chemical method were respectively 3.42, 4.50, 4.84 and 5.38 times less than ones produced by physical method. These figures indicate that the toxicity of AgNPs produced by physical method (laser ablation, in this study) is lesser than chemically produced AgNPs (chemical reduction, in this study) and so, our hypothesis is accepted.

This hypothesis was on this basis that chemically produced AgNPs contain chemicals which are used in bottom-up approaches for reducing the Ag ions to AgNPs. These chemicals may cause secondary effects and sometimes have toxic impacts on the cells and organisms but in the process of physical production of AgNPs, no additional chemicals are used. For example Uboldi *et al*. (2009) showed that purification grade, and presence or absence of contaminants (such as sodium citrate residues on the particle surface) might play a pivotal role in inducing cytotoxicity. Nanoparticle production by laser ablation in liquid is a physical method to produce AgNPs of high purity without additional stabilizing agents or toxic byproducts (Bärsch *et al*., 2009; Petersen & Barcikowski, 2009). A huge advantage of this ligand-free synthesis route is its independence from chemical precursors (such as metal-organic substances), avoiding the use of toxic substances or by-products that possibly adsorb onto the nanoparticle surface which can affect its toxicity (Barcikowski & Compagnini, 2013).

According to estimates, about 63 tons of nano silver is entering annually to the water bodies on the earth (Keller *et al*., 2013). Although the lowest observed effect concentrations of AgNPs in the present acute experiment (40 and 180 $\mu g.L^{-1}$, for chemically and physically produced AgNPs respectively) were much higher than the value currently predicted by the presence of nanoscale silver in aqueous environment (0.03 to 0.32 $\mu g.L^{-1}$, according to Batley *et al*., 2013), but results of chronic toxicity tests are also needed to determine the effects of environmentally relevant concentrations of AgNPs on aquatic organisms.

In general, 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, have toxic effects on aquatic organisms, so more attention should be paid to prevent from their accidental or intentional entrance into aquatic ecosystems.

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مقایسه سمیت نانوذرات نقره تولید شده به روش‌های فیزیکی و شیمیایی در بچه قورباغه مردابی (*Rana ridibunda*)

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چکیده

از جمله خطرات احتمالی ناشی از افزایش استفاده از نانومواد، موضوع ایجاد سمّیت این مواد در انسان و سایر موجودات است که در علم سم شناسی نانومواد به آن پرداخته می‌شود. علاوه بر اثرات سمّی ذاتی نانومواد، روش‌های شیمیایی متفاوت به کار رفته در تولید این مواد در روش‌های "پایین به بالا"، ممکن است باعث ایجاد اثرات سمّی ثانویه در موجودات زنده شود. در مقابل استفاده کمتر از مواد شیمیایی در روش‌های "بالا به پایین" ساخت نانومواد ممکن است این اثرات ثانویه را کاهش دهد. برای آزمون این فرضیه، اثر سمّیت دو نوع نانوذرات نقره تولید شده به روش‌های فیزیکی (بالا به پایین) و شیمیایی (پایین به بالا) بر بازماندگی بچه قورباغه مردابی (*Rana ridibunda*) مورد بررسی و مقایسه قرار گرفت. مقادیر غلظت‌های کشنده میانی (LC_{50}) ۴۸ ساعته نانوذرات نقره تولید شده به روش‌های شیمیایی و فیزیکی در این جانور به ترتیب ۰/۰۰۴ ± ۰/۰۵۵ و ۰/۰۸۵ ± ۰/۲۹۶ میلی‌گرم در لیتر بدست آمد. مقادیر بیشترین غلظت فاقد اثر سمّیت، کمترین غلظت ایجاد کننده سمّیت، حداکثر غلظت قابل قبول و غلظت کشنده میانی نانوذرات نقره تولید شده به روش شیمیایی نسبت به نانوذرات نقره تولید شده به روش فیزیکی به ترتیب ۳/۴۲، ۴/۵۰، ۴/۸۴ و ۵/۳۸ برابر کمتر بود. بنابراین در تأیید فرضیه فوق، آشکار است که نانوذرات نقره تولید شده به روش شیمیایی سمّیت بالاتری نسبت به نانوذرات نقره تولیدی به روش فیزیکی دارند. با این وجود، به نظر می‌رسد که صرف نظر از روش تولید، نانوذرات نقره دارای اثرات سمّی بر آبزیان بوده و بنابراین پیشگیری از ورود عمدی یا تصادفی این مواد به بوم سازگان‌های آبی باید مورد توجه جدّی قرار گیرد.

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