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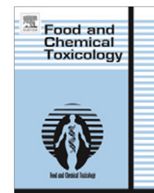

Evaluating cell specific cytotoxicity of differentially charged silver nanoparticles

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ABSTRACT

Silver nanoparticles (AgNPs) are one of the most commercially viable nanotechnological products, nevertheless; safety issues are raised regarding the use of such nanoparticles due to unintentional health and environmental impacts. In the present study, AgNPs were synthesized by chemically reducing silver nitrate alternatively with sodium borohydride, tannic acid, ascorbic acid and sodium citrate. AgNPs synthesized by reduction with tannic acid (TSNPs) and sodium borohydride (BSNPs) exhibited highest and lowest surface potential respectively. Therefore these two types of AgNPs were selected for their toxicity assessment in cellular environment. We treated skin epithelial A431, lung epithelial A549 and murine macrophages RAW264.7 cells with AgNPs over a range of doses (5–100 μg/ml). Toxicity was evaluated by measuring changes in cellular morphology, ROS generation, metabolic activity and expression of various stress markers. Interestingly, TSNPs exhibited a higher negative zeta-potential and also higher toxicity. Higher toxicity of TSNPs was attested by dose-dependent increase in cellular disruption and ROS generation. BSNPs showed cytotoxic effect up to the concentration of 50 μg/ml and thereafter the cytotoxic effect attenuated. TSNPs induced a dose dependent increase in the expression of stress markers pp38, TNF-α and HSP-70. Our report proposes that cytotoxicity of AgNPs changes with surface potential of nanoparticles and cells type.

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1. Introduction

The revolution set up by nanotechnology aims to bring advantages in areas of our lives as diverse as engineering, information technology and diagnostics. Wide applicability of this science raises the concern about safety when this technology comes out of the lab. For some nanoparticles (NPs), such as those in sun block creams, dermal exposure is already occurring and the range of different NPs in creams is likely to increase (Pinnell et al., 2000; Tan et al., 1996). It has been experimentally observed that UV-B damaged skin allows more penetration of TiO_2 and ZnO nanoparticle found in sunscreens (Monteiro-Riviere et al., 2011). Nanoparticles in food are reported to cross into gut lymphatic and redistribute to other organs more readily than the larger particles (Hillery et al., 1994; Jani et al., 1990). Huge classes of NPs are designed to be introduced directly into the body for diagnostic and therapeutic purpose (Borm and Kreyling, 2004). Recent research showed that nanoparticles from photocopiers were capable of inducing oxidative damage and inflammation in healthy volunteers (Khatri et al., 2012). Thus it becomes important to study the toxicity of such an extensively used nanotechnology product. Nanotoxicology is an emerging discipline which not only provides data for safety evaluation of engineered nanostructures but also helps to advance the field of nanomedicine by providing information about their undesirable properties and means to avoid them (Oberdorster et al., 2005). Research in this field helps to keep a check on the chronic health problems caused by the use of untested nanomaterials (Pumera, 2011).

The anti bacterial effects of silver are known since ages and silver is currently used to control bacterial growth in a variety of applications including dental work, catheters and burn wounds. AgNPs have provided a novel therapeutic direction for wound treatment in clinical practice (Tian et al., 2007). AgNPs are also used in washing clothes due to their anti-microbial property (Vigneshwaran et al., 2007). They are also used in clothing to inhibit the bacterial growth and thereby making the fabric odor resistant. It has been reported that such an extensive use of AgNPs in clothing can disrupt helpful bacteria in waste water treatment facilities and can also endanger aquatic species (Benn and Westerhoff, 2008). Thus before incorporating AgNPs into our daily lives, we should elaborate their toxic effects on biological systems.

AgNPs have been synthesized using various methods, the most preferred being the chemical reduction method (Tao et al., 2006; Wiley et al., 2005). Other methods include green synthetic

Abbreviations: AgNPs, silver nanoparticles; TSNPs, AgNPs synthesized by reduction with tannic acid; BSNPs, AgNPs synthesized by reduction with sodium borohydride; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide.

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methods such as mixed-valence polyoxometallates, polysaccharide, Tollens and irradiation (Sharma et al., 2009). Studies have shown that the size, morphology, stability and properties (chemical and physical) of the metal nanoparticles are strongly influenced by the experimental conditions, the kinetics of interaction of metal ions with reducing agents, and adsorption processes of stabilizing agent with metal nanoparticles (Knoll and Keilmann, 1999; Sengupta et al., 2005). Recent reports provide evidence for dependence of AgNP mediated toxicity, in drosophila, on the size of nanoparticles (Gorth et al., 2011). Hence, the design of a synthesis method in which the size, morphology, stability and properties are controlled has become a major field of interest (Wiley et al., 2007).

AgNPs have been found to be deposited in various organs in zebrafish embryos giving rise to distinct developmental defects (Asharani et al., 2008a). Recent reports on Ag NP toxicity have identified the mitochondria as primary target of silver nanoparticle in rat liver cells. Moreover Ag NPs were reported to act via reactive oxygen species (ROS) generation and glutathione depletion (Hussain et al., 2005). The depletion of antioxidants including glutathione and protein bound sulfhydryl groups and the increase in activity of various anti-oxidant enzymes indicative of lipid peroxidation have been implicated in oxidative damage of cell molecules (Ahamed and Siddiqui, 2007). A linear correlation between Ag NP toxicity and dissolved silver has also been reported (Yang et al., 2011).

Here we show that change in method of synthesis of nanoparticles may result in a subsequent change in surface charge potential which further leads to change in toxicity of nanoparticles in general. Moreover, the toxicity of nanoparticles also varies with the cell type. Different cells show different susceptibility towards AgNP mediated toxicity. Thus we suggest there cannot be a generalized toxicological profile for individual nanoparticles.

2. Materials and methods

2.1. Synthesis of AgNPs

Tri-sodium citrate, sodium borohydride and tannic acid were purchased from Loba-Chemie, Sigma–Aldrich and Merk respectively. Silver nitrate ($AgNO_3$) and ascorbic acid were obtained from Hi Media. All the reagents were used without further purification. The water used in all experiments was double distilled water. In a typical synthesis, 100 μl $AgNO_3$ (100 mM) was added to 9.7 ml double distilled water under magnetic stirring. Further 100 μl of tri-sodium citrate (300 mM) and 100 μl of sodium borohydride (500 mM) were added. Tri-sodium citrate coats the AgNPs and prevents their aggregation. The solution was stirred on a magnetic stirrer for 15 min, till the solution turns yellow in color. The nanoparticles were collected by centrifugation at 18000 rpm for 1 h. Ionic impurities were removed by washing the particles thrice with double distilled water. In other methods sodium borohydride was switched with tannic acid (10%) and ascorbic acid. Fourth group of AgNPs were synthesized simply by adding sodium citrate to $AgNO_3$ solution. The prepared AgNPs were sonicated in a bath sonicator for 30 min prior to use. UV–visible extinction spectra were collected on a UV–Visible spectrophotometer (UV Pharmaspec 1700- Shimadzu).

2.2. Characterization of Ag NPs

Dynamic light scattering for characterization of hydrodynamic size of Ag NPs dispersed in DMEM medium was performed on Nano-ZS, Malvern Instruments, Malvern, UK, taking the average of 5 measurements. Zetapotential was also measured to determine the amount of aggregation of particles. Ag NPs were analyzed under TEM (FEI TF-20) for size determination. Briefly, the stock solution of AgNPs synthesized by the above methods were diluted in DMEM medium (5 μg/ml and 100 μg/ml) and sonicated. 2 drops of the solution were dropped onto carbon coated copper grids and kept for drying. The dried sample was analyzed under the microscope at 200 kV. Only BSNPs and TSNPs were selected for further characterization and biological studies as these exhibited highest and the lowest aggregation respectively. The size and morphology of BSNPs and TSNPs was viewed under a scanning electron microscope (S-3400, Hitachi Ltd., Japan) operated at an excitation voltage of 25 kV. The nanoparticles were mounted onto steel stage using double sided adhesive tape and sputter coated with gold using ion sputter (E-1010, Hitachi Ltd., Japan). Mean size was calculated from measurement in a random field of view. From the images we can determine the morphology and aggregation of silver nanoparticles. BSNPs and TSNPs were also observed under AFM to determine the three dimensional structure. For AFM analysis, a drop of 100 μg/ml of TSNPs and BSNPs was dropped onto a mica sheet. On drying, this sample was observed under the microscope.

2.3. Cell culture

A431 human epithelium carcinoma, A549 human lung carcinoma and RAW264.7 murine macrophages were obtained from NCCS Pune. The cells were cultured in DMEM containing 10 % FBS and 1% penicillin–streptomycin. DMEM and FBS were obtained from Sigma Chemicals. Antibiotic was purchased from invitrogen. Cell cultures were maintained in flasks under standard conditions: incubation at 37 °C and 5% CO_2. All the subcultures were used prior to passage 15. Cells were routinely passaged using 0.25% trypsin/0.1% EDTA. For treatment cells were cultured in presence of increasing concentrations (5–100 μg/ml) of silver nanoparticles for 24 h.

2.4. Anti-microbial activity of silver nanoparticles

The anti-microbial activity of silver nanoparticles was evaluated against *E.coli* and *S.typhii*. by colony counting method. Approximately 10^7 colony forming units of each microorganism were inoculated in LB broth and incubated overnight in shaking conditions at 37 °C. LB broth containing bacteria is diluted so that we have 10^3 colony forming units in each tube. Each tube was treated with three concentrations of silver nanoparticles 5 μg/ml, 25 μg/ml, and 75 μg/ml. After overnight incubation of bacteria with silver nanoparticles, all samples were serially diluted and inoculated on Luria Bertani agar plates for direct colony counting. Ethanol was used as the positive control.

2.5. Change in cellular morphology after Ag NP treatment

A431, A549 and RAW264.7 cells were treated with both the types of silver nanoparticles at the concentration of 100 μg/ml for 24 h. Used media was discarded and the cells were washed with PBS to remove any unbound AgNPs. The cells were then observed under inverted microscope (Nikon Eclipse TE2000-S) at a magnification of 40X for change in morphology.

2.6. Intracellular detection of oxidative stress

We investigated intracellular ROS generation in A431, A549 and RAW264.7 cells exposed to Ag NPs. We used dichlorodihydrofluorescein diacetate H_2DCFDA (obtained from Cell Signaling). Cells were treated with 50 μg/ml and 100 μg/ml Ag NPs for 30 min. After incubation, media was removed and 1 ml DCFDA solution (1 μl DCFDA/ml PBS) was added to the plates. After 10 min of incubation, DCFDA solution was removed and the cells were observed under microscope for florescence.

2.7. Metabolic activity determination

Metabolic activity was determined by MTT assay (MTT obtained from Hi-Media). Cells were plated at 90% confluence and incubated in the presence or absence of increasing concentration of Ag NPs. After 24 h incubation, cells were treated with MTT solution for 4 h at 37 °C in a cell culture incubator at 37 °C and 5% CO_2. MTT which is a tetrazolium salt is converted into insoluble formazan by mitochondrial dehydrogenases in live cells. Formazan is dissolved in DMSO (Merk) and absorbance was measured at dual wavelength of 550 nm and 630 nm on an ELISA plate spectrophotometer (Biotrek instruments). The total number of viable cells relative to viable cells in untreated control is calculated.

2.8. Western analysis

A431, A549 and RAW264.7 cells were plated at confluence for western analysis. Confluent cells were treated with 5 μg/ml, 10 μg/ml, 25 μg/ml, 50 μg/ml, 75 μg/ml, 100 μg/ml Ag NPs for 24 h. Treated cells were then washed with chilled PBS. The cells were then scraped in lysis buffer and sonicated. The proteins were separated by centrifugation at 10,000 rpm for 8 min. Protein estimation was performed using Lowry's method. The samples were then prepared for SDS–PAGE. The samples were then loaded on 10% polyacrylamide gel and the proteins were transferred on a PVDF membrane which was incubated in presence of anti-phospho p-38, anti-p38, anti-TNF-α, anti-IGF-1Rβ, anti-Actin (1:1000 dilution; obtained from Santa Cruz Biotechnology) and anti-HSP70 (1:1000 dilution; obtained from Calbiochem) overnight. The antigen antibody complex was detected using HRP-coupled secondary antibodies (Santa Cruz Biotechnology) and ECL (Amersham).

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