Comparison of toxicity of uncoated and coated silver nanoparticles

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Abstract: This study compares toxic effects of uncoated (20, 40, 60 and 80 nm) and OECD (Organization for Economic Co-operation and Development) standard citrate- and polyvinylpyrrolidone (PVP)-coated (10, 50, and 75 nm) silver nanoparticles (Ag-NPs) in J774A.1 macrophage and HT29 epithelial cells. The cells were exposed to different concentrations (silver content) of Ag-NPs for 24 h. Analysis showed that uncoated Ag-NPs, at a concentration of 1 \( \mu \text{g/ml} \), decreased cell viability by 20-40\% and that 20 and 40 nm particles were 10\% more cytotoxic than the 60 and 80 nm particles. In exposures to coated Ag-NPs, cell viability dropped at 25 \( \mu \text{g/ml} \) or higher concentrations, and the effects were also size-dependent. PVP-coated particles induced greater cytotoxicity than citrate-coated particles. Changes in sub-cellular architecture were observed in J774A.1 cells upon exposure to test Ag-NPs. Furthermore, uncoated Ag-NPs (1 \( \mu \text{g/mL} \)) decreased the expression of selected cytokines including TNF-\( \alpha \), IL-1\( \beta \), and IL-12 (p70) in J774A.1 and IL-8 in HT29 cells. In contrast, both citrate- and PVP-coated Ag-NPs increased the expression of these cytokines at higher concentrations (25 \( \mu \text{g/mL} \)), and PVP-coated particles elevated cytokine levels the most. Moreover, while uncoated Ag-NPs resulted in decreased glutathione (GSH) content and increased superoxide dismutase (SOD) activity in test cells in a size-dependent manner at 1 \( \mu \text{g/mL} \), coated Ag-NPs caused non-significant changes in GSH and SOD, even at the highest test concentrations. Lastly, uncoated (20 and 40 nm) at 1 \( \mu \text{g/mL} \) and coated Ag-NPs (10 nm PVP) at 50 \( \mu \text{g/mL} \) slightly increased the production of reactive oxygen species (ROS). Our data showed that uncoated Ag-NPs are more toxic than coated Ag-NPs. While uncoated Ag-NPs appear to suppress inflammatory responses and enhance oxidative stress in the test cells, coated Ag-NPs induce toxic effects through up-regulation of cytokines. Our findings support the toxicity of Ag-NPs as being size- and coating-dependent while providing additional insight on the health impact of Ag-NPs.
1. Introduction

Silver-containing nanoparticles (Ag-NPs), like other nano-sized particles, exhibit special properties relative to their bulk counterparts partly due to their very small size and greater surface area. In addition, Ag-NPs possess unique optical activities that have been exploited in many applications such as catalysis, electronics, and biosensors [1, 2]. Further, due to their antimicrobial properties, these NPs have been used in a variety of consumer and medical products, leading to increased human and ecosystem exposures [3, 4]. Examples of Ag-NP-containing products include wound dressing, surgical instruments, disinfectants, clothing, cosmetics, paints, home appliances, food containers, toys, and computer accessories [5]. As a consequence of production and distribution of a large number of consumer products containing different types of Ag-NPs and new design applications under development, rigorous knowledge is needed about the potential impacts of these NPs on human health and the environment.

Over the past decade, research on biological effects of various Ag-NP types has been performed on microorganisms, various cell lines, aquatic organisms and animal models [6]. The antimicrobial activity of Ag-NPs is fairly well documented in a broad spectrum of bacteria and fungi [7]. *In vitro* toxicity studies have indicated effects of Ag-NPs in immune, skin, lung, liver, and neuron cells [8, 9, 13, 10, 11, 12]. Exposures of Ag-NPs *in vivo* have shown toxic effects in various non-mammalian models, including zebrafish (*Danio rerio*) [13], rainbow trout (*Oncorhynchus mykiss*) [14], nematode (*Caenorhabditis elegans*) [15], and crustacean (*Daphnia magna*) [16]. Moreover, studies in rats and mice have demonstrated toxicity of Ag-NPs in a variety of target organs including lung, liver and brain [17, 18, 19]. From these *in vitro* and *in vivo* studies, the proposed mechanisms of Ag-NP-induced toxicity are oxidative stress, damage to cellular components including mitochondria and DNA, and cytokine induction [20, 21, 22, 23]. Even though there is growing evidence that Ag-NPs are toxic in various test models, the exact mechanisms of Ag-NP toxicity remain unclear.

Toxicological studies of NPs have demonstrated that physicochemical properties of NPs including size, shape, surface coating, surface charge, solubility, and chemical composition could dramatically affect NP behaviour in biological systems and thus influence the toxicity of NPs [24, 25]. A number of studies reported that Ag-NP toxicity was dependent on their size and surface coating [20, 26, 27, 28]. Similarly to other NPs, the size of Ag-NPs has been linked to cellular uptake processes [27]. The surface coating has been shown to affect the affinity of Ag-NPs for the cell surface and the dissolution or the release of Ag⁺ ions from Ag-NPs, which was also proposed as a toxicity mechanism for Ag-NPs [28, 29]. The investigation of these physicochemical characteristics and their impact on the biological effects of Ag-NPs is important for their safe design, development, and applications.

In this study, we investigated and compared the toxicity of uncoated and coated Ag-NPs of different sizes in J774A.1 macrophage and HT29 epithelial cells. The comparative toxicity was assessed by metabolic activity, cell morphology, sub-cellular structures, inflammatory responses, ROS generation, and oxidative stress induction upon exposure to these Ag-NPs. The study provides scientific insight relating physical/chemical characteristics of Ag-NPs to cell damage and possible toxicity mechanisms of these NPs.

2. Materials and Methods

2.1. Materials:

Cell lines modelling murine macrophage (J774A.1) and human colonic epithelial (HT29) were obtained from the American Type Culture Collection (Manassas, VA). Uncoated Ag-NPs (20, 40, 60, and 80 nm) were purchased from Ted Pella, Inc. (Redding, CA). Ag-NPs marketed as OECD (Organization for Economic Co-operation and Development) standard material with either
a citrate- or a PVP-coating (10, 50, and 75 nm) were purchased from NanoComposix (San Diego, CA). Silver nitrate (AgNO₃), (3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyl tetrazolium bromide (MTT), menadione, DMSO, glutaraldehyde, paraformaldehyde, osmium tetroxide, and Triton-X 100 were obtained from Sigma-Aldrich (St. Louis, MO). Rhodamine-Phalloidin, Sytox-Red, dihydroethidium (DHE), and Prolong antifade were purchased from Molecular Probes-Invitrogen (Carlsbad, CA). Bio-Plex cytokine kits and reagents were purchased from Bio-Rad (Hercules, CA).

2.2. Ag-NP size characterization
Test Ag-NPs were characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS). For TEM, Ag-NP stocks were diluted 10-fold with ddH₂O, deposited on carbon-coated grids, which were air-dried overnight at RT and then examined with a JOEL JEM 1230 operating at 60 kV. DLS analysis was performed on a Zetasizer Nano (Malvern Instruments Ltd). Ag-NP stocks were diluted 10-fold with ddH₂O or Dulbecco’s modified Eagle’s medium (DMEM). One millilitre was added to a clean 12 mm polystyrene cuvette (DTS0012) and inserted into the instrument to obtain the readings. Each sample was measured at least 3 times.

2.3. Cell cultures and Ag-NP treatments
J774A.1 and HT29 cells were cultured in DMEM, supplemented with 10% fetal bovine serum (FBS), 1 mg/ml glutamine, and 100 µg/ml gentamicin at 37°C in a humidified atmosphere with 5% CO₂. Cells were treated with different concentrations (based on silver content) of Ag-NPs for 24 h. For assays involving measurement of MTT bioreduction, glutathione, and cytokines, cells were seeded into 96-well plates at a density of 5x10⁴ cells/well. For confocal microscopy, cells were seeded on cover-slips in 12-well plates at a concentration of 1x10⁵ cells/ml/well. Cells were pre-cultured for 24 h to 80% confluency and media were replaced before exposure regimes. AgNO₃ treatments of with equivalent silver content that used for Ag-NPs, were done in some assays for comparison purposes. The silver content of Ag-NPs was provided by the manufacturers. The silver content of AgNO₃ was calculated as the total silver mass, which is 63.5% of the total AgNO₃ molecular mass. Menadione (25 µM) was used as a positive control in ROS detection assays. Working solutions of Ag-NPs and AgNO₃ were prepared by diluting stock solutions in cell culture medium.

2.4. Cell viability – MTT assay
After exposure to Ag-NPs, the culture media was removed and replaced with fresh media (100 µl/well) and 10 µl/well of MTT stock (10 mg/ml), followed by incubation for 1 h at 37°C. Media was removed, cells were rinsed with PBS (100 µl/well), and the formed formazan was solubilized with DMSO (100 µl/well) and quantified spectrophotometrically (Molecular Devices) at 505 nm. All measurements were done in duplicate in three independent experiments.

2.5. Cell morphology by confocal microscopy
Cells, after being exposed to Ag-NPs, were fixed with 4% paraformaldehyde in PBS containing 1% Triton X-100 for 15 min and were washed twice with PBS for 5 min. Cells were stained for actin by treatment with rhodamine-phalloidin (1:40) for 1 h. After two washes with PBS, cells were stained for nuclei by treatment with Sytox-red (1:1000) for 15 min and were washed again with PBS. Each coverslip was inverted onto a drop of Prolong™ antifade (Molecular Probes) placed on a glass slide and was then cured overnight in the dark before observation with a Nikon TE2000 microscope attached to a C1 confocal unit.

2.6. Cell morphology by TEM
After treatments, cells were fixed with 2.5% glutaraldehyde in 66.7 mM cacodylate buffer (pH 7.4) for 1 h, washed twice with 100 mM cacodylate buffer for 10 min, and subsequently post
fixed with 1% osmium tetroxide in 100 mM cacodylate, pH 7.4. Following dehydration in a series of ascending alcohols the cell samples were infiltrated and processed in Spurr’s epoxy resin and embedded onto resin-filled Beem capsule molds. Specimen blocks were then ultrathin-sectioned on a Leica EM UC6 ultramicrotome and the resulting sections stained with uranyl acetate and lead citrate. Sections were analysed with a Jeol 1230 TEM equipped with AMT imaging software.

2.7. ROS detection
Cells were grown to 80% confluency on glass cover-slips inside 12-well cell culture plates. After treatment, cells were rinsed with PBS and incubated with dihydroethidium (DHE) at a concentration of 30 µM for 30 min. Cells were again washed with PBS and the cover-slip containing the monolayer of cells was mounted on a slide and viewed immediately with a Nikon TE2000 microscope attached to a C1 confocal unit (Nikon Canada Inc. Mississauga ON). Fluorescence areas from confocal micrographs were analysed using Nikon Imaging Software (NIS) element (Nikon Canada Inc. Mississauga ON). The average area of fluorescence of three micrographs was plotted to quantify the level of ROS production in the control and treatments.

2.8. Total GSH measurement
A Total GSH Colorimetric Assay kit (Oxford Biomedical Research) was used to estimate the total glutathione content in cell lysates. Briefly, after treatment, cells were washed with PBS pH 7.2. To each well, 100 µl of 5% metaphosphoric acid (MPA) was added, and the plate was frozen at −80°C and subsequently thawed at 37°C. After two freeze/thaw cycles, 50 µl of each cell lysate was transferred to new microtiter wells, and 50 µl each of 5,5′-dithiobis (2-nitrobenzoic acid) (DTNB, 0.5 mg/ml) and glutathione oxidoreductase solution was added to each well. The microtiter plate was incubated for 10 min at RT, and 50 µl of the reduced form of β-nicotinamide adenine dinucleotide phosphate (β-NADPH, 0.6 mg/ml) was added to each well. The absorbance was measured at 405 nm after a 3 min incubation at room temperature.

2.9. SOD activity measurement
After treatment, cells were collected, homogenized in cold 20 mM HEPES buffer (pH 7.2) containing 1 mM EGTA, 210 mM mannitol, and 70 mM sucrose, and centrifuged at 1,500 x g for 5 min at 4°C. The supernatants were collected and centrifuged at 10,000 x g for 15 min at 4°C to yield cytosolic SOD samples. The pellets were homogenized in cold HEPES buffer to yield mitochondrial SOD samples. SOD samples were assayed using a SOD colorimetric enzyme assay kit from Cayman Chemical (Ann Arbor, MI) according to the manufacturer’s protocol.

2.10. Cytokine measurement
Levels of cytokines/chemokines produced by the two cell lines were estimated by using multiplex bead assays based on mouse 23-plex cytokine kits and human 8-plex cytokine kits. The cytokine/chemokine levels were measured using the Luminex-based Bio-Plex array system (Bio-Rad, Hercules, CA) according to the manufacturer’s instructions. Briefly, anti-cytokine/chemokine conjugated beads were added to individual wells of a 96-well filter plate. Beads were then sedimented using vacuum filtration and washed briefly with kit wash buffer. After washing, 50 µl of pre-diluted standards or cell culture supernatants were added and incubated for 30 min at RT with gentle shaking. The filter plate wells were then washed before adding 25 µl/well of pre-diluted detection antibody and incubating for 30 min at RT. After a further washing, 50 µl/well of pre-diluted streptavidin-conjugated phycoerythrin was added and the plate was shaken for 10 min. The wells were again washed and 125 µl/well of assay buffer was added. The plate was shaken for one minute and analyzed with the Bio-Plex 100 Array System.
2.11. Statistical analysis
Results were compared by one-way analysis of variance (ANOVA) followed by Dunnett’s multiple comparison test for comparison to the controls. All data were expressed as mean ± standard deviation. A value of p<0.05 was considered statistically significant.

3. Results

3.1. Ag-NP size distribution characterization
Ag-NPs from both sources were characterized for size distribution and shape by TEM image analysis (Figure 1). Uncoated Ag-NPs appeared as individual spherical particles. The size was obtained by measuring the minimum and maximum dimensions of each NP. The average particulate size in either dimension for uncoated Ag-NPs was slightly greater than the size indicated by the supplier (Table 1). Coated Ag-NPs also appeared as spherical particles and many particles existed as aggregates. The average particulate sizes for coated Ag-NPs are also shown in Table 1. TEM images showed forms of agglomerated NPs for all coated Ag-NPs (Figure 1).
Figure 1: TEM micrographs of test Ag-NPs. (A) uncoated Ag-NPs (20, 40, 60, and 80 nm). (B) coated Ag-NPs (10 nm-citrate, 10 nm-PVP, 50 nm-citrate, 50 nm-PVP, 75 nm-citrate, and 75 nm-PVP).

DLS results showed bell-curved size distributions for all Ag-NPs (data not shown). The mean sizes for the uncoated and coated Ag-NPs in ddH$_2$O were similar to claims made by respective manufacturers, except for the 10 nm coated Ag-NPs whose mean sizes were generally larger than the sizes provided by the manufacturer. For most test particles, DLS particle sizes in cell medium appeared larger than those measured in ddH$_2$O indicating agglomeration (Table 1).
Table 1: Average Ag-NP sizes determined by TEM and DLS

| Ag-NP      | TEM (nm) | DLS in water (nm) | DLS in cell medium (nm) |
|------------|----------|-------------------|-------------------------|
| 20 nm-citrate | 9.86 ± 1.22 | 16.57 ± 6.12 | 14.99 ± 7.34 |
| 10 nm-PVP   | 11.95 ± 1.55 | 15.90 ± 3.11 | 10.06 ± 3.21 |
| 50 nm-citrate | 50.58 ± 4.97 | 45.85 ± 6.74 | 50.30 ± 4.12 |
| 50 nm-PVP   | 52.60 ± 3.23 | 48.31 ± 5.53 | 53.75 ± 1.22 |
| 75 nm-citrate | 78.60 ± 8.66 | 72.32 ± 6.43 | 74.17 ± 1.68 |
| 75 nm-PVP   | 78.38 ± 3.02 | 74.19 ± 3.96 | 82.87 ± 7.67 |

3.2. Cell viability
Cell viability was assessed by measuring the cellular capacity to reduce MTT to its formazan form. Decreased viability in J774A.1 and HT29 cells in response to Ag-NPs exposure was dose-dependent (Figures 2A, B, C and D). Uncoated Ag-NPs resulted in cell viability changes at levels as low as 1 µg/ml of silver content (Figures 2A and C), while coated Ag-NPs resulted in changes starting at 25 µg/ml (Figures 2B and D). With both uncoated and coated Ag-NPs,

Figure 2: Cell viability of Ag-NPs treated cultures relative to the controls. J774A.1 macrophages (A, B) and HT29 epithelial cells (C, D) were exposed to uncoated Ag-NPs (A, C) or coated Ag-NPs (B, D) for 24 h. The asterisks (*) indicate statistically significant differences compared to the controls (p<0.05).
smaller sized particles induced a greater loss in viability, compared to their larger counterparts. For coated Ag-NPs, PVP-coated NPs were 10-20% (p<0.05) more cytotoxic compared to citrate-coated NPs of the same size (Figures 2B and D). Moreover, at the equivalent concentrations of silver that were used for uncoated Ag-NPs, AgNO$_3$ caused no observable changes in cell viability indicating that test uncoated Ag-NPs were more cytotoxic than their bulk material. By contrast, at the same silver contents of test coated Ag-NPs, AgNO$_3$ treatments resulted in greater cytotoxic effects (Figure 2).

3.3. Cell morphology
The effects of test Ag-NPs on J774A.1 cell morphology were examined using confocal microscopy and TEM. At 1 µg/ml of silver content, 20 and 40 nm uncoated Ag-NPs caused changes in cell shape and structure (Figures 3A and B). Cell damage including shrinkage, deformation, and enlargement of mitochondria were observed in response to NP treatments. Both the 10 and 50 nm citrate- and PVP-coated Ag-NPs also induced changes in cell morphology at 50 µg/ml (Figures 3A and B). Cells treated with these coated NPs appeared elongated and enlarged. Similar to uncoated particles, these coated versions also resulted in enlarged mitochondria. Both uncoated and coated Ag-NPs were visible inside the cells on the TEM images (arrows in Figures 3B).

3.5. ROS generation
The effect of Ag-NPs on the production of ROS was studied by quantifying the fluorescence from confocal micrographs of oxidised DHE in J774A.1. Treatments with uncoated 20 and 40 nm Ag-NPs at 1 µg/ml resulted in slightly increased intensity and area (1.2-1.3 fold, p<0.05) of fluorescence from DHE oxidation compared to the controls, indicating slight ROS generation (Figures 4A and B). Similar observation was made for treatments with 10 nm citrate-coated Ag-NPs (50 µg/ml). PVP-coated 10 and 50 nm Ag-NPs also induced slight increases in ROS generation, but the effects were not statistically significant (Figures 4A, and C). Treatment with menadione was used as the positive control for the assay (Figure 4A).
**Figure 3**: Change in cell morphology and sub-cellular structure induced by Ag-NPs. (A) Confocal micrographs of J774A.1 cells treated with uncoated and coated Ag-NPs for 24 h. The cells were stained for F-actin (pink); nucleus is stained (blue). (B) TEM micrographs of J774A.1 cells treated with uncoated and coated Ag-NPs for 24 h. “m” stands for mitochondrion. Arrows point to the presence of intracellular Ag-NPs.

**Figure 4**: ROS production in J774A.1 cells. (A) Representative micrographs of control cells, cells treated with menadione, and cells treated with test Ag-NPs. The presence of ROS is indicated by red fluorescence. (B, C) The total area of red fluorescence (pixels squared: px²) was analysed with image analysis software (NIS Elements) using three micrographs for each sample. Each data point represents the mean ± standard deviation. The asterisks (*) indicate statistically significant differences compared to the control.
3.6. Oxidative stress induction
The total GSH levels and SOD activities were measured to examine oxidative stress induction in cells exposed to Ag-NPs. At 1 µg/ml, uncoated Ag-NPs significantly reduced GSH levels (1.2-1.7 fold, p<0.05) in both J774A.1 and HT29 cells. At the same dose, uncoated Ag-NPs also significantly increased SOD activities (1.6-1.8 fold, p<0.05) in J774A.1, but not in HT29 cells. These effects appeared to be size-dependent (Figures 5A and C). In contrast, citrate- and PVP-coated Ag-NPs did not affect GSH levels or SOD activities even at the highest test concentrations (Figures 5B and D).

3.4. Cytokine production
The effects of Ag-NPs on selected cytokine levels in test cells were investigated. Treatments of cells with 20 and 40 nm uncoated Ag-NPs at 1 µg/ml resulted in decreased TNF-α levels (1.2-1.4 fold, p<0.05) in J774A.1 cells and in IL-8 levels (1.3-1.4 fold, p<0.001) in HT29 cells, compared to the control (Figures 6A and C). In contrast, treatments of cells with coated NPs at 25 µg/ml led to elevated (1.2-1.5 fold, p<0.05) levels of these cytokines (Figures 6B and D). Similar effects were observed for IL-1β, and IL-12 (p70) levels in J774A.1 (Data not shown).

Figure 5: Effects of Ag-NPs on biomarkers of oxidative stress. (A) GSH levels in J774A.1 and HT29 cells after treatment with uncoated Ag-NPs for 24 h. (B) SOD activities in J774A.1 cells after treatment with uncoated Ag-NPs for 24 h. (C) GSH levels in J774A.1 cells after treatment with coated Ag-NPs for 24 h. (D) SOD activities in J774A.1 cells after treatment with coated Ag-NPs for 24 h. Data points represent the means of three independent experiments done in duplicate ± standard deviations. The asterisks (*) indicate statistically significant differences compared to the control at p<0.05.

4. Discussion and Conclusions
This study aimed at investigating the impact of physicochemical properties, such as surface coating and size, on the toxicity of Ag-NPs in two different mammalian cell lines, namely
J774A.1 macrophage and HT29 epithelial cells. These cell lines are well-characterised cell models and have been previously used to assess the pathogenic effects of bacteria [30] and the toxicity of other NPs such as quantum dots [31].

Initial work showed that reduction in cell viability by uncoated Ag-NPs occurred at lower concentrations than for coated Ag-NPs, revealing that uncoated NPs are more cytotoxic than their coated counterparts. For coated Ag-NPs of the same size, PVP-coated NPs resulted in greater loss in cell viability compared to citrate-coated NPs, indicating surface coating-dependent toxicity. Our findings agree with a previous study, which compared the toxic effects of citrate-, PVP- and gum arabic (GA)-coated Ag-NPs on the nematode Caenorhabditis elegans. The authors reported that PVP-coated Ag-NPs were more toxic than citrate-coated Ag-NPs and demonstrated a linear correlation between Ag-NP toxicity and dissolved Ag$^+$ ions. The study also suggested that citrate from the coating was protective by forming chelation complexes with free Ag$^+$ ions and thus reducing availability of dissolved Ag$^+$ [29].

The greater cytotoxicity of uncoated Ag-NPs compared to coated Ag-NPs observed in the current study is contrary to a recent study which reported that uncoated Ag-NPs were less toxic than coated Ag-NPs in similar cell lines such as macrophage RAW264.7 and lung epithelial C-10 [28]. This previous study compared poly(diallyldimethylammonium) chloride, bionic, oleate-coated and uncoated colloidal Ag-NPs sized from 4.0 to 9.0 nm and found that uncoated Ag-NPs were least toxic compared to coated counterparts. The reasons for the discrepancy between our findings and this report could be the difference in the composition of the coating materials and the

![Figure 6](image_url): Effects of a 24 h exposure to Ag-NPs on the production of cytokines in test cells. (A) Levels of TNF-$\alpha$ in J774A.1 cells after treatment with uncoated Ag-NPs. (B) Levels of IL-8 in HT29 cells after treatment with uncoated Ag-NPs. (C) Levels of TNF-$\alpha$ in J774A.1 cells after treatment with coated Ag-NPs. (D) Levels of IL-8 in HT29 cells after treatment with coated Ag-NPs. The asterisks (*) indicate statistically significant differences compared to the control (p<0.05).
size of test NPs examined. A number of studies have proposed that the toxicity of Ag-NPs is associated with the release of Ag\(^+\) ions from Ag-NPs [32, 33, 34, 35]. While the dissolution of Ag-NPs was not measured in this study, AgNO\(_3\) was used as the Ag\(^+\) ion source for comparative purposes. Our study showed that at equivalent silver concentrations, uncoated Ag-NPs showed greater toxicity than AgNO\(_3\). This suggests that silver dissolution cannot be the only factor contributing to uncoated Ag-NP-induced effects in the test cells. Additionally, coated Ag-NPs were less toxic than AgNO\(_3\) at equivalent silver concentrations, suggesting that coatings might have protective effects on the release of Ag\(^+\) ions or the mechanisms of toxicity of the Ag-NPs are different from the bulk material.

Coated and uncoated Ag-NPs induced loss in cell viability in a dose- and particle size-dependent manner. Our observations support claims from previous reports on dose- and size-dependent cytotoxicity of Ag-NPs [20, 26, 27]. A study in alveolar macrophages reported that exposure to 15 nm hydrocarbon-coated Ag-NPs led to a significant decrease in metabolic activity, as assessed by the MTT redox assay, compared to a marginal or non-significant effect from exposures to 30 and 55 nm particle sizes [20]. Another study examined the toxicity of Ag-NPs of different sizes (5, 20, and 50 nm) on human cell models including human lung adenocarcinoma A459, gastric carcinoma SGC-790, liver hepatocellular HepG2, and breast adenocarcinoma MCF-7 and reported that 5 nm Ag-NPs exposure resulted in highest cell viability loss (also assessed by MTT assay) and greater changes in cell morphology compared to its larger sized counterparts [26]. Similar findings were reported in a recent study that examined cellular toxicity of differently sized Ag-NPs on mouse osteoblastic MC3T3-E1 and rat adrenal pheochromocytoma PC12 cell lines [27].

Changes in gross cell morphology as examined by confocal microscopy corroborated our observations on the loss in cell viability. However, while uncoated Ag-NPs resulted in cell shrinkage, coated Ag-NPs induced cell elongation and enlargement, suggesting different mechanisms of cell damage. Examination of sub-cellular structures using TEM revealed mitochondrial enlargement after exposures to both uncoated and coated Ag-NPs at high test concentrations. Mitochondria are known to play a role in NP toxicity. For example, following NP exposures, mitochondrial structural damage, loss of mitochondrial membrane integrity, opening permeability transition pores, ROS production, and cell death may occur [36, 37]. Our observations support previous studies that linked Ag-NP toxicity to mitochondrial damage [10, 11, 21, 38, 39]. Further studies are needed to determine whether mitochondrial damage is the primary effect of Ag-NPs or is secondary to cell death stimulation from other cellular targets of the NPs.

Oxidative stress is one of the proposed mechanisms of Ag-NP toxicity [20, 40, 41]. Oxidative stress can be the consequence of the generation of intracellular ROS [38, 20, 10]. The depletion of GSH and a reduction of anti-oxidant enzyme activity might also be causes of oxidative stress in cells and animals [41, 42]. Ag-NPs have been reported to increase ROS generation from Ag\(^+\) ions released from the NPs or from the Ag\(^+\) on the NP surface [11, 20, 40, 35]. Alternatively, there is evidence that Ag-NPs are able to bind to thiol groups with high affinity which are found in thiol proteins/peptides such as GSH restricting the availability of these molecules for ROS neutralization and resulting in an oxidant-mediated response to ROS [2, 43]. A recent study using Chang liver cells also reported that Ag-NPs decreased GSH levels through the inhibition of GSH synthesizing enzymes [42]. In the present study, uncoated Ag-NPs at high test concentrations reduced GSH content and slightly increased ROS generation. This may be explained by the possible direct interaction of these Ag-NPs with GSH or by the inhibition of GSH-synthesizing enzymes by the Ag-NPs leading to an accumulation of ROS. The increase in SOD activities observed with uncoated Ag-NPs could be a result of excess ROS and oxidative stress in cells. In contrast, coated Ag-NPs exposure lead to non-significant changes in GSH and SOD suggesting oxidative stress might not be the primary toxic mechanism of these Ag-NPs.
Ag-NPs have been shown to have both stimulatory and suppressive effects on the production of cytokines [9, 20, 44, 45, 46]. In the present study, uncoated Ag-NPs decreased pro-inflammatory cytokines such as TNF-α, IL-1β, IL-12 (p70) and IL-8, while cells exposed to coated Ag-NPs had elevated levels of these cytokines. Inhibition of cytokine production by Ag-NPs has been reported [45, 46]. These reports found that at doses similar to ours, uncoated Ag-NPs strongly inhibited cytokine production of IL-1β, IL-6, and TNF-α in J774A.1 cells [45]. The inhibitory effects of Ag-NPs on the production of IL-8 and IL-11 were also reported in human mesenchymal stem cells (hMSCs) and on the production of IL-5, IFN-γ, and TNF-α in the peripheral blood mononuclear (PBMC) cell line [44, 46]. The inhibitory effects of Ag-NPs on cytokines have been suggested to result from the interaction of Ag-NPs with toll-like receptors (TLRs) located on the cell surface or in the endocytic compartments [47]. However, Ag-NPs were also found to increase the production of TNF-α, MIP-2 and IL-1β in alveolar macrophages [20]. Similarly, exposures of Ag-NPs to human epidermal cells led to an increase in IL-1β, IL-6, IL-8 and TNF-α [9]. These differential effects on cytokine production have been suggested to be dependent on dose and cell type [48]. In our study, for the same cell type, the threshold doses used for uncoated Ag-NPs were much lower than those used for coated particles. This difference in dose could explain the different effects observed on cytokine production. These effects need to be further investigated.

In summary, the present study demonstrates the effects of physicochemical properties on the toxicity of Ag-NPs. Our data suggest that uncoated Ag-NPs are more toxic than coated Ag-NPs and that the size and coat-type are also important contributing factors to NP toxicity. Cells treated with uncoated Ag-NPs appeared to undergo oxidative stress while coated-Ag-NPs seemed to induce cell damage through inflammatory pathways by up-regulating selected cytokines. This study provides toxicological data which might be helpful not only for assessing the potential health and environmental risks of Ag-NPs, but also for the safe design and use of these NPs.

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References

[1] Murphy C J, Sau T K, Gole A M, Orendorff C J, Gao J, Gou L, Hunyadi S E and Li T 2005  *J Phys Chem B* **109** 13857–13870.
[2] Lewis L N 1993  *Chem Rev* **93** 2693–2730.
[3] Marambio-Jones C and Hoek E MV 2010  *J Nanopart Res* **12** 1531–1551.
[4] Chen X and Schluesener H J 2008  *Toxicol Lett* **176** 1–12.
[5] Maynard AD 2006 Washington DC: WoodrowWilson International Center for Scholars.
[6] Ahamed M, Alsahi M and Siddiqui M 2010  *Clinica Chimica Acta* **411** 1841–1848.
[7] Lara H H, Garza-Trevino E N, Ixtepan-Turrent L and Singh D K 2011 J. *Nanobiotechnology*, 9 30.
[8] Eom H J and Choi J 2010  *Environ Sci Technol* **44** 8337–8342.
[9] Samberg M E, Oldenburg S J and Monteiro-Riviere N A. *Environ Health Perspect* **118** 407–413.
[10] Asharani P V, Mun G L K, Hande M P and Valiyaveetil S 2009  *ACS Nano* **3** 279–290.
[11] Hussain S M, Hess K L, Gearhart J M, Geiss K T and Schlager J J 2005 Toxicol In Vitro 19 975–983.

[12] Yang Z, Liu Z W, Allaker R P, Reip P, Oxford J, Ahmad Z and Ren G 2010 J R Soc Interface 7 411–422.

[13] Asharani P V, Wu Y L, Gong Z and Valiyaveettil S 2008 Nanotechnology 19 255102.

[14] Scown T M, Santos E M, Johnston B D, Gaiser B, Baalousha M, Mitov S, Lead J R, Stone V, Fernandes T F, Jepson M, van Aerle R and Tyler C R 2010 Toxicol Sci 115 521–534.

[15] Roh J Y, Sim S J, Yi J, Park K, Chung K H, Ryu D Y and Choi J 2009 Environ Sci Technol 43 3933–3940.

[16] Asghan S, Johan S A, Lee J H, Kim Y S, Jeon Y B, Choi H J, Moon M C and Yu I J 2012 J. Nanobiotechnol 10 14.

[17] Hyun J S, Lee B S, Ryu H Y, Sung J H, Chung K H and Yu I J 2008 Toxicol Lett 182 24–28.

[18] Sung J H, Ji J H, Yoon J U, Kim D S, Song M Y, Jeong J, Han B S, Han J H, Chung Y H, Kim J, Kim T S, Chang H K, Lee E J, Lee J H and Yu I J 2008 Inhal Toxicol 20 567–574.

[19] Rahman M F, Wang J, Patterson T A, Saini U T, Robinson B L, Newport G D, Murdock R C, Schlager J J, Hussain S M, Ali S F 2009 Toxicol Lett 187 15–21.

[20] Carlson C, Hussain S M, Schrand A M, Braydich-Stolle L K, Hess K L, Jones R L and Schlager J J 2008 J Phys Chem B 112 13608–13619.

[21] Hsin Y H, Chen C F, Huang S, Shih T S, Dai P S and Chueh P J 2008 Toxicol Lett 179 130–139.

[22] Ahamed M, Karna M, Goodson M, Rowe J, Hussain S M, Schlager J J and Hong Y 2008 Toxicol Appl Pharmacol 233 404–410.

[23] Samberg M E, Oldenburg S J and Monteiro-Riviere N A 2010 Environ Health Perspect 118 407–414.

[24] Oberdörster G, Oberdörster E and Oberdörster J 2005 Environ Health Perspect 113 823–839.

[25] Maynard A D, Warheit D B and Philbert M A 2011 Toxicol Sci 120 S109–S129.

[26] Liu W, Wu Y, Wang C, Li H, Wang T, Liao C Y, Cui L, Zhou Q F, Yan B and J G B 2010 Nanotoxicology 4 319–330.

[27] Kim T-H, Kim M, Park H-S, Shin U S, Gong M-S and Kim H-W 2012 J Biomed Mater Res Part A 100A 1033–1043.

[28] Suresh A K, Pelletier D, Wang W, Morrell-Falvey J L, Gu B, and Doktycz M J 2012 Langmuir 28 2727–2735.

[29] Yang X Y, Gondikas A P, Marinakos S M, Auffan M, Liu J, Hsu-Kim H and Meyer J N 2012 Environ. Sci. Technol. 46 1119–1127.

[30] Tayabali A F and Seligy V L 2000 Environ Health Perspect 108 919-930.

[31] Nguyen K C, Seligy VL and Tayabali AF 2012 Nanotoxicology Early Online 1-10.

[32] Park E J, Yi J, Kim Y, Choi K and Park K 2010 Toxicol In Vitro 24 872-878.

[33] Miura N and Shinozawa Y 2009 Biochem Biophys Res Commun 390 733–737.

[34] Meyer J N, Lord C A, Yang X Y, Turner E A, Badireddy A R, Marinakos S M, Chilkoti A, Wiesner M R and Auffan M 2010 Aquat Toxicol 100 140–150.

[35] Foldbjerg R, Olesen P, Hougaard M, Dang D A, Hoffmann H J and Autrup H 2009 Toxicol Lett 190 156–162.

[36] Li N, Sioutas C, Cho A, Schmitz D, Misra C, Sempf J, Wang M, Oberley T, Froines J and Nel A 2003 Environ Health Perspect 111 455–460.

[37] Xia T, Kovochich M and Nel A E 2007 Front Biosci 12 1238–1246.

[38] Foldbjerg R, Dang D A, and Autrup H 2010 Arch Toxicol 85 743–750.

[39] Teodoro J S, Simões A M, Duarte F V, Rolo A P, Murdoch R C, Hussain S M and Palmeira
C M 2011 Toxicol. In Vitro 25 664–670.

[40] Kim S, Choi J E, Choi J, Chung H, Park K, Yi J and Ryu D Y 2009 Toxicol In Vitro 23 1076–1084.

[41] Arora S, Jain J, Rajwade J M, and Paknikar K M 2008 Toxicol Lett 179 93–100.

[42] Piao M J, Kang K A, Lee I K, Kim H S, Kim S, Choi J Y, Choi J and Hyun J W 2011 Toxicol. Lett. 201 92–100.

[43] Navarro E, Piccapietra F and Wagner B, Marconi F, Kaegi R, Odzak N, Sigg L and Behra R 2008 Environ Sci Technol 42 8959–8964.

[44] Yen H J, Hsu S H and Tsai C L 2009 Small 5 1553–1561.

[45] Greulich C, Kittler S, Epple M, Muhr G and Koller M 2009 Langenbecks Arch Surg 394 495–502.

[46] Shin S H, Ye M K Kim H S and Kang H S 2007 Int Immunopharmacol 7 1813–1818.

[47] Castillo P M, Herrera J L, Fernandez-Montesinos R, Caro C, Zaderenko A P, Mejias J A and Pozo D 2008 Nanomed 3 627–635.

[48] Stensberg M C, Wei Q, McLamore E S, Porterfield D M, Wei A and Sepúlveda M S 2011 Nanomedicine 6 879–898.