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Mammalian cells exhibit a range of sensitivities to silver nanoparticles that are partially explicable by variations in antioxidant defense and metallothionein expression

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Abstract

While it is well known that there are interspecies differences in Ag sensitivity, we have also observed differences in the cytotoxic responses of mammalian cells to silver nanoparticles (Ag NPs). In order to explore these response outcomes, six cell lines, including epithelial cells (Caco-2, NHBE, RLE-6TN and BEAS-2B) and macrophages (RAW 264.7 and THP-1) of human and rodent origin were exposed to 20 nm citrate- and PVP-coated AgNPs with Au cores as well as 20 nm citrate-coated particles without cores. A MTS assay showed that while Caco-2 and NHBE cells were resistant to particles over a 0.1- 50 $\mu\text{g/mL}$ dose range, RAW 264.7, THP-1, RLE-6TN and BEAS-2B cells were more susceptible. While there were small differences in dissolution rates, there were no major differences in the cytotoxic potential of the different particles. However, we did observe differences in anti-oxidant defense and metallothionein expression among different cell types, which can partially explain differential AgNP sensitivity. So it is important to consider these differences in understanding the potential heterogeneous effects of nano Ag on mammalian biological systems.

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Keywords

silver nanoparticles; cytotoxicity; sensitivity; oxidative stress; heavy metal stress response

1. Introduction

We have included one sentence in “Introduction” to introduce the application of Ag NPs in biological and biomedical research: Silver nanoparticles (Ag NPs) are among the most commonly used nanomaterials, and have been widely applied in food, consumer products and medical products categories, such as wound dressing,^[1] catheter,^[2] prosthesis,^[3] athletic socks,^[4] bandage,^[5] laundry detergent.^[6] Numerous *in vitro* studies revealed that AgNPs are capable of cytotoxic effects in a broad range of mammalian cells, including germline stem cells,^[7] mesenchymal stem cells,^[8-9] BRL3A rat liver cells,^[10] NIH3T3 cells,^[11] HepG2 human hepatoma cells,^[12] normal human lung fibroblasts,^[13] human glioblastoma cells,^[14] human normal bronchial epithelial cells,^[15-16] mouse leukaemic monocyte macrophage,^[16] and Hela cells.^[17] However, there appears to be some variability in the sensitivity of different cell types^[18-19], including cells of the same lineage^[20] that has not been properly explained from a mechanistic perspective. This response heterogeneity was also underscored by response differences among a number of mammalian cell types under investigation by participants in the NIEHS Centers for Nanotechnology Health Implications Research (NCNHIR) consortium, which have been provided with a shared library of AgNPs to conduct safety studies. At first glance, the response differences cannot be explained simply according to the species of origin (human, mouse, rat), cell lineage (epithelial, macrophage, endothelial, hepatocyte, fibroblast, etc.), or primary vs transformed cell lines. Among the cell types studied by the UCLA Center of Nano Biology and Predictive toxicology, we hypothesized that mammalian cellular response differences could possibly reflect differences in the dissolution characteristics and Ag shedding by the nanoparticles, cellular uptake and bioavailability or variation in biological defense against metal ion toxicity or the generation of oxidative stress, which play key roles in nano Ag toxicity.^[14, 16, 19, 21]

For the purpose of this study, we chose six cell lines from three species (human, mouse and rat) to investigate the cytotoxic potential and cellular defense against nano Ag in epithelial cells (Caco-2, NHBE, RLE-6TN and BEAS-2B) and macrophages (RAW 264.7 and THP-1). We used three commercially available AgNPs, namely 20 nm citrate- and PVP-coated AgNPs synthesized with gold (Au) cores, as well as a 20 nm citrate-coated AgNPs without Au cores. Our results showed that while Caco-2 and NHBE cell lines were resistant to AgNP-induced cytotoxicity, RAW 264.7, THP-1, RLE-6TN and BEAS-2B cell had variable susceptibility to the impact of the particles on cell viability. These differential cellular responses could be shown to be correlated to cellular antioxidant defense and metallothionein expression.

2. Results

2.1. Physicochemical characterization of citrate- and PVP-coated AgNPs

Three commercially available AgNPs, including citrate- and PVP-coated AgNPs synthesized with gold cores as well as citrate-coated AgNPs without Au cores, were selected for study. Au nanoparticles are frequently used as seeds for the synthesis of AgNPs with uniform size. PVP and citrate are two of the most common coating agents to obtain colloiddally stable particles. TEM showed all the particle types had spherical shapes, with uniform primary sizes of ~20 nm. More specifically, the citrate-coated and PVP-coated particles with Au cores have primary sizes of 19.4 ± 3.0 nm and 18.7 ± 3.7 nm, respectively, while the size of the AgNPs without cores were 22.0 ± 3.5 nm (Figure 1 and Table 1). STEM and EDX results confirmed that the presence of ~10 nm Au cores in the former two materials (Figure 1). All AgNPs exhibited narrow size distributions in water, with hydrodynamic sizes of 26.6 ± 0.2 nm, 30.1 ± 0.3 and 34.0 ± 0.2 nm for citrate- and PVP-coated nanoparticles with cores and citrate-coated NPs without cores, respectively (Table 1). Their mono-dispersiveness could be attributed to the electrostatic repulsion of the negatively charged citrate and PVP layers on the surface. This was reflected by zeta potential values of -20.6 mV, -28.4 mV and -35.9 mV, respectively (Table 1). We also determined particle dispersion and size distribution in the cell culture media to be used in this study, namely MEM (Caco-2), BEGM (BEAS-2B and NHBE), DEME (RAW264.7), RPMI-1640 (THP-1) and F12 medium (RLE-6TN) (Table 2). Because of the salt content of the culture media leading to charge neutralization and the shrinkage of the electric double layer, particle agglomeration could be seen, as shown in Table 2. However, all the hydrodynamic sizes remained in the nanoscale range with relatively narrow size distributions, and therefore demonstrate good particle dispersiveness. Moreover, all the particles maintained a negative zeta potential in culture medium (Table 3). Finally, we also used ICP-OES to measure particle dissolution in water, BEGM and DMEM media. This demonstrated that although the dissolution rates were similar in water and DMEM, it was slightly higher in BEGM (Figure 1D). There were minimal differences between particles with and without Au cores.

2.2. Differential cytotoxicity to nano Ag among comparison cell lines

We used the MTS assay to assess the cytotoxic effects of the AgNPs in 6 cell lines, using a dose range of 0-50 $\mu\text{g/mL}$ for 24 hr (Figure 2A-C). This demonstrated that Caco-2 and NHBE cells were relatively resistant to particle treatment, regardless of the surface coating or the presence or absence of a Au core. However, the AgNPs did significantly reduce the viability of RAW 264.7, THP-1, RLE-6TN and BEAS-2B cells, with RLE-6TN and BEAS-2B showing particular sensitivity to all particle types (Figure 2).

2.3. Use of ICP-OES to assess cellular uptake of AgNPs

We chose citrate-coated AgNPs with Au cores to investigate the impact of cellular uptake and Ag bioavailability on the cytotoxic response to this material. We chose a particle dose of 6.25 $\mu\text{g/mL}$ over 24 hr to avoid the impact of cell death on cellular uptake. ICP-OES analysis was used to quantify the elemental Ag content of the cells after thorough washing of the cell pellets. While there were significant differences in the cellular Ag content, the resistant cell lines, Caco-2 and NHBE, showed the highest Ag content while the most

sensitive cells, RLE-6TN and BEAS-2B, showed a significantly lower values (Figure 3). Moreover, the less sensitive THP-1 and RAW 264.7 cells showed the lowest Ag content. The same trend was seen with assessment of cellular Ag content after treatment with PVP-coated Ag NPs with Au cores (Figure S1, Supporting information). All considered, these data show that, in spite of the differences in cellular uptake, the ICP-OES results do not correlate with the cytotoxic effects of the particles, suggesting that mechanisms other than bioavailability are responsible for the differential cytotoxicity.

2.4. Use of GSH levels and heme oxygenase 1 (HO-1) expression to compare the role of anti-oxidant defense in AgNP toxicity

One of the principal mechanisms for AgNP-induced toxicity is the generation of oxidative stress.^[14, 16, 19, 21] However, we have previously shown that oxidative stress is a hierarchical series of events that involve three tiers of oxidative stress.^[22-24] Tier 1 is equivalent to an anti-oxidant response that is controlled by the erythroid-related 2-like 2 (Nrf-2) transcription factor. Through its interaction with the antioxidant response element (ARE), Nrf-2 is capable of inducing the expression of over 200 phase II enzymes, which play a role in the homeostatic control of oxidative stress.^[22, 24] These include enzymes that control the cellular content of glutathione (GSH), such as GSH synthetase, as well as hemeoxygenase 1 (HO-1), which has antioxidant and anti-inflammatory effects.^[22, 24] Since the tripeptide, GSH, plays a key role in the homeostatic defense against oxidative stress, we assessed the cellular GSH content by a luminescence-based GSH-Glo™ assay. Cells were treated with 0-50 µg/mL citrate-coated AgNPs for 24 hours. The GSH-Glo™ assay showed a significant and dose-dependent decline of GSH levels in RAW264.7, THP-1, RLE-6TN and BEAS-2B cells without a significant effect on Caco-2 or NHBE cells (Figure 4A). Interestingly, Caco-2 and NHBE cells also express a significantly higher total cellular GSH content under basal conditions (Figure 4B). All considered, the GSH data are consistent with the cytotoxic effects of AgNPs, as determined by the MTS assay. To further demonstrate the involvement of redox equilibrium, we also performed an analysis, in which the cells were pre-treated with buthionine sulfoximine (BSO), an inhibitor of gamma-glutamylcysteine synthetase (γGCS).^[25-26] BSO is theoretically helpful in determining whether a reduction of cellular GSH levels could lower the threshold of toxicity to pro-oxidative materials, such as nano Ag.^[25-26] Utilizing the MTS assay, we showed that BSO alone does not cause cytotoxicity, with concentrations as high as 40 mM (Figure S2A, Supporting information). Pretreatment of our cell lines with 5 mM BSO for 6 h showed that exposure to 0-50 µg/mL citrated-coated Ag NPs with Au cores resulted in a significant increase of cytotoxicity in THP-1, RLE-6TN and BEAS-2B cells at doses 25 µg/mL. In contrast, the cytotoxicity of NHBE and RAW264.7 only began to increase at 50 µg/mL (Figure S2B, Supporting information). For Caco-2 cells there was no significant change in cellular susceptibility at all dose levels.

We also assessed HO-1 expression through the use of Western blotting (Figure 5). The cells were exposed to 6.25 or 12.5 µg/mL AgNPs for 24 hr. The results showed that AgNPs exposure could induce HO-1 expression in resistant (Caco-2 and NHBE) as well as susceptible cells (RAW 264.7, THP-1, RLE-6TN and BEAS-2B) in dose-dependent fashion (Figure 5). Different from the GSH results, however, we did not observe any evidence of

response differences between resistant vs susceptible cell lines. This could be indicative of AgNP-induced effects on the generation of oxidative stress beyond tier 1.

2.5. Differential metallothionein (MT) expression among AgNP-exposed cell lines

In addition to oxidative stress, Ag and nano Ag are also capable of binding to and activating the metal-regulatory transcription factor 1 (MTF-1) to activate metallothionein (MT) gene expression via the metal response element (MRE).^[27-29] One molecule of MT is capable of complexing up to 12 Ag atoms.^[30-32] Immunoblotting was used to determine MT expression in cell lines exposed to 0, 6.25 and 12.5 µg/mL AgNPs for 24 hours. Caco-2 and NHBE cells showed a dose-dependent increase in MT expression (Figure 6A) while RAW 264.7, THP-1 and RLE-6TN cells did not show increased expression (Figure 6B). Interestingly, however, the latter cell types showed MT expression after CdCl₂ treatment (Figure S3, Supporting information). This is in favor of a failure of a specific defense against AgNPs in these cell lines, but not for BEAS-2B cells, which showed nano Ag susceptibility in spite of MT expression.

3. Discussion

In this study, we investigated possible mechanisms that are involved in the differential susceptibility to AgNP toxicity in six different cell types, including Caco-2, NHBE, RAW 264.7, THP-1, RLE-6TN and BEAS-2B cells. Cellular exposure to three 20 nm particle types did not reveal a significant impact of differences in the the surface coating, presence of Au cores or dissolution rates in determining cell lethality. Caco-2 and NHBE cells were resistant to the particles up to 50 µg/ml while RAW 264.7, THP-1, RLE-6TN and BEAS-2B cells showed variable susceptibility. The possible mechanism(s) contributing to these response differences were further investigated using the citrate-coated particles with Au cores. While there were small differences in dissolution rate and significant differences in cellular uptake, these variables did not correlate with cytotoxicity. Instead, we observed that differences in GSH levels, the cellular response to particle-induced oxidative stress and MT expression show some correlation with particle toxicity, to the degree that the protective effect of these pathways appear to contribute to the finding susceptibility to nano Ag in mammalian cells. Although we did not determine the Ag ion toxicity in this study, we did focus on the effects of the particle supernatants in our previous work,^[16] which demonstrated that Ag⁺ shedding contributes to particle toxicity. While Ag ion shedding also contributed to toxicity in this study, we do not think that different rates of dissolution in different cell and tissue culture media played a role in explaining the cellular differences in this study, because the dissolution rates of these 3 particles were similar in different media (Figure 1D).

There is good evidence to suggest that generation of ROS and oxidative stress play a major role in AgNP-induced cytotoxicity.^[14, 16, 19, 21] This could involve direct AgNP particle effects as well as dissolved Ag ions.^[33-34] Ag ions bind directly to GSH,^[35-37] which could lead to its depletion and making cells more vulnerable to additional sources of oxidative stress such as ROS.^[26] Moreover, Ag ions can also bind the thiol groups of a host of proteins, which can impair their function, including promoting release Fe²⁺ from metal

binding proteins.^[38] The Fe^{2+} could generate ROS through the Fenton reaction. Regarding direct particle effects, recent studies have shown that the surface of AgNPs could participate in Fenton-like reactions under acidic conditions, with the ability to generate hydroxyl radicals.^[39-40] ROS generation can also take place in mitochondria as a result of AgNP uptake into endosomes/lysosomes and subsequent release of Ag ions, which could interfere in electron transfer.^[14] It is also important to consider the impact of particle shape and surface defects, as reported for Ag nanoplates.^[21] For instance, we have demonstrated that crystalline defects, such as stacking and point defects, on the surface Ag nanoplates contribute directly to ROS generation and particle reactivity, which can be passivated by defect coating with cysteine.^[21]

Detailed investigation has been performed to explain the role of NP-induced ROS generation in terms of the hierarchical oxidative stress hypothesis.^[22, 24] Briefly, at the lowest level of oxidative stress (Tier 1), the induction of antioxidant and detoxification enzymes is mediated by the transcription factor, Nrf2, which induced the expression of phase II enzymes. At higher levels of oxidative stress (Tier 2), this protective response transitions to pro-inflammatory effects based on the activation of redox-sensitive signaling pathways such as MAP kinase and NF- κ B cascades. At the highest level of oxidative stress (Tier 3), perturbation of mitochondrial electron transfer and permeability transition pore can trigger cellular apoptosis and cytotoxicity.^[41] The tier 1 response is mediated by the release of the transcription factor, Nrf2, to the nucleus where interaction with the antioxidant response element in the promoters of >200 phase II enzyme genes is capable of inducing protective effects against oxidative stress. This includes the expression of HO-1 as well as numerous phase II enzymes that are responsible for the maintenance of GSH and other redox equilibrium pathways. GSH, HO-1, and phase II enzymes act synergistically to neutralize the ROS and to restore redox equilibrium. A previous study has demonstrated that the Nrf2 pathway is important in cellular susceptibility to nano Ag.^[28] The present study also demonstrates that all cell lines show increased HO-1 expression (Figure 5). Although we did not observe any correlation between HO-1 expression and cytotoxicity, this does not mean that all phase II enzymes are controlled identically. It is well known that the expression profile of individual phase II enzymes differ across different cell types.^[42] Moreover, we did observe differences in the total cellular GSH content, which did show good correlation to the cytotoxicity profiling to nano Ag (Figure 4). Numerous phase II enzymes are involved in the homeostatic regulation of the cellular GSH content.^[43-45] However, the exploration of the integrated response profile of phase II enzymes in relation to AgNP exposure will warrant an independent study.

Besides the antioxidant defense, heavy metal detoxification system has also been reported as another protective mechanism against Ag and nano Ag toxicity.^[46-47] Initiation of this defense pathway is dependent on the activation of the MTF-1 transcription factor, which is responsible for MT protein expression and the regulation of cellular responses to heavy metal-induced stress.^[46-47] Each MT molecule contains 20 cysteine residues,^[30-32] which could play a role in chelation and removal of potentially 12 Ag ions per MT molecule, in addition to the ability of neutralizing ROS.^[30-32] Different abilities to activate the MTF-1 signaling pathway and to induce MT expression could therefore determine the sensitivity of different cell types to Ag NPs. This is compatible with a previous report that MT expression

plays an important role in protection against AgNP toxicity,^[27] as well as the current data showing that the resistant cell lines (Caco-2 and NHBE), show increased MT expression while three of four susceptible cell lines (RAW 264.7, THP-1 and RLE-6TN) failed to respond similarly (Figure 6). However, the full explanation is likely to be more complicated in light of the finding BEAS-2B cells, a nano Ag sensitive cell line, also show increased MT expression. Thus, MT expression is likely only one of multiple components that contribute to nano Ag susceptibility. It is also worth pointing out that there could be pathways unrelated to oxidative stress and heavy metal defense, such as autophagy, which could play a role in the susceptibility to nano Ag.

Differential cellular sensitivity to specific nanomaterial compositions has also been demonstrated for the materials other than nano Ag. For instance, polystyrene NPs have been shown to be toxic in RAW macrophage and BEAS-2B epithelial cells, but not in endothelial cells at comparable doses.^[23] Also, palladium NPs are toxic to bronchial epithelial but not to A549 cells,^[48] while ceria NPs preferentially kill SCL-1 squamous carcinoma cells but not dermal fibroblasts.^[49] While the mechanistic explanation of these differences goes to the heart of studies on nanomaterial safety, comparatively few mechanistic studies have been undertaken to explain cellular response differences to specific NP formulations. The present study, which only addressed limited number of NPs and cell lines, serves to illustrate the incompleteness of the database about the heterogeneity of responses at the cellular nano/bio interface. Our study also points out the importance of the necessity to undertake microarray-based global gene expression analysis, as well as involving other omics approaches to fill the data gap that is required for a more comprehensive mechanistic and predictive toxicological approach to the understanding of nanomaterial safety.

4. Conclusions

We have investigated a limited number of mechanistic cellular responses to a small collection of Ag NPs to show response heterogeneity among 6 different cell lines, with some but not absolute evidence for a correlation to differences in protective cellular responses, including antioxidant defense and MT expression. Differences in the cellular responsiveness did not show a relationship to surface coating, presence of an Au core, cellular uptake or dissolution of the NPs. While this does not address all the possible factors that can contribute to response heterogeneity, antioxidant defense and metal chelation do seem to present important response variables that need to be considered in developing a complete understanding.

5. Experimental Section

5.1. Chemicals

All chemicals were reagent grade and used without further purification or modification unless otherwise indicated. Reagent grade water used in all experimental procedures was obtained from a Milli-Q water purification system (Millipore, Bedford, MA). Citrate-coated Ag NPs with Au cores, PVP-coated Ag NPs with Au cores and citrate-coated Ag NPs without a core were obtained from nanoComposix (San Diego, CA) and made available to participants of the NIEHS Consortium (NCNHIR).

5.2. Physicochemical characterization of silver nanoparticles

TEM was performed on samples dispensed from aqueous suspension onto a carbon-coated TEM grid using a JEOL 2010 microscope operating at 200 keV. Particle size and zeta potential in solution were measured by using a ZetaSizerNano (Malvern Instruments Ltd., Worcestershire, UK).

5.3. Cell culture

We used the following cell lines: Caco2 (human colonic carcinoma, ATCC, Manassas, VA), NHBE (normal human bronchial epithelial cells, Walkersville, MD), BEAS-2B (human bronchial epithelial cell lines, Lonza), RLE-6TN (rat alveolar type II epithelial cell lines, ATCC, Manassas, VA), RAW 264.7 (mouse macrophages cell lines, ATCC, Manassas, VA) and THP-1 (human monocytic leukemia cell lines, ATCC, Manassas, VA). These cells were cultured in vented T-75 cm² flasks (Corning, Fisher Scientific, Pittsburgh, PA) at 37°C in a humidified 5% CO₂ atmosphere, and passaged at 70–80% confluency every 2–4 days. Caco-2 cells were cultured in Minimum Essential Medium (MEM) containing 20% fetal bovine serum (FBS). RAW 264.7 and THP-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) and RPMI 1640 medium containing 10% FBS, respectively. RLE-6TN cells were cultured in Ham's F12 medium, supplemented with L-glutamine, bovine pituitary extract (BPE), insulin, insulin growth factor (IGF)-1, transferrin, and epithelial growth factor (EGF), and 10% FBS. NHBE and BEAS-2B cells were cultured in bronchial epithelial basal medium (BEBM) (Lonza, Walkersville, MD), supplemented with growth factors from the SingleQuot kit (Lonza) to make BEGM. All these medium were supplemented with 100 U/mL penicillin, 100 µg/mL streptomycin, and 2 mM L-glutamine.

5.4. Cell viability assessment

Cell viability was determined by a MTS assay, which was carried out with a CellTiter 96® AQueous (Promega Corporation) kit. 1×10^4 cells in 100 µL of culture medium were plated in each well of a 96 multi-well black plate (Costar, Corning, NY) for overnight growth. The medium was removed and cells treated for 24 h with 100 µL of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.25, 12.5, 25 and 50 µg/mL nanoparticle suspensions. Subsequently, the cell culture medium was removed and the plates washed three times with PBS. Each well received 100 µL of culture medium containing 16.7 % of the MTS stock solution for an hour at 37°C in a humidified 5% CO₂ incubator. The plate was centrifuged at $2000 \times g$ for 10 min in the Eppendorf 5430 with microplate rotor to spin down the cell debris. 80 µL of the supernatant was removed from each well and transferred into a new 96 well plate. The absorbance of formazan was read at 490 nm on a SpectraMax M5 microplate spectrophotometer (Molecular Devices Corp., Sunnyvale, CA, USA).

5.5. Assessment of cellular Ag content by ICP-OES analysis

The THP-1 Ag content was determined by ICP-OES analysis.^[16] 4×10^4 THP-1 cells were seeded in each well of a 24-well plate and grown overnight in 400 µL culture medium, containing 6.3 µg/mL nanoparticles for 6 h. After treatment, the cells were gently washed three times with PBS and harvested in 500 µL PBS. The cell suspension was digested in 3 mL concentrated nitric acid at 90° C for 3 h. The digested solution was dried by evaporation

at 120° C, and 8 mL of 5% nitric acid was added for ICP-OES analysis, using by a Shimadzu ICP-OES.

5.6. Western blot analysis for HO-1 and MT expression

1.6×10⁵ cells from each line were seeded into the wells of six-well plates (Costar, Corning, NY). After overnight culture, each well received 1.6 mL of the appropriate culture medium containing 6.25 and 12.5 µg/mL of citrate-coated AgNPs suspension for an additional 24 hours. Untreated cells were used as a control. Cells were washed with PBS three times and harvested by scraping. The cell pellets were resuspended in cell lysis buffer containing Triton X-100 and protease inhibitors. After sonication and centrifugation, the protein content of the supernatants were measured by the Bradford method and 30 µg protein from each sample was electrophoresed by 10% SDS-PAGE and transferred to a PVDF membrane. After blocking, the membranes were incubated with anti-HO-1 monoclonal antibody (1:1000) (ENZO Life Sciences, Plymouth Meeting, PA, USA) or anti-metallothionein monoclonal antibody (1:1000) (Abcam, Cambridge, MA, USA).^[50] The membranes were overlaid with biotinylated secondary antibody (1:1000) before the addition of HRP-conjugated avidin-biotin complex (1:10,000). The proteins were detected using the ECL reagent according to the manufacturer's instructions.

5.7. Assessment of cellular GSH content

1×10⁴ cells from each line were plated in 100 µL of the appropriate culture medium into 96 well white plates (Costar, Corning, NY) for 14hr. The medium was removed and cells treated with 100 µL of 0.1, 0.2, 0.4, 0.8, 1.6, 3.2, 6.3, 12.5, 25 and 50 µg/mL nanoparticle suspension for 24 h. The culture medium was removed, and each well was washed three times with PBS. Following the addition of 100 µL of the GSH-Glo™ reagent (Promega Corporation) for 0.5 h at room temperature, 100 µL of the Luciferin Detection Reagent was added for an additional 15 min at room temperature. The luminescence intensity of each well was recorded on a SpectraMax M5 microplate spectrophotometer.

5.8. Statistical analysis

All data were expressed as mean ± SD. All values were obtained from three independent experiments. Statistical significance was evaluated using two-tailed heteroscedastic Student's *t*-tests according to the TTEST function in Microsoft Excel. The significant difference between groups was considered statistically significant when the *p*-value was lower than 0.05.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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C

D

Figure 1. Physicochemical characterization and dissolution analysis of Ag NPs

(A) TEM, STEM and EDX analysis of citrate-coated Ag NPs with Au cores; (B) TEM, STEM and EDX analysis of PVP-coated Ag NPs with Au cores; (C) TEM, STEM and EDX analysis of citrate-coated Ag NPs without Au cores; (D) Dissolution analysis of Ag NPs in water, BEGM and DMEM medium, using ICP-OES. 1 mL of 200 $\mu\text{g/mL}$ Ag NPs (three types) in these media were kept at 37 $^{\circ}\text{C}$ for 24 hours, respectively. The supernatants, which were harvested after centrifugation at 10,000 rpm for 30 minutes, were digested with concentrated nitric acid at 90 $^{\circ}\text{C}$ for 3 h. A diluted solution was used for ICP-OES analysis. Each experiment had three replicates and each data were expressed as mean $\pm$ standard deviation.

C Citrate Ag NPs without Au cores

Figure 2. MTS assay to study the cytotoxic potential of AgNPs in different cell lines

Cells were treated with 0.1-50 μg/mL of each of the following Ag NPs for 24 h: (A) cells exposed to citrate-coated Ag NPs with Au cores; (B) cells exposed to PVP-coated Ag NPs with Au cores; (C) cells exposed to citrate-coated Ag NPs without Au cores. Cell viability was determined by the MTS assay. Each experiment had three replicates and each data were expressed as mean ± standard deviation. * $p < 0.05$ compared to control.

Figure 3. Cellular Ag content by ICP-OES after exposure to citrate-coated Ag NPs with cores
Cells were treated with 6.25 µg/mL particles for 24 h. After washing in PBS, cells were harvested using 0.05% trypsin, and the cell suspension digested with concentrated nitric acid at 90° C for 3 h. The digested solution was diluted for ICP-OES measurement. Each experiment had three replicates and each data were expressed as mean± standard deviation.
* $p < 0.05$ compared to Caco-2; # $p < 0.05$ compared to NHBE.

Figure 4. GSH assessment in Caco-2, NHBE, RAW 264.7, THP-1, RLE-6TN and BEAS-2B cells after exposure to citrate-coated Ag NPs with cores

(A) Percent change of the GSH content in cells after treated with 0-50 µg/mL of citrated-coated AgNPs for 24 hours. (B) GSH content of different cell lines. Cellular GSH levels were determined by the luminescence-based GSH-Glo™ assay. GSH levels were expressed as a percentage of the luminescence intensity compared to control cells (100%). Each experiment had three replicates and each data were expressed as mean± standard deviation. * $p < 0.05$ compared to control.

Figure 5. HO-1 expression determined by immunoblotting

All cell lines were treated with 0, 6.25 and 12.5 μg/mL citrate-coated Ag NPs for 24 h. Cell extracts were used to assess HO-1 expression by immunoblotting. (A) HO-1 expression in resistant cell lines (Caco-2 and NHBE cell lines); (B) HO-1 expression in susceptible cell lines (RAW 264.7, THP-1, RLE-6TN and BEAS-2B cell lines).

Figure 6. Metallothionein expression in different cell lines after exposure to citrate-coated Ag NPs with Au cores
 (A) metallothionein expression in resistant cell lines (Caco-2 and NHBE cell lines); (B) metallothionein expression in susceptible cell lines (RAW 264.7, THP-1, RLE-6TN and BEAS-2B cell lines). Cells were treated with 0, 6.25 and 12.5 μ g/mL citrate-coated Ag NPs for 24 h. The expressed MT was determined by western blotting.

Table 1
Physicochemical properties of Ag NPs

Ag nanoparticles	Primary size (nm)	Hydrodynamic size in water (nm)	Zeta potential in water (mV)
Citrate-Ag NPs with Au cores	19.4±3.0	26.6±0.2	-20.6±2.3
PVP-Ag NPs with Au cores	18.7±3.7	30.1±0.3	-28.4±1.0
Citrate-Ag NPs/without Au cores	22.0±3.5	34.0 ± 0.2	-35.9 ± 1.3

Table 2
Hydrodynamic sizes (nm) of Ag NPs in different cell culture media

Ag nanoparticles	EMEM (20%FBS)	BEGM (2mg/mLBSA)	DMEM (10%FBS)	RPMI 1640 (10%FBS)	F12 (10%FBS)
Citrate-Ag NPs with Au cores	133.1±1.0	275.4±2.9	93.2±0.1	91.4±2.6	94.3±0.1
PVP-Ag NPs with Au cores	90.5±1.2	233.7±5.0	77.7±1.1	104.3±5.1	111.3±6.1
Citrate-Ag NPs without Au cores	98.5 ± 0.4	337.7 ± 2.7	100.8 ± 1.1	148.4±0.8	90.0 ± 0.2

Table 3
Zeta potential (mV) of Ag NPs in different cell culture medium

Ag nanoparticles	EMEM (20%FBS)	BEGM (2mg/mLBSA)	DMEM (10%FBS)	RPMI 1640 (10%FBS)	F12 (10%FBS)
Citrate-Ag NPs with Au cores	-17.0±1.2	-12.87±0.9	-11.0±3.9	-13.2±2.4	-8.6±1.2
PVP-Ag NPs with Au cores	-8.2±2.2	-8.8±1.9	-8.8±2.6	-8.2±3.8	-6.9±2.1
Citrate-Ag NPs without Au cores	-17.6±4.2	-12.5±1.7	-14.0± 0.8	-12.8±2.4	-6.6±1.9