Fast intracellular dissolution and persistent cellular uptake of silver nanoparticles in CHO-K1 cells: implication for cytotoxicity

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Abstract
Toxicity of silver nanoparticles (Ag NPs) has been reported both in vitro and in vivo. However, the intracellular stability and chemical state of Ag NPs are still not very well studied. In this work, we systematically investigated the cellular uptake pathways, intracellular dissolution and chemical species, and cytotoxicity of Ag NPs (15.9 \pm 7.6 nm) in Chinese hamster ovary cell subclone K1 cells, a cell line recommended by the OECD for genotoxicity studies. Quantification of intracellular nanoparticle uptake and ion release was performed through inductively coupled plasma mass spectrometry. X-ray absorption near-edge structure (XANES) was employed to assess the chemical state of intracellular silver. The toxic potential of Ag NPs and Ag\textsuperscript{+} was evaluated by cell viability, reactive oxygen species (ROS) production and live–dead cell staining. The results suggest that cellular uptake of Ag NPs involves lipid-raft-mediated endocytosis and energy-independent diffusion. The degradation study shows that Ag NPs taken up into cells dissolved quickly and XANES results directly indicated that the internalized Ag was oxidized to Ag–O-- species and then stabilized in silver–sulfur (Ag–S--) bonds within the cells. Subsequent cytotoxicity studies show that Ag NPs decrease cell viability and increase ROS production. Pre-incubation with N-acetyl-L-cysteine, an efficient antioxidant and Ag\textsuperscript{+} chelator, diminished the cytotoxicity caused by Ag NPs or Ag\textsuperscript{+} exposure. Our study suggests that the cytotoxicity mechanism of Ag NPs is related to the intracellular release of silver ions, followed by their binding to SH-groups, presumably coming from amino acids or proteins, and affecting protein functions and the antioxidant defense system of cells.

Introduction
Due to their antibacterial activity, silver nanoparticles (Ag NPs) are broadly employed in a variety of house-hold goods and healthcare products (Chopra, 2007; Cohen et al., 2007; Fu et al., 2006). Widespread use of Ag NPs has caused concerns about their potential harmful effects on both the environment and humans. Studies have revealed that Ag NPs induce cytotoxicity and genotoxicity in vitro and in vivo (Ahamed et al., 2008; AshaRani et al., 2008; Flower et al., 2012; Jiang et al., 2013; Lima et al., 2012). The mechanism of Ag NP toxicity has been discussed, and several hypotheses have been formulated. Among these, Ag\textsuperscript{+} release (Kittler et al., 2010; Liu & Hurt, 2010) and reactive oxygen species (ROS) production (Cheng et al., 2013; Foldbjerg et al., 2011) have been suggested as the most likely. Several papers have reported Ag\textsuperscript{+} release from Ag NPs as causing toxicity. Studies suggest that Ag NPs induce toxicity through a so-called Trojan-horse mechanism, where NPs are taken up by cells and subsequently release ions intracellularly, leading to cell death (Lubick, 2008). In addition, our previous results showed that the initial Ag\textsuperscript{+} fraction in Ag NPs suspensions contributes greatly to the cytotoxicity of Ag NPs (Beer et al., 2012). Recently, Gui-bin Jiang’s group used a Triton X-114-based cloud point extraction method to separate intracellular Ag NPs and released Ag\textsuperscript{+}. They found 10.3% of silver as Ag\textsuperscript{+} in cells after 24 h exposure, compared to 5.2% Ag\textsuperscript{+} in the pristine Ag NPs suspension (Yu et al., 2013). N-acetyl-L-cysteine (NAC) acts as an antioxidant and was also proposed as a silver ion scavenger, as thiol groups have high affinity for Ag\textsuperscript{+}. Studies showed that pre-incubation with NAC reduced the toxicity of Ag NPs exposure (Foldbjerg et al., 2011; Yang et al., 2011).

In this study, the Triton X-114-based cloud point extraction method and XANES were applied in order to study the intracellular dissolution of Ag NPs and the chemical species resulting from binding of the dissolved silver ions inside cells. To gain further understanding of the toxicity mechanism of Ag NPs and derivatives, the cytotoxicity of Ag NPs was compared to that of Ag\textsuperscript{+}.

Keywords
Degradation, N-acetyl-L-cysteine, reactive oxygen species, silver nanoparticles, uptake pathway, XANES

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Materials and method

Materials

Ham’s F-12K (Kaighn’s) medium (F-12K medium), heat-inactivated fetal bovine serum (FBS), l- alanyl-l-glutamine, penicillin and streptomycin were purchased from Invitrogen (Taastrup, Denmark), 2′,7′-dichlorodihydrofluorescein diacetate (H2DCF-DA), 7-AAD, 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), dimethyldisulfide (DMSO), HEPEs, phosphate buffered saline (PBS), NaCl, CaCl2, AgNO3, HNO3, NaBH4, sodium citrate and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (Copenhagen, Denmark). Calcein-AM and propidium iodide (PI) were purchased from Donjindo (Beijing, China). Triton X-114 was purchased from Solarbio (Beijing, China). Na2S2O3 and H2O2 were purchased from Beijing institute of chemical reagent (Beijing, China). NAC was purchased from Amresco (Solon, OH).

Silver nanoparticles

Ag NPs were synthesized by chemical reduction of silver nitrate with sodium borohydride in sodium citrate as described previously (Beer et al., 2012). The particles were characterized by transmission electron microscopy (TEM), UV-visible spectrophotometry, atomic absorption spectroscopy (AAS) and dynamic light scattering. Technical details of primary and in situ characterization methods are described in our previous publications (Beer et al., 2012; Foldbjerg et al., 2009).

Cell culture

The Chinese hamster ovary cell subclone K1 (CHO-K1) cell line was purchased from the German Collection of Microorganisms and Cell Cultures (DSMZ, ACC-110, Braunschweig, Germany). Cells were cultured in F-12K medium supplemented with 5% heat-inactivated FBS, penicillin (100 U/mL), streptomycin (100 μg/mL) and glucose (2 mM), hereafter referred to as cell culture medium, and maintained at 37°C in a humidified atmosphere of 5% CO2.

For all exposure experiments, cells were seeded in culture dishes or well plates one day prior to the exposure in cell culture medium. For exposure studies, Ag NPs stock suspension (or water for controls) was diluted in cell culture medium. The silver concentrations used in the study refer to the mass concentration of silver atoms.

Inductively coupled plasma mass spectrometry analysis

Inductively coupled plasma mass spectrometry (ICP-MS; Thermo Elemental X7, ThermoFisher Scientific, Inc., Roskilde, Denmark) was employed to quantify the silver from Ag NPs taken up by cells. Briefly, cells were exposed to 10 μg/mL Ag NPs for 3, 6, 12 and 24 h, respectively. After incubation, the cells were washed with PBS at least three times, trypsinized and collected by centrifugation (TLA 100 rotor, Beckman Coulter Danmark ApS, Copenhagen, Denmark). The cell number was assessed with a blood cell counting chamber. The cell pellet was soaked in 70% HNO3 overnight and heated to about 140°C until the solution was colorless and clear, indicating the complete evaporation of nitrogen oxides. The samples were diluted up to 3 mL with a 2% nitric acid aqueous solution, and then the silver content was analyzed using ICP-MS, with indium (20 ng/mL) as an internal standard. The concentration of elemental silver in solution was obtained, and the amount of silver taken up by 10^6 cells was calculated.

Uptake pathway

To study the internalization pathway of Ag NPs in CHO-K1 cells, several uptake pathway inhibitors were used, including chlorpromazine hydrochloride (1 μg/mL), methyl-β-cyclo-dextrin (MβCD) (10 mM), nystatin (5 μg/mL), dynasore (80 μM). For all the inhibitors, we used a concentration that did not induce significant cell death. Briefly, cells were seeded in 6-cm plates until 70% confluency and pre-incubated either with FBS-free medium at 4°C or with uptake inhibitors at 37°C for 1 h, prior to nanoparticle exposure. Subsequently, cells were washed once with PBS and incubated with Ag NPs either in FBS-containing medium at 4°C or together with uptake inhibitors at 37°C for 3 h. After incubation, cells were washed with PBS three times to remove most of the Ag NPs on the cell surface. The cells were then trypsinized, counted and the silver content was quantified by ICP-MS.

The release of silver ions in cell culture medium

The ion release behavior of citrate-capped, BSA pre-coated Ag NPs was evaluated at different pH values and various time points using cell culture medium relevant for in vitro studies. In the experiments, particles were spun down at 50,000 rpm and 4°C for 30 min (TLA 100 rotor, Beckman Coulter Danmark ApS, Copenhagen, Denmark) using an OptimatTM TLX Personal Benchtop Ultracentrifuge (Beckman Coulter Danmark ApS, Copenhagen, Denmark). The supernatant was discarded to eliminate any ions released during storage, and the particles were re-dispersed in the cell culture medium (F-12K supplemented with 5% FBS). This system will be further referred to as ‘particle stock dispersions’. Samples (from here on referred to as ‘pH working samples’) were prepared for incubation by mixing 700 μL of medium with 100 μL of particle stock dispersion and obtaining the desired pH value (4.5 or 7) by adding the required amount of 1% HNO3 aqueous solution (titration curves for the cell culture medium were previously obtained). The pH was measured using a S2K712 pocket-sized pH meter (Isfetcom, Japan) with a silicon micro sensor.

The pH working samples were incubated at 37°C and 5% CO2 for times ranging from 1 to 24 h. After incubation, they were spun down as described above, and the supernatants were further processed for subsequent measurement of silver ions. Each sample was measured in duplicate. Samples were prepared by digesting 300 μL of supernatant with 300 μL of 65% HNO3 for 30 min, followed by dilution with MilliQ water up to a volume of 4 mL, appropriate for measurements. Silver content was measured by flame atomic absorption spectroscopy (F-AAS) on a Perkin Elmer Analyst 300 AAS mounted with a silver lumina hollow cathode lamp (Perkin Elmer, Copenhagen, Denmark).

The ion release was expressed as percentage of total silver considering the concentrations of the particle stock dispersions. All experiments were performed in triplicate.

Triton X-114-based cloud point extraction for intracellular Ag NPs and Ag+ isolation

The Triton X-114-based cloud point extraction for intracellular Ag NPs and Ag+ isolation was performed as described elsewhere (Yu et al., 2013), with minor modifications. CHO-K1 cells were seeded in 6-cm cell culture dishes until 70% confluency was reached. They were subsequently exposed to 10 μg/mL Ag NPs for 1, 3, 6, 12 and 24 h, respectively. Following the exposure period, the cell culture medium was discarded, and the cells were washed with PBS at least three times. Then, the cells were trypsinized and centrifuged at 2000 rpm for 3 min. The cells were resuspended in 1 mL PBS, and cell numbers were assessed using a blood cell counting chamber. The suspensions (1 mL) were collected in a 10 mL centrifuge tube, and the cells were disrupted by ultrasonication. Control cells were cultured without Ag NP exposure, and the nanoparticles were added to the cells after...
ultrasonication. The samples were adjusted to 9 mL by adding ultrapure water, and the pH was adjusted to 3.5 with 10% aqueous HNO₃ solution. Subsequently, 0.2 mL of 1 mol/L Na₂S₂O₃ and 0.2 mL of 10% (w/v) TX-114 were added to the mixture in this order. The components were mixed together, and the resulting system was incubated in a water bath at 40 °C, for 30 min, and then centrifuged at 3000 rpm at room temperature for 5 min to facilitate phase separation. The nanoparticles in the triton phase were soaked by adding HNO₃ and H₂O₂, followed by silver quantification with ICP-MS. The fractions of Ag⁺ and Ag NPs were calculated according to the amounts of silver in the water and Triton phase, respectively. Silver uptake per 10⁵ cells were calculated based on the total silver amount and the cell number.

**XANES**

XANES was employed to study the chemical species of the internalized and adsorbed Ag. Briefly, cells were seeded in several 20 cm cell culture dishes until 70% confluency and exposed to different concentrations of Ag NPs or Ag⁺ for 3, 6, 12 and 24 h, respectively. After exposure, the cells were washed three times with PBS to remove the Ag adsorbed onto the cell surface as much as possible. Then the cells were trypsinized, pelleted through centrifugation and freeze dried (ThermoFisher Scientific, Yokohama, Japan, RVT4104 and SPD121P). The dried cells were stored in a sealed tube filled with nitrogen gas to protect them from the oxidation in air and finally pressed into a pellet with a 3 M tape film (3 M, Tokyo, Japan) before measurement. XANES data of silver K-edge were recorded at the beamline BL-14W1 of the Shanghai Synchrotron Radiation Facility in China. The transmission mode was used for the reference samples (Ag foil, Ag₂S, Ag₂O, AgCl and AgNO₃, obtained from Sigma Aldrich, Tokyo, Japan), while the fluorescence mode was adopted for the dried powder of BSA-Ag NPs and cell samples exposed to Ag NPs with a 32 element germanium solid-state detector. All of the XANES spectra data were normalized and then analyzed with least squares fitting (LSF) to calculate the ratio of silver species using IFEFFIT Athena software (CARS, the Consortium for Advanced Radiation Sources, at The University of Chicago).

**MTT assay**

Viability of CHO-K1 cells after Ag NP exposure was analyzed using the MTT assay, as described previously (Foldbjerg et al., 2011). Cells were seeded in 96-well plates with 8 × 10⁴ cells per well and exposed to Ag NPs for 3, 6, 12 and 24 h, respectively. After exposure, cells were washed with PBS and incubated with 0.5 mg/mL MTT in cell culture medium for 2 h at 37 °C. Following incubation, the MTT solution was discarded, DMSO (100 μL/well) was added and the plate was shaken for 2 min. The optical density was read on a microplate reader at 570 nm, with a reference at 655 nm (EL800, Bio-Tek Instruments, Inc., Winooski, VT). The cell viability of Ag NP-treated cells was normalized to the control (without Ag NPs exposure).

**Reactive oxygen species**

Intracellular ROS were measured using the oxidation-sensitive fluoroprobe H₂DCF-DA, employing a method described previously (Hayashi et al., 2012) with minor modifications. Briefly, cells were seeded in six-well plates with 5 × 10⁴ cells per well and exposed to different concentrations of Ag NPs or Ag⁺ for 3, 6, 12 and 24 h, respectively. Following exposure, the cells were washed once with PBS, then H₂DCF-DA (10 μM) fluorescent marker was added to each well and samples were incubated at 37 °C for 30 min. Subsequently, the cells were washed with PBS, harvested, resuspended in 500 μL PBS containing 1 μg/mL 7-AAD and analyzed by flow cytometry (Cell Lab Quanta SCML, Beckman Coulter Danmark ApS, Copenhagen, Denmark). The 488 nm laser was used for excitation, and fluorescence was detected in FL-1 using a 525 BP filter and FL-3 using a 600 LP filter. For each sample, the mean fluorescence of 2 × 10⁴ cells was determined.

**Effect of NAC on cytotoxicity induced by Ag NPs or Ag⁺ exposure**

Cells were seeded in 96-well plates until 70% confluency was reached. Prior to exposure, the cells were pre-incubated with 10 mM NAC for 1 h. Following pre-incubation, the cells were washed with PBS and exposed to Ag NPs or Ag⁺ for 12 h, respectively. Cell viability was analyzed with the MTT assay (as described above) and live–dead cell staining. For the live–dead assay, the cells were incubated with calcein-AM (2 μM) and PI (2 μg/mL) for 30 min at 37 °C. Images were collected using a fluorescence microscope (OLYMPUS, 1 × 71, Ballerup, Denmark) under 100 × magnification. The Ex/Em for calcein is 494/517 nm and the Ex/Em of PI is 535/617 nm.

**Statistical analysis**

All experiments were performed in at least three independent experiments. Data are expressed as mean ± standard deviation of the independent experiments. The statistical significance was determined by one-way analysis of variance followed by Dunnett test. Statistical analysis was performed in Origin Pro 8.

**Results**

**Cellular uptake kinetics of Ag NPs in CHO-K1 cells**

Ag NPs (mean particle size = 15.9 ± 7.6 nm, n = 490) were used in this study (the characterization of the particles has been described previously (Beer et al., 2012)). ICP-MS was employed to quantify the amount of silver associated with and taken up by CHO-K1 cells after 3, 6, 12 and 24 h exposure. Although the cells were extensively washed, upon analysis it cannot be excluded that some Ag NPs were still bound or attached to the cell surface. As shown in Figure 1, the profile of Ag NPs associated with the reference samples (Ag foil, Ag₂S, Ag₂O, AgCl and AgNO₃, obtained from Sigma Aldrich, Tokyo, Japan), while the fluorescence mode was adopted for the dried powder of BSA-Ag NPs and cell samples exposed to Ag NPs with a 32 element germanium solid-state detector. All of the XANES spectra data were normalized and then analyzed with least squares fitting (LSF) to calculate the ratio of silver species using IFEFFIT Athena software (CARS, the Consortium for Advanced Radiation Sources, at The University of Chicago).

![Figure 1](image)

**Figure 1. Time-dependent cellular uptake of Ag NPs in CHO-K1 cells.** Cells were incubated with 10 μg/mL Ag NPs for 3, 6, 12 and 24 h, respectively. The data are presented as mean value ± standard deviation of three independent experiments.
Figure 2. Cellular uptake pathway for Ag NPs internalization in CHO-K1 cells. Prior to Ag NP exposure, cells were pre-treated with uptake inhibitor chlorpromazine hydrochloride, MβCD, nystatin, dynasore or kept at 4°C. Cellular uptake of Ag NPs was quantified by ICP-MS. The data are presented as mean value ± standard deviation of three independent experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA), and asterisks denote significant difference from the control (only Ag NP treated) (p < 0.05).

Figure 3. Time and pH-dependent ion-release study of Ag NPs. Ag NPs were incubated in cell culture medium for 1, 3, 6, 12 and 24 h, respectively. After centrifugation, the Ag⁺-containing supernatants were collected and measured by F-AAS. The data are presented as mean value ± standard deviation of three independent experiments.

Uptake pathway of Ag NPs in CHO-K1 cells
To get a better understanding of the mechanisms involved in the uptake process, the uptake pathway of Ag NPs into CHO-K1 cells was studied in more detail. Several uptake inhibitors, including chlorpromazine hydrochloride, MβCD, nystatin, dynasore and low temperature were used to block cellular uptake pathways. Chlorpromazine hydrochloride is a specific inhibitor of clathrin-mediated endocytosis. MβCD and nystatin inhibit lipid raft-mediated endocytosis. Dynasore is a specific inhibitor of dynamin-mediated lipid-raft endocytosis and low temperature inhibits energy-dependent endocytosis pathways. Internalized and cell-associated silver was quantified and compared to the control. The results show that internalization of Ag NPs was significantly inhibited by MβCD (42%), dynasore (62.7%) and low temperature (70.1%), but not by other uptake inhibitors, suggesting that lipid-raft mediated endocytosis, energy-dependent uptake pathways but also energy-independent diffusion are involved in the uptake of Ag NPs (Figure 2).

Silver ion release in culture medium
We studied the kinetics of Ag⁺ release from nanoparticles by incubating Ag NPs in cell culture medium for 1, 3, 6, 12 and 24 h, respectively. Experiments were performed at pH 4.5 and 7.0, with the lower pH value mimicking the intracellular lysosomal environment, while physiological pH corresponds to that of the cell culture medium and the cytoplasm. Our results show that more silver ions are released at pH 4.5 than pH 7.0. However, the overall ion release is low, with only 7.5% Ag⁺ at pH 4.5 and 5% at pH 7.0, after 24 h incubation in cell culture medium (Figure 3).

Silver ion release inside cells
To further investigate the real conditions of silver ion release in cells, we incubated cells with 10 μg/mL Ag NPs for 1, 3, 6, 12 and 24 h. After isolating both nanoparticulate and ionic silver from cells by Triton X-114-based cloud point extraction and quantification with ICP-MS, the Ag⁺ fraction was calculated by dividing the amount of silver ions by the sum of nanoparticulate and ionic silver. As seen in Figure 4(A), the cellular uptake of total silver increases over time, from 5.03 ± 0.91 (ng) Ag/10⁵ cells after 1 h incubation to 152.4 ± 46.1 (ng) Ag/10⁵ cells after 24 h incubation. Figure 4(B) shows that, after 12 h, only 5.7% of Ag NPs introduced in the cell culture medium was present as Ag⁺, while 83.2% of Ag NPs that were taken up by cells were present as Ag⁺ inside cells. Figure 4(C) shows that Ag NPs dissolved very rapidly after being taken up into cells, with 55% of total silver dissolved inside cells after 1 h incubation and 80% after 24 h.

XANES detects the chemical species of intracellular Ag
Assessment of the intracellular chemical species of silver was done using XANES. The XANES spectra are shown in Figure 5. The data were normalized and then analyzed with LSF to calculate the ratio of silver species. As shown in Table 1, the content of silver existing as Ag⁺ decreased from 80.7% in the stock suspension to 38.5% in cells after 24 h incubation. This observation is coupled with an increase in the amount of silver bound to oxygen (Ag−O−) to 14.2% after 12 h incubation and Ag−O− was no longer detectable after 24 h incubation. Silver existing as Ag−S− increased from 18.3% in the stock solution to 61.5% in the cells after 24 h incubation. These results indicate that the chemical species of silver in cells change overtime from Ag⁺ to Ag−O− to Ag−S− form.

Cell viability and intracellular ROS
The MTT assay was used to measure the mitochondrial activity as sign for Ag NP-induced toxicity in CHO-K1 cells at different time-points. Results show that Ag NPs induce a time- and
concentration-dependent decrease in cell viability; however, the concentration dependency is much less pronounced (Figure 6A). Furthermore, we used flow cytometry to measure the intracellular ROS production as a consequence of Ag NP or Ag+ exposure. As seen in Figure 6(B), exposure to either Ag NPs or Ag+ elevates the intracellular ROS level. Furthermore, at the same silver concentration and exposure duration, silver ions increase ROS production two times more than Ag NPs.

**Effect of NAC on cytotoxicity induced by Ag NPs or Ag+ in CHO-K1 cells**

NAC is a ROS scavenger and Ag+ chelating agent. To measure the effect of NAC on the cytotoxicity of Ag NPs or Ag+ to CHO-K1 cells, MTT and live–dead cell staining were employed. As shown in Figure 7(A) and (B), the decrease in cell viability was reduced when cells were pre-incubated with NAC. This effect is seen with both nanoparticles and ions, but it is more pronounced for Ag+. Furthermore, at a NAC concentration of 10 mM, only 3.9% and 24.81% cytotoxicity was observed after exposure to 10 μg/ml Ag NP or Ag+ for 12 h, respectively.

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Table 1. The content of Ag in different chemical state before and after expose to CHO-K1 cells.

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As shown in Figure 7(B), exposure to either Ag NPs or Ag+ elevates the intracellular ROS level. Furthermore, at the same silver concentration and exposure duration, silver ions increase ROS production two times more than Ag NPs.
Discussion

Studies of the hazard potential of nanomaterials are of great importance due to their widespread production and application. Ag NPs have been utilized in various products, especially in food packaging and healthcare products, due to their broad spectrum antibacterial properties. However, Ag NPs have been shown to induce cytotoxicity and genotoxicity, including increased levels of intracellular ROS, decreased cell viability, induction of DNA breakage and chromosome damage (AshaRani et al., 2008; Foldbjerg et al., 2009, 2011; Jiang et al., 2013; Kim et al., 2010).

In this study, we investigated the uptake pathway and intracellular fate of Ag NPs in more detail. We have previously used TEM to show that Ag NPs are taken up by CHO-K1 cells (Jiang et al., 2013). The uptake of Ag NPs into cells is a time-dependent process, with increased amount of nanoparticles in cells over time. However, Ag NPs may have been adsorbed onto the surface of cell membranes, due to the binding to molecules containing –SH group. Even though the cells were washed several times after Ag NPs incubation in order to remove nanoparticles attached to cell surface, it cannot be guaranteed that all the nanoparticles were washed away. Therefore, the real uptake of Ag NPs may be lower than the values obtained in this study. Several authors have reported that different pathways, such as clathrin-mediated and caveole-mediated endocytosis, phagocytosis and pinocytosis (Nel et al., 2009; Verma & Stellacci, 2010; Zhao et al., 2011), are involved in the uptake process of nanomaterials into cells. To investigate the cellular uptake pathway for Ag NPs internalization into CHO-K1 cells, several chemical uptake inhibitors and low temperature were used in this study. Our results show that cells incubated at 4°C or treated with MβCD or dynasore exhibit a significant decrease in uptake of Ag NPs, indicating that Ag NPs are mainly taken up via an energy-dependent and lipid-raft-mediated endocytosis pathway. However, at 4°C, cells are still able to take up Ag NPs, suggesting that energy-independent uptake pathways may also be involved in the internalization of Ag NPs. A previous study showed that both clathrin- and lipid raft-dependent endocytosis are involved in the internalization of gold nanorods in A549, 16HBE and MSC cells (Wang et al., 2011). Parameters such as nanoparticle composition, size and surface chemistry may play a significant role in establishing the preferred uptake pathways for a specific nanomaterial (Zhao et al., 2011).

Our previous study, looking at the gene expression of A549 cells after Ag NPs and Ag⁺ exposure, indicated that Ag NPs, due to their particulate form, affect exposed cells in a more complex way than Ag⁺ (Foldbjerg et al., 2012). Several studies suggest that the mechanisms for Ag NPs-induced toxicity involve silver ion release inside cells (Kittler et al., 2010; Yang et al., 2011). Smaller Ag NPs are more toxic than larger nanoparticles due to a higher surface to volume ratio, which may facilitate silver ion release (Kim et al., 2012; Wang et al., 2014). This is also supported by our finding that a silver ion chelating agent, NAC, reduces the cytotoxicity of the nanoparticles. Among the suggested mechanisms, a Trojan-horse model was proposed to explain the mode of Ag NPs toxicity. Nanoparticles are taken

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Figure 6. Cytotoxicity of Ag NPs in CHO-K1 cells over time. CHO-K1 cells were exposed to Ag NPs for 3, 6, 12 and 24 h, respectively. (A) After exposure, cell viability was evaluated by MTT assay. (B) Intracellular ROS levels were analyzed with fluorescent dye H2DCF-DA by flow cytometry. The data are presented as mean value ± standard deviation of three independent experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA), and asterisks denote significant difference from the control ($p < 0.05$).

Figure 7. Cytotoxicity of Ag NPs (A) or Ag⁺ (B) with or without pre-incubation with 10 mM NAC. The cells were pre-incubated with/without 10 mM NAC for 1 h and then exposed to Ag NPs or Ag⁺ for 12 h. Cell viability was evaluated by MTT assay. The data are presented as mean value ± standard deviation of three independent experiments. Statistical significance was calculated by one-way analysis of variance (ANOVA), and asterisks denote significant difference between NAC treated and untreated control ($p < 0.05$).
up and then release Ag⁺ inside cells, which induce intracellular ROS production, decreased mitochondrial activity and induce cell death. In order to investigate the silver ion release from Ag NPs and to mimic the intracellular environment, we incubated Ag NPs in cell culture medium at pH 4.5 and pH 7.0 and measured the Ag⁺ release by F-AAS. We found that Ag⁺ release in the medium was lower than expected, with only 7.5% at pH 4.5 and 5% at pH 7.0 after 24 h incubation in culture medium. However, pH is not the only parameter that is different inside cells compared to our simple experimental setting: the presence of small and macro molecules and the dynamic exchanges within cells can influence ion release as well. The Triton X-114-based cloud point extraction method described by Gui-bin Jiang’s group (Yu et al., 2013) allows the separation of silver ions released from intracellular Ag NPs.

Using this method, we found that 10.3% of intracellular silver is released as Ag⁺ after 24 h, suggesting that pH has only a minor role in Ag NP dissolution. In addition, our results show that increase in exposure time leads to more particles that are taken up, which, in turn, subsequently causes an accumulation of Ag⁺ inside cells because of nanoparticle dissolution. This process may induce a time-dependent cytotoxicity of Ag NPs.

A recent study demonstrated that sulfidation of Ag NPs could significantly decrease the toxicity to killifish and duckweed, as a result of decreased Ag⁺ concentration due to the lower solubility of Ag₂S relative to elemental Ag (Ag₀) (Levard et al., 2013). XANES has been applied as a very good method to study the degradation and bio-interaction of nanoparticles in biological systems (Qu et al., 2011; Wang et al., 2013). Therefore, in this study, we used XANES to study the intracellular fate of Ag NPs in CHO-K1 cells after different incubation duration. Our finding suggests that 14.2% of the silver taken up into cells was oxidized to Ag−O− after 12 h incubation. Subsequently, upon longer incubation (24 h), 61.5% of the silver is present as Ag−S−, suggesting that binding of Ag to sulfide groups of amino acids and proteins may be involved in the dissolution of Ag NPs. This intracellular Ag−S− binding can be toxic to cells as, first of all, binding of Ag⁺ to proteins may disrupt the protein structure and function thereby resulting in toxic effects. Secondly, the intracellular ROS scavenger, GSH, which was shown to be an efficient Ag⁺ chelator (Lok et al., 2007), is an important redox balancer in cells. Binding of Ag⁺ to GSH could reduce the amount of available GSH in cells, which will, in turn, induce imbalance of intracellular redox levels. Some speculated that the toxicity of Ag NPs is induced by increased intracellular ROS levels (Kim et al., 2011; Navarro et al., 2008), while other scientist believe that toxicity could be caused through both ROS-dependent and ROS-independent pathways (Chairuangkitti et al., 2012). To investigate the cytotoxicity of Ag NPs over time, the MTT assay was employed in this study. Results showed that the cytotoxicity of Ag NPs increased overtime, which is in agreement with the time-dependent intracellular silver ion release. ROS production has been suggested as the toxicity mechanism for most nanoparticles. In regards to silver, there are two distinct opinions: one proposed mechanism claims that Ag directly increases intracellular ROS levels, while the other claims Ag leads to a decrease of antioxidants, such as GSH, thereby indirectly elevating ROS amounts. Our results also showed increased intracellular ROS after CHO-K1 cells were exposed to either Ag NPs or Ag⁺. NAC and vitamin C (Vit C) were reported as efficient ROS scavengers, and NAC is also a good Ag⁺ chelating agent (Guo et al., 2013). A study comparing NAC with Vit C showed that both NAC and Vit C completely reverse the generation of ROS upon Ag NPs; however, only NAC and not Vit C could significantly protect cells from loss of MMP, DNA damage and apoptosis, suggesting silver ion chelation is critical.
in reducing Ag toxicity (Guo et al., 2013). Therefore, we here pre-incubated cells with NAC and then exposed them to either Ag NPs or Ag+. Results showed an important increase in the viability of cells exposed to Ag NPs or Ag+, more significantly for the Ag+ treated ones. In agreement with the MITT data, the live–dead cell staining experiments also showed a decrease in the number of dead cells for the NAC pre-incubated systems. This suggests that NAC is chelating Ag+, thus diminishing the amount of silver ions available for bio-interaction and subsequently decreasing cytotoxicity.

Conclusions

In this work, we systematically studied the cellular uptake, intracellular dissolution and cytotoxicity of Ag NPs in CHO-K1 cells. Our results suggest that Ag NPs are taken up into CHO-K1 cells by lipid-raft-mediated endocytosis and energy-independent diffusion. More than half (55%) of the internalized Ag NPs dissolved into Ag+ after 1-h incubation and dissolution increased over time. Furthermore, the intracellular silver was shown to oxidize to Ag—O— after 12-h incubation and finally stabilize as Ag—S— after 24-h incubation. Subsequent cytotoxicity studies showed the time-dependent decrease in cell viability and increase in ROS production. Cytotoxicity can be reduced by adding NAC as Ag+ chelating agent and ROS scavenger. We propose a toxicity mechanism that involves the uptake of Ag NPs into cells followed by their dissolution, oxidation and binding of Ag+ to —SH moieties of intracellular proteins, leading to a disturbance of the cellular redox balance, thereby generating an increase in the intracellular ROS levels and cytotoxicity. Future studies of the interaction of Ag NPs or Ag+ with —SH containing molecules should be performed to further understand the mechanisms of toxicity of Ag NPs and Ag+.

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Declaration of interest

The authors declare not to have any conflict of interest.

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