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Frontier article

Quantitative multimodal analyses of silver nanoparticle-cell interactions: Implications for cytotoxicity

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ABSTRACT

This study demonstrates the value of combining complementary analytical methods to assess the surface binding and internalization of AgNPs differing in primary size (10–70 nm) and surface (bPEI, PEG, citrate) in human T-lymphocytes, and to link this information with cytotoxicity of those AgNPs. Enhanced dark-field microscopy and imaging flow cytometry were used to semi-quantitatively assess cell–AgNP interactions while inductively coupled plasma mass spectrometry was used to quantitate those interactions. Extracellularly bound and intracellular AgNPs were distinguished by selective removal of cell surface bound NPs by chemical etching.

By taking advantage of the combined analytical methods, we were able to quantify the cell surface bound and intracellular Ag NPs which indicated significantly higher extracellular binding and cellular uptake of cationic bPEI-AgNPs compared to citrate- and PEG-AgNPs. Although the toxicity (IC50) of AgNPs was size and surface coating dependent, the intracellular Ag concentration at each IC50 was similar, varying only from 35–62 fg Ag/cell. This suggests that intracellularization is the main determinant of AgNPs toxicity in mammalian cells.

While this study demonstrates the benefits of the multimodal analysis to reveal cellular interactions with AgNPs, the methodology has a potential to be used for other types of NPs and for further applications in nanotoxicology and nanomedicine.

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1. Introduction

The rising prevalence of nanomaterials in consumer products makes assessment of the health risks associated with nano-sized particles imperative. Silver nanoparticles (AgNPs) are currently one of the most widely used group of NPs; they are present in about 30% of the consumer products listed in relevant NP-product inventories (www.nanotechproject.org; www.nanodb.dk). Noticeable toxicity of AgNPs towards various organisms as well as to human cells *in vitro* has been shown in a range of studies (Bondarenko et al., 2013). However, although the number of nanotoxicology-focussed papers on AgNPs is increasing, uncertainties exist in understanding the mechanisms of AgNP toxicity. It is not fully known whether the toxic effects of AgNPs are caused by particles present on the cell surface (and their local dissolution) or by intracellular AgNPs (AshaRani et al., 2009) (Gliga et al., 2014). This incomplete picture of cellular interactions with NPs is mainly due to the lack of suitable analytical methods enabling the visualization and quantification of those interactions. Although a general picture of the level of cell-NP association can be obtained using light microscopy (Ostrowski et al., 2015) or dark-field microscopy (Gibbs-Flournoy et al., 2011), these methods do often not achieve the required nanoscale resolution or sensitivity. Fluorescence microscopy in confocal and/or super-resolution modes can be used to visualize extra- and intracellular NPs at high resolution (Müller et al., 2012) but for that, NPs need to be specifically labelled. The most accurate demonstration of the mode of cellular interaction with NPs can be gleaned from electron microscopy analysis (Ostrowski et al., 2015). However, since the preparation cells for TEM samples involve fixation, drying and preparation of ultrathin slices, the process is laborious, with limited throughput and may result in the introduction of experimental artefacts. Most importantly, all the microscopy methods listed above enable only qualitative evaluation of cell–NP interactions and therefore cannot be directly linked to quantitative toxicological results. Flow cytometry has previously been proposed as a promising method to characterize the interactions between

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mammalian cells and NPs of TiO_2 (Suzuki et al., 2007) (Zucker et al., 2010) (Vranic et al., 2013), Ag (Suzuki et al., 2007) (Zucker et al., 2013), Fe_3O_4 (Suzuki et al., 2007), SiO_2 (Suzuki et al., 2007) (Vranic et al., 2013) (Choi et al., 2014) and carbon nanotubes (Marangon et al., 2012) but most of the studies pursuing this approach have only obtained qualitative information on cell-associated NPs.

In the study reported here, we applied a multimodal approach by using a combination of state-of-the-art analytical capabilities to advance the quantitative and qualitative understanding of AgNP–cell binding and internalization, and to correlate those processes with measured cytotoxicity effects. For quantitative analysis of cell–NP interactions (surface association and intracellularization), ICP-MS was used; semi-quantitative assessment of cell–NP interactions was carried out by imaging flow cytometry and these data were complemented by qualitative imaging with enhanced dark-field microscopy which is specifically designed for viewing of nanosize particles and depending of material, allows to visualize particles as low as tens of nanometers (Uchiyama et al., 2014). Extra- and intracellular particles were distinguished by means of a selective chemical etching procedure. This combination of analytical methods: ICP-MS that exhibits high sensitivity (detection of Ag at ng/mL range), imaging flow cytometry that is a relatively new method in cell-NP studies but enables high-throughput format, and dark-field microscopy that allows visualization at higher magnification than flow cytometry, enabled us to create novel visual and quantitative data for extracellularly bound and internalized AgNPs. This is a highly novel approach as most of the previous studies (Suzuki et al., 2007) (Zucker et al., 2010) (Zucker et al., 2013) (Vranic et al., 2013) (Choi et al., 2014) have relied on the assumption that NPs detected by flow cytometry analysis are localized in intracellular space. Furthermore, with the exception of Zucker et al. (2013) and Böhme et al. (2014), quantification of cell-associated NPs was not attempted in the previous studies. We studied a set of AgNPs with different surface coatings and primary sizes that were expected to exhibit different propensities for cell binding and uptake. The selected AgNPs included particles with primary sizes of 10, 30 and 70 nm with either cationic surfaces (branched polyethylene imine, bPEI), anionic surfaces (citrate), or near-neutral surfaces (polyethylene glycol, PEG). Since exposure of NPs *via* ingestion, dermal transfusion or inhalation eventually leads to systemic distribution throughout the blood stream (potentially compromizing the circulatory system) we decided to study cell–NP interactions using a human T-lymphocyte cell line (Jurkat) that is known to be sensitive to exogenous agents (Vecchio et al., 2014). An additional advantage of this choice is that this is a suspension cell line and therefore, does not require trypsinization that may alter cellular permeability and membrane structure and potentially introduce artefacts (Johnson and Johnstone, 1981). This comprehensive experimental design and the selection of complementary techniques allowed us to elucidate the impact of AgNP extracellular binding and internalization on cytotoxic effects.

2. Materials and methods

2.1. Chemicals and NPs

1000 μg/mL aqueous dispersions of branched polyethylene imine (bPEI), citrate and polyethylene glycol (PEG) coated AgNPs with 10, 30 and 70 nm primary sizes was purchased from Nanocomposix. The particles were diluted either in water (for TEM, hydrodynamic diameter and ζ-potential analysis) or cell culture medium (hydrodynamic diameter analysis and cellular assays). $AgNO_3$, components of the cell culture medium (RPMI-1640 medium, L-glutamine, Na-pyruvate, penicillin-streptomycin solution, glucose), Dulbecco's phosphate buffered saline (PBS), propidium iodide and fluorescein diacetate were from Sigma-Aldrich; fetal bovine serum (FBS) was from hyclone. HNO_3 and HCl were from Fisher Chemicals; potassium hexacyanoferrate(III) ($K_3Fe(CN)_6$) was from J. T. Baker and sodium thiosulfate pentahydrate ($Na_2S_2O_3x5H_2O$) was from Ajax Finechem, Univar. Water purified with a MilliQ system (18.2 mΩ) was used throughout the study.

2.2. Characterization of NPs

Transmission electron microscopy (TEM) images were taken using a JEM2100F (JEOL) at 200 kV. Suspensions of 50 μg AgNPs/mL in water were placed onto 300 mesh Cu grids covered by carbon film (ProSciTech) and dried overnight before imaging. The primary diameter of the AgNPs was measured using TEM images (Fig. A1) from which 20–30 NPs were counted and sized using the Gatan software (Gatan Inc.).

Hydrodynamic diameters (D_h) and surface charges (ζ-potential) of AgNPs were measured by dynamic light scattering (DLS) instrument PSS Nicomp 380 (particle sizing systems) in triplicate. D_h of NPs was measured from 20–80 μg AgNPs/mL dispersions in water and in cell culture medium (10% FBS). The dispersions in cell culture medium were incubated for 24 h at 37 °C and 5% CO_2 before measurement, to imitate the actual assay conditions with human cells. For ζ-potential measurements, ~100 μg AgNPs/mL water dispersions were used.

Dissolution of AgNPs in cell culture medium (10% FBS) was measured after incubation of 3 μg AgNPs/mL dispersions for 24 h at 37 °C and 5% CO_2. The dispersions were centrifuged using a Beckman Coulter Allegra 64R centrifuge (Beckman Coulter Inc.) at 57,000 *g* for 1.5 h and the upper half of the supernatant was carefully removed from the tubes (not to disturb the sediment in the lower half of the centrifuge tube). An aliquot of the supernatants (1.5 mL) was digested using 17% HNO_3 and 4% HCl (4 mL) in Teflon tubes for 10 min at 170 °C and 1800 W using a MARS6 microwave reaction system (CEM). The Ag content in the samples was measured by triple quadrupole ICP-MS (Agilent 8800) using single quad scan, He as collision gas (5 mL/min) and 1500 W plasma power. Experiments to measure the AgNP dissolution were carried out in duplicate. Statistical differences between dissolved Ag fractions for different NP primary sizes were analyzed using *t-test*.

2.3. Exposure of AgNPs to human T-lymphocyte cells

The Jurkat human T-lymphocyte cell line (Clone E6-1, ATCC® TIB-152™) was maintained in RPMI 1640 medium that contained 4.5 g glucose/L and was supplemented with Na-pyruvate (1 mM), L-glutamine (2 mM), penicillin–streptomycin (100 U/mL and 100 μg/mL, respectively) and 10% FBS. The cells were subcultured every 2nd–3rd day and the cell density was kept between 2×10^5 and 2×10^6 cells/mL. For AgNP exposures, 100 μL of cell suspension at 6×10^5 cells/mL was pipetted onto a transparent 96-well Nunclon Delta microplate (Thermo Scientific) and 100 μL of AgNPs dispersion at suitable concentration in cell culture medium was added. $AgNO_3$ was used as a control for dissolved Ag. In non-exposed controls, cell culture medium was added instead of AgNP suspensions. After the addition of test chemicals, the microplates were incubated at 37 °C and 5% CO_2 for 24 h and then analyzed for the specified endpoints (cytotoxicity and cellular interactions; see below).

2.4. Cytotoxicity assay

Reduction of resazurin to fluorescent resorufin (Ahmed et al., 1994) was used as an assay for cell viability. The cells were exposed to 0.1–100 μg AgNPs/mL cell culture medium or to 0.05–25 μg Ag^+ (in the form of $AgNO_3$)/mL cell culture medium. After 24 h exposure, resazurin dye was added to each well to give a final concentration of 150 μg/mL. To take into account the possible background fluorescence of the resazurin dye, the dye was also added to cell culture medium and cell culture medium with the respective concentration of test chemical but without cells. The microplates were incubated for 4 h at 37 °C, 5% CO_2. Then, the amount of resazurin metabolized to fluorescent resorufin was quantified with a fluorescence plate reader (Synergy, Biotek)

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