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Silver nanoparticle-induced cytotoxicity in rat brain endothelial cell culture

Susann Grosse [1], Lars Evje, Tore Syversen *

Department of Neuroscience, Norwegian University of Science and Technology, P.O. Box 8905 MTFS, NO-7491 Trondheim, Norway

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ABSTRACT

Silver nanoparticles (AgNPs) are among the most widely commercialised engineered nanomaterials, because of their antimicrobial properties. They are already commonly used in medical devices, household products and industry. Concerns have been raised about potential adverse health effects due to increasing dispersion of AgNPs in the environment. The present study examined the cytotoxic effects of spherical, citrate-coated AgNPs (10, 50 and 100 nm) in rat brain endothelial (RBE4) cells and investigated whether the observed effects can be explained by the intrinsic toxicity of the particles or the silver ions released from the particles. The results indicated that exposure of RBE4 cells to AgNPs lead to significant reduction in dye uptake as measured with the Neutral red (NR) assay. The effect was found to be related to particle size, surface area, dose and exposure time. In contrast, silver ions increased NR uptake (ca. 10%) in RBE4 cells after 1 h, while a reduction in NR uptake was observed after 24 h exposure at high concentrations (20–30 μM). Colony formation, as an indicator of proliferation ability, was completely inhibited by AgNPs at concentrations higher than 1 μg/ml. Silver ions had less effect on the colony formation of RBE4 cells than AgNPs.

1. Introduction

Nanotechnology is a fast growing field and engineered nanomaterials (ENMs) are already found in a vast variety of consumer products as well as industrial and medical applications (Salata, 2004; Thomas et al., 2006). ENMs are defined as particles with at least one spatial dimension equal to or <100 nm (Oberdorster et al., 2005). In addition to the size difference, nanomaterials also differ in other physicochemical properties from larger-sized materials of the same chemical composition. Unique properties associated with nanoparticles (NPs) are their shape, size and size distribution, surface structure and reactivity, agglomeration/aggregation state and chemical composition (Nel et al., 2006). These properties make them attractive additives in e.g. electronic devices. However, their effect in living organisms may differ from that observed during exposure to larger-sized materials of equal chemical composition (Maynard et al., 2011; Oberdorster, 2010). To assure a safe use of NPs engineered by nanotechnology, it is important to evaluate potential toxic effects of such particles in humans as well as the environment.

Humans can be exposed to NPs through different exposure routes, such as inhalation, ingestion, injection and dermal penetration. NPs are able to translocate from the site of entry to other organs like liver and kidneys (Oberdorster et al., 2002). It has also been reported that NPs can cross the blood–brain-barrier (Koziara et al., 2004; Oberdorster et al., 2004) and the testis–blood-barrier (Borm and Kreyling, 2004), as well as the placental barrier (Kulvietis et al., 2011; Wick et al., 2010).

AgNPs are widely used and commercialised today with a further increasing number of products containing nano-silver (Wijnhoven et al., 2009). AgNPs are most of all used in health care, medical devices and surgical instruments, because of their antimicrobial properties (Chen and Schluesener, 2008; Lansdown, 2006). They are also used as additives in textiles, food packaging materials, room sprays, refrigerators and various other consumer applications (Wijnhoven et al., 2009).

The toxicity of AgNPs towards microbial organisms, various cell lines and eukaryotic organisms can be found in the literature (Bilberg et al., 2010; Cha et al., 2008; Choi and Hu, 2008; El Badawy et al., 2011; Samberg et al., 2010; Sinha et al., 2011). Recently, several *in vitro* and *in vivo* studies on possible mechanisms of AgNP toxicity have been published. Among other effects, it has been found that AgNPs induce oxidative stress mediated cytotoxicity (Foldbjerg et al., 2009; Hussain et al., 2005), impairment of mitochondrial function (Teodoro et al., 2011), decreased viability and apoptosis (Arora et al., 2009; Miura and Shinohara, 2009) and genotoxicity (Hackenberg et al., 2011). Silver ions (Ag^+ ions) released from AgNPs are considered as an important parameter in the toxicity mechanism of these metal nanoparticles. So far, there are a few studies that investigated the role of Ag^+ ions in AgNP toxicity (Foldbjerg et al., 2011; Park et al., 2011; Powers et al., 2011),

* Corresponding author. Tel.: +47 7359 8848; fax: +47 7359 6879.
E-mail address: tore.syversen@ntnu.no (T. Syversen).
[1] Present address: Department of Surgical Sciences, Faculty of Medicine and Dentistry, University of Bergen, PO Box 7804, NO-5020 Bergen, Norway.

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but no clear conclusions about the degree of involvement of Ag^+ ions in AgNP toxicity have yet been drawn.

The aim of the present study was to investigate potential cytotoxic effects of spherical, citrate-coated AgNPs on a rat brain endothelial (RBE4) cell line which represent cells important for proper function of the blood–brain-barrier. Furthermore, this study aimed to examine the importance of Ag^+ ions – released from the particles – in the toxicity mechanism.

2. Materials and methods

2.1. Chemicals

Three different sizes (10 nm (AG10-BP), 50 nm (AG50-BP) and 100 nm (AG100-BP)) of spherical, citrate-coated AgNPs were purchased from Nanocomposix, Inc., San Diego. $AgNO_3$ (Cat. No. 101512, Merck) was used as Ag^+ ion-source. Lactate dehydrogenase (LDH) *in vitro* toxicology assay kit (TOX7), Giemsa staining (G-5637) and Neutral red dye (Cat. No. N7005) were bought from Sigma.

2.2. Characterisation of AgNPs

No characterization of the AgNPs (hereafter: Ag10, Ag50 and Ag100) beyond that of the manufacturer was done in our laboratory.

2.3. Dispersion of test materials in solution

The original AgNP-solutions (1 mg/ml) were supplied in 2 mM citrate buffer and diluted with ultrapure water (Purelab Ultra Analytic, Elga) to a stock solution of 100 μg/ml. This AgNP solution was vortexed vigorously (2 min at 2200 rpm) before further preparing the test solutions. From the stock solution, the final test concentrations were prepared in cell growth medium (MEM alpha, Cat. No. 22571-020, Invitrogen) supplemented with 10% foetal bovine serum (FBS, Cat. No. A15-152, PAA). The final preparation of the AgNP test solutions was made immediately before each experiment. The solutions were vortexed 2 times for 1 min at 2200 rpm to assure proper dispersion and reduce agglomeration of the NPs.

2.4. Cell culture

The RBE4 cell line has been derived from rat brain endothelium and was a gift from Michael Aschner (Vanderbilt University, Nashville, TN, USA). The RBE4 cells were cultured in MEM alpha medium, containing ribonucleosides and desoxyribonucleosides and supplemented with 10% heat-inactivated FBS, penicillin/streptomycin (5 ml in 500 ml medium; Cat. No. 15140-122, Invitrogen), geneticin sulphate (1 ml in 500 ml medium; Cat. No. 11811-064, Invitrogen) and basic fibroblast growth factor (1 ml in 500 ml medium; Cat. No. 13256-029, Invitrogen). Cells were grown on collagen type-I coated-surfaces (25 cm^2 and 75 cm^2 flasks, Cat. No. 354484 and Cat. No. 354485, BD, respectively). The cells were maintained in a humidified incubator at 37 °C and 5% CO_2 and used for experiments until sub-passage number between 25 and 30.

2.5. Neutral red (NR) uptake assay

Membrane permeability as an indicator of cytotoxicity was determined by the NR uptake assay modified after Borenfreund and Puerner (1985). Briefly, RBE4 cells were seeded in 24 well plates (Cat. No. 356408, BD) at a density of 60,000 cells/well. After reaching 80–90% confluency (usually after 2 days), the cells were exposed to 500 μl test solutions (AgNPs; $AgNO_3$; medium/10% FBS; medium/10% FBS/20 μM citrate) for time periods up to 24 h. Following exposure, the medium was replaced by HEPES buffer (pH 7.4) containing 0.05 mg/ml Neutral red dye. The plates were incubated at 37 °C for 90 min. Subsequently, the cells were fixated with 1% $CaCl_2$–0.5% formaldehyde. The dye was extracted in 500 ml 1% acetic acid–50% ethanol by shaking the plates for 20 min and transferring 200 μl liquid in a 96 well plate (Cat. No. 269620, NUNC). Absorbance was measured at 570 nm using a microplate reader (GENios Plus, Tecan). Cells incubated with cell growth medium only (i.e. medium/10% FBS) served as control. Background measurements were done with cells incubated with citrate suspension buffer diluted in growth medium (i.e. medium/10% FBS/20 μM citrate). All test and background values were corrected against the control. The assay was done in triplicate for each experimental condition. The NR uptake upon exposure to the test materials was calculated as percentage (NR uptake) of the control.

2.6. Lactate dehydrogenase (LDH) assay

The LDH leakage assay is a measure of cell membrane integrity. RBE4 cells were seeded in 96 well plates (Cat. No. 353070, BD) and grown until 80–90% confluency. Following exposure to 100 μl test solutions (AgNPs; $AgNO_3$; medium/10% FBS; medium/10% FBS/20 μM citrate), 50 μl of cell-free supernatant was transferred to a new 96 well plate and LDH leakage was measured according to the standard protocol of the Sigma assay kit. The percentage of LDH leakage was calculated using the following equation: $[(A - C)/(P - C)] \times 100$ where A is the mean absorbance in the test wells, C is the low control (medium/10% FBS) and P is the high (i.e. positive) control (Triton X-100; Cat. No. T8787, Sigma).

2.7. Colony formation assay

The protocol used in the present study was slightly modified from previously published work (Gellein et al., 2009). After seeding the cells in 6 well plates at a density of 150 cells/well, the cells were allowed to attach for 4 1/2 h. Growth medium was then replaced by 2 ml test solutions (AgNPs; $AgNO_3$; medium/10% FBS; medium/10% FBS/20 μM citrate) and the cells were incubated for 5 days at 37 °C and 5% CO_2. After the incubation period, the cells were fixed with ice-cold methanol, stained with Giemsa and examined under the microscope. Microscope pictures were taken with Spot Advanced Camera and Software (Version 4.1, Diagnostic Instruments, Inc.) and are shown in Fig. 7. Additionally, digital pictures of the whole well were recorded using a Sony DSC-T200 and image analysis was carried out using NIS Elements Advanced Research 3.0 Software (Nikon).

2.8. Separation of Ag^+ ions released from AgNPs in cell-free supernatant and cultured cells

Following exposure of RBE4 cells to the test solutions (Ag10, Ag50 and Ag100, medium/10% FBS, medium/10% FBS/20 μM citrate), the cells were removed and transferred to a centrifuge tube. The cells were loosened from the plastic by addition of 0.02% trypsin with 0.05% EDTA (Cat. No. L11-004, PAA) followed by incubation at 37 °C for 20 min. The plates were then frozen at −80 °C for further 20 min to ensure that all cell material was fractured. Both cell samples and cell-free samples were incubated for 1 h at room temperature with 50 μl mercaptoethanol to break the protein-silver bonds. Subsequently, Ag^+ ions were separated from AgNPs in both sample types using centrifugation (Sorvall RC6 Plus ultra, Thermo) at 45,500*g* for 30 min. The supernatant (500 μl), containing the Ag^+ ions, was carefully removed.