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Surface coating affects uptake of silver nanoparticles in neural stem cells

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ABSTRACT

The rapid development and widespread applications of nanotechnology necessitates the design towards safe nanoparticles. Surface structure is among the most important physicochemical characteristics of metallic nanoparticles affecting their mode of action in certain biological or environmental compartments. This study aimed to investigate how different surface coatings affect the cytotoxicity and cellular uptake of silver nanoparticles (AgNPs) in murine neural stem cells (mNSCs). Different AgNPs were prepared by stabilisation with surface coatings encompassing sodium bis(2-ethylhexyl)-sulfosuccinate (AOT), cetyltrimethylammonium bromide (CTAB), poly(vinylpyrrolidone) (PVP), poly-L-lysine (PLL), and bovine serum albumin (BSA). The obtained results revealed that AgNPs stabilized with different surface coating caused different cytotoxicity effects and internalization pattern in mNSCs. Macropinocytosis was determined as the main uptake mechanism in mNSCs for all of the tested AgNP types. These findings contribute to the overall knowledge essential to the safety assessment of novel nanomaterials.

1. Introduction

Nanotechnology stimulates the development and new applications of engineered materials in numerous industrial and biomedical applications. Utilising metals in the form of nanoparticles (NPs) progressively expands due to the unique physicochemical properties and increased reactivity of a substance at nano-sized level. There is a plethora of different marketable options for engineered metal-based NP including electronics, food, cosmetic, and the pharmaceutical industry. Silver NPs (AgNPs) are widely applied in treating burns, textiles, water purification devices, toothbrushes, shampoo, deodorants, and food packaging materials due to their biocidal effects [1]. Such rapid development of nanotechnology has raised concerns about the risk of unintentional exposure to NPs [2]. Despite numerous studies reporting the toxicity effects of AgNPs *in vitro*, reliable and comprehensive knowledge of their biological impacts is still lacking [3–7]. It has been well-established that AgNPs are able to induce oxidative stress and other similar negative consequences on cellular functions, which is strongly dependent on their size and surface characteristics, but also on the processes involved in the cellular uptake and intracellular distribution of AgNPs [[3–7],7–18]. Quality, efficacy and safety of biomedical innovation based on metallic NPs will be accelerated by improving the ability to control and manipulate their uptake and the impacts on living cells. Many biomedical applications of nanotechnological products endeavour to reach cytosolic cellular space, while others are designed to avoid crossing cell membranes. For most mammalian cells, NP internalisation is mediated by one of the pinocytotic pathways (Table 1), typically classified into macropinocytosis, clathrin- and caveolae-mediated endocytosis, and clathrine- and caveolae-independent endocytosis [6–18].

The negative health effects or undesirable environmental impacts of metallic NPs can be diminished by gaining an increased understanding of the role of NPs' physicochemical characteristics on their biological fate [19].

After the lessons learned from technological mistakes like asbestosis or silicosis, it is of the utmost importance to gather all information necessary to conduct the risk assessment of engineered NPs before they become a common health problem. Developing organisms are of special concern. There is evidence that some NPs may pass biological barriers and affect the function of organs like the brain, reproductive organs and foetal organisms [20–23]. The neuronal system is pronouncedly vulnerable to intoxication, which may lead to the development of neurodegenerative diseases [24]. This concern is even more striking for a developing nervous system, which is characterized by the immature blood brain barrier and the proliferation process of cells. Due to the ever increasing number of products based on AgNPs, the evaluation of

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Table 1
Classification of cellular uptake mechanisms [6–17].

Pathway	Description	Inhibitor (used in this study)	Size of vesicles
Phagocytosis	Actin-dependent process for uptake of solid particles	cytochalasin D	> 0.5 μm
Pinocytosis	Absorption of extracellular fluids	–	~100 nm
Macropinocytosis	Absorption of extracellular fluids and molecules	nocodazole, amiloride, cytochalasin D	< 5 μm
Clathrin-mediated endocytosis	Energy-dependent internalization of materials into "coated pits"	phenylarsine oxide	60–200 nm
Caveolae-mediated endocytosis	Energy-dependent clustering of lipid membrane components into caveolin	filipin	> 200 nm
Clathrin- or caveolae- independent endocytosis	Various mechanism of receptor-dependent endocytosis	–	~50 nm

their capability to reach, translocate into and cause detrimental effects in neuronal cells is crucial for a risk/benefit ratio assessment of silver-based nanomaterials. A limited number of *in vitro* nanotoxicological studies have been reported on neural-like cells [18,25–27]. Embryonic neural stem cells represent a good model for the evaluation of developmental neurotoxicity caused by NPs [18,25–27].

To the best of our knowledge, this study is the first to comprehensively investigate the impact of surface coating on the cytotoxicity and uptake mechanisms of well-characterised AgNPs in murine neural stem cells (mNSCs). For this purpose, differently coated and well-characterised AgNPs were prepared by surface functionalization using sodium bis(2-ethylhexyl)-sulfosuccinate (AOTAgNPs), cetyltrimethylammonium bromide (CTABAgNPs), poly(vinylpyrrolidone) (PVPAgNPs), poly-*L*-lysine (PLLAgNPs), and bovine serum albumin (BSAAgNPs). Surface coating agents are commonly used in preparation of AgNPs. Chemicals applied as surface coating agents can protect NP from direct interaction with the environment, but also from reactions between NPs themselves, thus stabilizing NP suspensions from aggregation [28,29]. For example, surface coating agents may prevent the oxidation of Ag atoms to Ag oxides on the surface of AgNPs, or the dissociation of ionic Ag in acidic aqueous solutions [30,31]. The aqueous AgNP dispersions can be stabilised either sterically using polymer non-ionic surfactants or electrostatically using anionic or cationic surfactants. Among the numerous stabilizers that can be used for AgNPs, a series of coating agents were selected in this study. This includes two cationic materials (PLL and CTAB), one anionic surfactant (AOT), non-ionic polymer (PVP) and one protein (BSA). The selection of coating agents was based on their molecular structure and charge. We aimed to include both surfactant and polymeric coatings as well as to test the positive, negative and neutral surface charge of AgNPs. The PVP was included in this selection as one of the most commonly used stabilizers for AgNPs.

According to our analysis of the Web of Science (WoS) database (performed on June 12, 2017) on the use of coating/capping agents for the stabilisation of AgNPs, where we found 22 493 publications altogether, the citrate and PVP were almost equally used as coating agent for AgNPs (~5% of all published papers), while the other coating agents studied here were represented by ~2.5% of all materials used for surface functionalisation of AgNPs. Thus, we considered PVPAgNPs as the reference compound throughout this study.

Cytotoxicity effects of different AgNPs on mNSCs were evaluated by means of cell viability and cell mortality, while the mechanism of cellular uptake was studied using different pharmaceutical inhibitors that block specific endocytic pathways as presented in Table 1 [6–14,17].

2. Materials and methods

2.1. Chemicals and materials

Cell viability cell counting kit 8 (CCK-8) (No. 96992, Sigma Aldrich) and Molecular Probes LIVE/DEAD™ Viability/Cytotoxicity kit (Invitrogen, Fisher Scientific) were used. The glassware and plastic used for chemical analysis were from Sarstedt (Germany), other plastics for cell culture were from Eppendorf (Wien, Austria) and TPP (Trasadingen, Switzerland). Medium for the cell culture growth was phenol-red free Dulbecco's modified Eagle's medium (DMEM/F12) with addition of GlutaMax (alternative to L-glutamine, Invitrogen, No. 31331093), Pen/Strep (100x) (PAA, cat. no. P11-010), and additional growth factors for cell proliferation: N2 (100x, No. 17502048), B27 (50x, No. 17504044), epidermal growth factor (EGF, stock solution 1000 ng/mL, No. PMG8041), fibroblast growth factor (FGF, stock solution 500 ng/mL, No. PMG0035), as well as StemPro Accutase for cell dissociation (No. A1110501); all from Invitrogen (Thermo Fisher Scientific, Schwerte, Germany). This medium, including all additives, was used and referred to throughout the manuscript as cell proliferation medium (CPM).

For TEM preparation, osmium tetroxide (Agar Scientific, Stansted, UK) and medium hard TAAB epoxy resin (Aldermaston, Berkshire, UK) were used. All necessary dilutions were done with high purity deionised Milli-Q water (18.2 MΩcm, Milli-Q system, Merck Chemicals GmbH, Darmstadt, Germany). All other chemicals used in the experiments were purchased from Sigma-Aldrich Chemie GmbH (Munich, Germany).

2.2. Synthesis and characterization of AgNPs

Differentially coated AgNPs were prepared by surface stabilization with AOT, CTAB, PVP, PLL and BSA following established procedures described elsewhere [28]. Characterization of AgNPs was performed in both ultrapure water (UW) and in cell proliferation medium (CPM) in order to compare freshly synthesized AgNPs dispersed in UW and stability of AgNPs in medium used for cells. As CPM was used for cell culture growth and cell culture experiments, all AgNPs were dispersed in CPM prior to any cell culture experiment. Total silver concentrations in AgNPs were determined using an Agilent Technologies 7500cx inductively coupled plasma mass spectrometer (ICPMS) (Waldbronn, Germany) as described elsewhere [28]. The size and charge of AgNPs were measured by dynamic (DLS) and electrophoretic light scattering (ELS), respectively, using a Zetasizer Nano ZS (Malvern, UK). Intensity of scattered light was detected at an angle of 173°. All measurements were conducted at 25 °C. The data processing was done by Zetasizer software 6.32 (Malvern instruments). Results for AgNP size distributions are given as an average value of 10 measurements and obtained as volume distributions. The charge was evaluated by measuring the electrophoretic ζ potential of AgNPs, and the results are reported as an average value of 5 measurements. Transmission electron microscope (TEM, Zeiss 902A, Oberkochen, Germany) was employed for visualization of AgNPs. TEM samples were prepared by depositing a drop of the AgNPs suspension on a Formvar® coated copper grid. Samples were air-dried at room temperature.

2.3. Neural stem cell culture establishment and treatment

Murine neural stem cells were isolated from wild type C57Bl/6NCrl mouse foetuses according to the rules of the Ethics Code of the Croatian Society for Laboratory Animal Science and EU Directive 2010/63/EU on the protection of animals used for scientific purposes [18,25].