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Uptake and intracellular distribution of silver nanoparticles in human mesenchymal stem cells

C. Greulich [a,*], J. Diendorf [b], T. Simon [c], G. Eggeler [c], M. Epple [b], M. Köller [a]

[a] Bergmannsheil University Hospital/Surgical Research, Ruhr University Bochum, Bürkle-de-la-Camp-Platz 1, 44789 Bochum, Germany
[b] Inorganic Chemistry and Center for Nanointegration Duisburg-Essen, University of Duisburg-Essen, Universitaetsstrasse 5-7, 45117 Essen, Germany
[c] Faculty of Mechanical Engineering/Institute for Materials, Ruhr University Bochum, Universitaetsstrasse 150, 44789 Bochum, Germany

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ABSTRACT

Silver nanoparticles (Ag-NP) are widely used due to their well-known antibacterial effects. In medicine Ag-NP have found applications as wound dressings, surgical instruments and bone substitute biomaterials, e.g. silver-containing calcium phosphate cements. Depending on the coating technique, during resorption of a biomaterial Ag-NP may come into close contact with body tissues, including human mesenchymal stem cells (hMSC). Despite the widespread uses of Ag-NP, there is a serious lack of information concerning their biological effects on human cells. In this study the uptake of Ag-NP into hMSC has been analyzed and the intracellular distribution of Ag-NP after exposure determined. Non-agglomerated (dispersed) Ag-NP from the cell culture medium were detected as agglomerates of nanoparticles within the hMSC by combined focused ion beam/scanning electron microscopy. The silver agglomerates were typically located in the perinuclear region, as determined by light microscopy. Specific staining of cellular structures (endo-lysosomes, nuclei, Golgi complex and endoplasmatic reticulum) using fluorescent probes showed that the silver nanoparticles occurred mainly within endo-lysosomal structures, not in the cell nucleus, endoplasmic reticulum or Golgi complex. Quantitative determination of the uptake of Ag-NP by flow cytometry (scattergram analysis) revealed a concentration-dependent uptake of the particles which was significantly inhibited by chlorpromazine and wortmannin but not by nystatin, indicating clathrin-dependent endocytosis and macropinocytosis as the primary uptake mechanisms.

1. Introduction

During the past decades nanomaterials have been introduced in various areas, e.g. in the food, electronics, car and clothing industries and medical applications. It is expected that the use of nanoparticles will further increase in future. Silver nanoparticles (Ag-NP) are currently the most commercialized among nanomaterials, mainly due to their well-known antiseptic properties [1]. Distinct silver compounds have been used clinically to reduce skin infections in the treatment of burns (e.g. silver sulfadiazine) and as coatings on various surfaces, such as catheters [2–5]. In previous studies we have shown that silver nanoparticles exert cytotoxic effects on hMSC at high concentrations, but also lead to cell activation at sublethal concentrations [6].

One major toxicological concern is the possible uptake of Ag-NP into cells. This is an effect that seems to be specific for particles with dimensions in the range 1–200 nm. Nanoparticles can be imported into cells via endocytosis for smaller particles and phagocytosis for larger particles [7]. However, the uptake of nanoparticles by cells not only depends on the size of the particles, but also on the surface charge and the surface functionalization of the particles [8]. Endocytosis occurs in all cells and includes macropinocytosis, clathrin-dependent endocytosis and non-clathrin-dependent endocytosis [9]. The specific endocytotic mechanism by which cells internalize Ag-NP has been hitherto unknown. Previous studies have demonstrated that exposure to nanoparticles may occur by inhalation, ingestion, injection for therapeutic purposes or by direct physical contact [10]. Injected Ag-NP are translocated to the blood circulation and distributed throughout the main organs, especially the kidney, liver, spleen and brain [11]. If nanoparticles are taken up by human cells, this results in interaction of these nanoparticles with different subcellular components and organelles, leading to their delivery into different intracellular organelles. Therefore, further information is required to assess the biological properties of Ag-NP to improve their application with different biomaterials.

To analyze the uptake mechanisms of Ag-NP into human cells we used human mesenchymal stem cells (hMSC) as an experimental in vitro model. hMSC are neither a transformed cell line nor immortalized cells but represent primary pre-tissue cells which can be cultured over several passages. hMSC are found in different

* Corresponding author. Tel.: +49 0234 302 4724.
E-mail address: christina.greulich@rub.de (C. Greulich).

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tissues, such as bone marrow, fat and muscle [12], and this cell type is intimately involved in tissue regeneration and tissue repair. It is likely that mesenchymal stem cells will come into close contact with any Ag-NP-coated implants. Furthermore, due to their high differentiation capacity, these cells represent an optimal cellular model in which to analyze a possible influence of Ag-NP on cell differentiation [13].

Here we have analyzed the uptake mechanisms of Ag-NP into hMSC and examined whether more than one endocytotic pathway is involved by a combination of focused ion beam/scanning electron microscopy (FIB/SEM), light and fluorescence microscopy and flow cytometry.

2. Materials and methods

2.1. Synthesis of silver nanoparticles

Polyvinylpyrrolidone (PVP)-coated silver nanoparticles were synthesized by reduction with glucose in the presence of PVP according to Wang et al. [14]. Briefly, 2 g glucose and 1 g PVP were dissolved in 40 g water and heated to 90 °C. Then 0.5 g $AgNO_3$ dissolved in 1 ml of water were quickly added. The dispersion was kept at 90 °C for 1 h and then cooled to room temperature. The particles were collected by ultracentrifugation (30,000 r.p.m., 30 min), redispersed in pure water and collected again by ultracentrifugation. Thereby NO_3^-, excess glucose and its oxidation products, excess PVP and excess Ag^+ were removed. The silver nanoparticles were then redispersed in water. The yield with respect to Ag was about 5%. The final silver concentration in all dispersions was determined by atomic absorption spectroscopy (AAS) (Thermo Electron Corp., M-Series). The hydrodynamic diameter and the ζ-potential of the dispersed particles were measured by dynamic light scattering (DLS) with a Malvern Zetasizer Nano ZS. The *z*-average value was used as the average particle diameter. The polydispersity index (PDI) was below 0.3 in all cases, indicating an absence of aggregates. SEM (FEI Quanta 400 ESEM instrument) revealed a spherical shape of the Ag-NP used, with a metallic core of 50 ± 20 nm (Fig. 1). The hydrodynamic diameter of the nanoparticles was 80 nm, as measured by DLS. Note that the hydrodynamic diameter included the polymer layer and the hydration shell. It was therefore always larger than the pure metal diameter of the silver core as determined by electron microscopy under high vacuum.

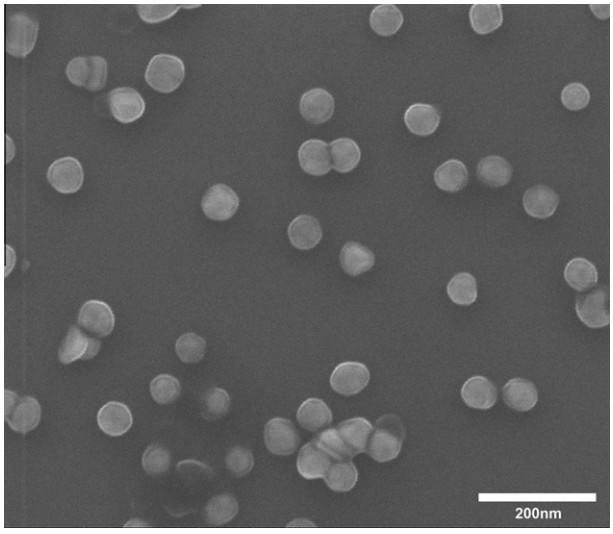

Fig. 1. Scanning electron micrograph of the spherical PVP-coated silver nanoparticles used, with a metallic core of 50 ± 20 nm.

PVP (PVP K30, Povidon 30, Fluka, molecular weight 40,000 g mol^{-1}), tri-sodium citrate dihydrate (Fluka, p.a.), silver nitrate (Fluka, p.a.) and D-(+)-glucose (Baker) were used. Ultrapure water was prepared with an ELGA Purelab ultra instrument.

2.2. Cell culture

hMSC (passages 3–7, Lonza Inc., Walkersville, MD) were cultured in cell culture medium RPMI 1640 (GIBCO Invitrogen GmbH, Karlsruhe, Germany) containing 10% fetal calf serum (FCS) (GIBCO Invitrogen GmbH) and L-glutamine (0.3 g l^{-1}, GIBCO Invitrogen GmbH) using 24-well cell culture plates (Falcon, Becton Dickinson GmbH, Heidelberg, Germany). Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2. hMSC were sub-cultivated every 7–14 days depending on cell proliferation. Adherent cells were washed with phosphate-buffered saline (PBS) (GIBCO Invitrogen GmbH) and detached from the culture flasks by addition of 0.2 ml cm^{-2} 0.25% trypsin/0.1% ethylene diamine tetraacetic acid (EDTA) (Sigma–Aldrich, Taufkirchen, Germany) for 5 min at 37 °C. Subsequently, the hMSC were collected and washed twice with RPMI 1640 containing 10% FCS.

2.3. Light microscopy

Subconfluently growing hMSC were incubated at 37 °C in the presence or absence of different concentrations of Ag-NP or silver ions (silver acetate solution, normalized for silver content) for 24 h under cell culture conditions. PVP-coated spherical Ag-NP (hydrodynamic diameter 80 nm) were dispersed in sterile ultrapure water at 1 mg ml^{-1} as a stock solution. The final concentration of 20 μg ml^{-1} for the Ag-NP and 2 μg ml^{-1} for the silver ions were achieved by the addition of 50 μl of Ag-NP/Ag ion dispersion to 1 ml cell volume prior to cultivation. The working solutions were prepared by serial dilution of a 1 g l^{-1} stock solution with sterile ultrapure water. Silver acetate solutions containing the same silver concentrations were prepared similarly with ultrapure water. All silver concentrations given here refer to the amount of silver as determined by AAS. The particle uptake into hMSC was assessed with a BX61 microscope (Olympus) and a F-view II camera (digital camera) using phase contrast microscopy. Subsequently the images were digitally processed by contrast enhancement (DCE filter) using cellP image software (Olympus).

2.4. Focused ion beam, scanning electron microscopy and EDX analysis

The FIB system (type FEI Quanta 200 3D) consisted of a dual beam unit equipped with an electron column and an ion column. Both the ion column and the electron column operated at accelerating voltages of up to 30 kV. Gallium was used as a liquid metal ion source to produce cross-sections of adherent hMSC. The SEM investigations were complemented by energy-dispersive X-ray spectroscopy (EDX) (ISIS EDX System, Oxford Instruments, Germany) for chemical analysis. To analyze the uptake of Ag-NP subconfluently growing hMSC were incubated at 37 °C in the presence of 50 μg ml^{-1} Ag-NP in medium for 24 h under cell culture conditions. After incubation the hMSC were washed with PBS and fixed with 3.7% glutaraldehyde (Fluka) for 15 min and washed again with PBS. Subsequently the cells were dehydrated in an ethanol series for 5 min each (50%, 70% and 100%). After drying the samples were sputtered with gold as per the standard SEM procedure. Before the milling process the specimens were coated with a thin protective layer of tungsten on the surface in order to protect them from ion contamination.

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