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Death and cell cycle progression are differently conditioned by the AgNP size in osteoblast-like cells

F. Rosário[a], P. Hoet[b], C. Santos[c,*], H. Oliveira[a,*]

[a] CESAM & Department of Biology, University of Aveiro, 3810-193 Aveiro, Portugal
[b] Occupational and Environmental Toxicology, KU Leuven, Leuven, Belgium
[c] Department of Biology, Faculty of Sciences, University of Porto, Rua do Campo Alegre, Porto, Portugal

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ABSTRACT

Silver nanoparticles (AgNPs) are useful to a wide range of consumer's and medical products, due to their antimicrobial and anti-inflammatory activities. AgNPs have been used to prevent the microbial colonization, therefore decreasing the risk of infection, on implantable devices, tumor prostheses, bone cement and surgical instruments. However, the putative toxicity of AgNPs to bone cells is still poorly understood. Therefore, this study aimed to contribute to enlighten the role of ionic silver release of small sized NPs on the biological outcomes of bone cells, in particular to what concerns to induction of cytotoxic and genotoxic effects. To achieve that goal osteoblast-like MG-63 cells were exposed to well characterized PVP coated AgNPs of two different primary sizes (10 nm and 20 nm) and evaluated after 24 and 48 h.

Our results showed that, the smaller sized AgNPs (10 nm) are more reactive and prone to form large aggregates, being therefore mandatory to provide a careful characterization of the particles, before the toxicity assessment. We also demonstrate that for short period exposures (up to 48 h) ionic silver (from $AgNO_3$) is more toxic than the corresponding dose of AgNP. However, when assessing longer term exposures by the clonogenic assay, we demonstrated the inverse effect, the AgNPs turn out being more toxic, completely inhibiting plate efficiency. Therefore, AgNPs toxicity cannot be attributed to the dissociated Ag^+ alone. Also, when comparing size-dependent effects, we demonstrate that AgNP20 were found to induce a cell cycle arrest at G0/G1 and apoptosis, while AgNP10 did not induce a cytostatic effect, but rather induced necrosis. Finally, combining the chemical and toxicological profiles of both AgNP sizes, we hypothesize that the size dependent AgNP toxicity may be associated in part with the NPs interference with the cell membranes and consequent uptake/adsorption processes.

1. Introduction

Nanomaterials have at least one dimension with a length ranging from 1 to 100 nm, which provides a high surface/volume ratio, that leads to specific characteristics (e.g. Electrical and optical properties), compared to bulk materials (Chaloupka et al., 2010; Lewinski et al., 2008). These unique properties make MNPs useful to a wide range of fields and consequently being increasingly produced and released to the environment. Along with the constant discovery of new applications of MNPs, it was estimated that their production will increase to 58,000 metric tons per year until 2020 (Lewinski et al., 2008; Maynard, 2006) and the estimated global annual production of Ag NPs is ~55 tons (Piccinno et al., 2012). So far, silver nanoparticles (AgNPs) are the most frequently used nanomaterial in consumers' products (435 products, or 24%) and also the most advertised nanomaterial component (207 products, or 14.5%), mostly due to their antimicrobial (Rai et al., 2009) and anti-inflammatory activities (Vance et al., 2015); see the Woodrow Wilson consumer products inventory (www.nanotechproject.org/cpi). For example, AgNPs are used in wound dressings and topical creams, acting as a potent anti-inflammatory agent which provided a major breakthrough for treatment of burns and various infections (Chaloupka et al., 2010; Feng et al., 2000; Sibbald et al., 2007; Tian et al., 2007). In addition, most medical devices are prone to bacterial adhesion and biofilm formation. Therefore, to protect and prevent from infections, these nanoparticles have been receiving much interest in the field of orthopedics, used as coating for implantable devices,

* Corresponding authors.
E-mail addresses: csantos@fc.up.pt (C. Santos), holiveira@ua.pt (H. Oliveira).

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(eg., catheters, joint replacement prostheses) (Wilcox et al., 1998; Sibbald et al., 2001; Furno et al., 2004; Necula 2013), as coating of surgical instruments (Li et al., 2013a,b) and as additive compound in bone cement (Joseph et al., 2003).

The use of AgNPs as additives in bone cements or prostheses has reduced the infection rates and also decreasing the joint replacement initial mortality of 2.7%–18% (Ahlberg et al., 1978; Alt et al., 2004; Joseph et al., 2003; Saravanan et al., 2011).

In spite of the wide usage of AgNPs in the medical field, there is a distinct lack of information on the amount of silver ions (Ag^+) released from the products and it is not clear if their toxicity is attributed to NPs intrinsic toxicity and/or from the released ions (Kawata et al., 2009; Navarro et al., 2008). In vivo toxicity studies in rats demonstrated a distribution of AgNPs among lungs, liver, heart, kidney, spleen and brain after oral and blood administration of these NPs (Lankveld et al., 2010; Lee et al., 2013; Park et al., 2010a,b, 2011a,b). Few other studies described pulmonary toxicity after AgNPs up to 4 mg/m^3, inhalation and argyria-like symptoms after wound dressing usage on different organisms (Samberg et al., 2010; Stebounova et al., 2011; Sung et al., 2009; Trop et al., 2006). In addition, (Kim et al., 2008) found no genotoxicity after 28 days of oral administration up to 1 mg/kg of AgNP, despite a gender dependency in the accumulation of Ag in rat tissues. Moreover, Kim et al. (2011) found that after 28 days of inhalation up to 1.32×10^6 AgNP particles/cm^3, there were no significant changes in the hematology and blood biochemical values in either the male or female rats.

In vitro exposure to AgNPs also induced toxicity in several human cell lines. For example, it was observed a decrease of cell viability and cell proliferation in human liver cells (Piao et al., 2011) and keratinocyte cell line HaCaT (Zanette et al., 2011) after exposure up to 10 μg/mL and 333 μM, respectively. Also, AshaRani et al. (2009) found an increase in apoptosis and changes in cell cycle dynamics in normal human lung fibroblasts (IMR-90 cell line) and glioblastoma cells (U251) upon exposure to AgNPs. It was also documented an AgNP size-dependent toxicity effect, independently of the cell line (Gliga et al., 2014; Kim et al., 2012; Liu et al., 2010; Park et al., 2011a,b). For exemple, Kim et al. (2012) reported that the AgNPs cytotoxicity was size-dependent, stimulating apoptosis in the mice osteoblastic cell line MC3T3-E, but induced necrosis in mice adrenal medulla cells PC12. Liu et al. (2010) reported a decreased cytotoxicity (e.g., morphology, viability, membrane integrity) with increased AgNPs size (5, 20 and 50 nm) in four human cell models (A549, SGC-7901, HepG2 and MCF-7). Similarly AgNPs (20, 80 and 113 nm) showed different toxicity (e.g., membrane damage) in mice L929 fibroblasts and in murine RAW264.7 macrophages (Park et al., 2011a,b). Studies correlating AgNPs size with cytotoxicity used large size ranges with expectable different effects in the cell. It remains therefore unclear if AgNPs with smaller differences in size also differ in their toxicity levels, and trigger different or similar pathways in the cell.

Despite metal and non-metal nanoparticles toxicity, has been widely studied in vitro, few studies focused on bone cells. including osteoblasts (human CRL-11372 cell line) exposed to alumina and TiNPs (Gutwein and Webster, 2002), pre-osteoblasts (mice L929 and MC-373 cell lines) exposed to TiO_2 (Bernier et al., 2012) and osteoblast-like (human MG-63) exposed to TiO_2 (Bacakova et al., 2007; Niu et al., 2012). Also, a cell line retaining osteosarcoma behaviour (mice UMR 106) were exposed to TiO_2 and Al_2O_3 (Di Virgilio et al., 2010; Gerhardt et al., 2007).

However, despite its importance to prosthesis and dental surgery, AgNP cytotoxicity has not been addressed in bone cells. The few available studies (Cao et al., 2011; De Giglio et al., 2013; Ye et al., 2011) used embedded AgNPs, which therefore masks the putative toxicities due to AgNPs alone, as well as the effective release of Ag^+ from these NPs.

To our knowledge, until now, there are no studies reporting the toxic potential of different-sized AgNP to bone cells and the role of subsequent ionic silver dissociation on their toxicity.

In the current work we hypothesize that even minimal differences on AgNPs size are sufficient to induce different toxicity profiles and may differently condition the pathways involved in cell death response. For that, we exposed the osteoblast – lmike cell line MG63 to small size AgNPs (10 and 20 nm) and to $AgNO_3$ (control for Ag^+). Different cell parameters were investigated: uptake of AgNP, cell viability and proliferation, cell-cycle dynamic, micronuclei induction and apoptosis and necrosis. Also, we aimed to determine the contribution of ionic silver (due to AgNP dissociation) on the toxicity of AgNPs.

2. Materials and methods

2.1. Silver nanoparticles and physicochemical characterization

Sterile, purified and endotoxin-free silver nanoparticles (Biopure AgNPs 1.0 mg/mL) with polyvinylpyrrolidone (PVP) coating and nominal size of 10 nm and 20 nm (here designated as AgNP10 and AgNP20, respectively), were obtained by NanoComposix Europe (Prague, Czech Republic). Morphology and size were analyzed by scanning electron microscopy (SEM – Hitachi, model SU-70, Japan). Approximately 10 μL of each stock solution was added to a carbon sheet and let dry, on atmospheric conditions. After the samples were dry, the SEM images were taken. Later, analyzed with KLONK Image Measurement software (https://www.imagemeasurement.com/en/). The hydrodynamic diameter of nanoparticles in culture medium 1 h and 24 h after preparation were measured by Dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern I, UK). Hydrodynamic size from samples with heterogenic sizes, where presented by the mean size of the peak with the highest percentage.

Putative dissociation of silver ions from AgNPs with time was measured. For that, AgNP10 and AgNP20 suspensions in culture medium (0, 50, 100 μg/mL) were prepared and incubated for 1 h, 24 h and 48 h in culture conditions (controlled humid atmosphere at 37 °C and 5% CO_2). After each time point, the samples were collected and ultracentrifuged (40,000*g* for AgNP10 and 25,000 g for AgNP20) in order to separate the nanoparticles from the supernatant that contains the dissociated ionic silver. The supernatant (300 μL) was diluted in 2700 mL of ultra-pure water and it was digested with *aqua regia* solution (HNO_3 (68%): HCL (37%)). The Ag^+ content in solution was determined by inductively coupled plasma mass spectrometry (ICP-MS Thermo X Series).

2.2. Cell culture

The human osteosarcoma MG-63 cell line (ATCC, Manassas, VA, USA) was cultured in complete growth medium (α-Minimum Essential Medium supplemented with 10% (v/v) fetal bovine serum (FBS), 2.5 μg/mL penicillin-streptomycin and 2.5 μg/mL fungizone) (all medium components from Life Technologies, Carlsbad, CA, USA) at 37 °C, 5% CO_2, in a humidified atmosphere. Cell confluence and morphology were daily observed under an inverted phase contrast microscope Nikon Eclipse TS100 (Japan). Cells were subcultured when confluence reached 80% using 0.25% trypsin/1 mM EDTA (Life Technologies, Carlsbad, CA, USA). Depending on the assay, cells were seeded in 96, or 6 well plates and left 24 h for adhesion. After that the culture medium was replaced with fresh medium containing AgNP solutions. MG-63 cells were cultured in the referred conditions for 24 h and 48 h.