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Coating independent cytotoxicity of citrate- and PEG-coated silver nanoparticles on a human hepatoma cell line

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ABSTRACT

The antibacterial potential of silver nanoparticles (AgNPs) resulted in their increasing incorporation into consumer, industrial and biomedical products. Therefore, human and environmental exposure to AgNPs (either as an engineered product or a contaminant) supports the emergent research on the features conferring them different toxicity profiles. In this study, 30 nm AgNPs coated with citrate or poly(ethylene glycol) (PEG) were used to assess the influence of coating on the effects produced on a human hepatoma cell line (HepG2), namely in terms of viability, apoptosis, apoptotic related genes, cell cycle and cyclins gene expression. Both types of coated AgNPs decreased cell proliferation and viability with a similar toxicity profile. At the concentrations used (11 and 5 μg/mL corresponding to IC50 and ~IC10 levels, respectively) the amount of cells undergoing apoptosis was not significant and the apoptotic related genes *BCL2* (anti-apoptotic gene) and *BAX* (pro-apoptotic gene) were both downregulated. Moreover, both AgNPs affected HepG2 cell cycle progression at the higher concentration (11 μg/mL) by increasing the percentage of cells in S (synthesis phase) and G2 (Gap 2 phase) phases. Considering the cell-cycle related genes, the expression of cyclin B1 and cyclin E1 genes were decreased. Thus, this work has shown that citrate- and PEG-coated AgNPs impact on HepG2 apoptotic gene expression, cell cycle dynamics and cyclin regulation in a similar way. More research is needed to determine the properties that confer AgNPs at lower toxicity, since their use has proved helpful in several industrial and biomedical contexts.

Introduction

AgNPs (silver nanoparticles) are being extensively used in a wide range of applications, from medicine and industry to the most common consumer products (Behra et al., 2013; Eckhardt et al., 2013; Franci et al., 2015; Rai et al., 2015). Consequently, the possible risks associated with their release into the environment and human exposure has also increased. Indeed, while researchers have stressed the need to establish whether the presence of nanosilver in those products is essential (Nowack and Bucheli, 2007), and several studies showed the toxic potential of AgNPs, their usage remains widespread (Chen et al., 2015; Dusinska et al., 2013; McShan et al., 2014).

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Previous studies have shown that the physico-chemical characteristics of AgNPs will influence cellular uptake, intracellular fate and, consequently, the toxicity of these NPs (Caballero-Díaz et al., 2013; Gliga et al., 2014; Wang et al., 2014; Zhang et al., 2014, 2015). In particular, the size (Gliga et al., 2014; Kim et al., 2012; Park et al., 2011) and surface chemistry (Lu et al., 2010), as well as the type of formulation (Boonkaew et al., 2014), period of exposure (Comfort et al., 2014) and storage conditions (Ahlberg et al., 2014) have all been shown to play a determinant role in AgNPs toxicity.

AgNPs are often coated to promote stability and avoid aggregation. Citrate is the most common reducing agent used to stabilize AgNPs, providing the particles a negative surface charge (Gutierrez et al., 2015; Pillai and Kamat, 2004; Sharma et al., 2009). Polyethylene glycol (PEG) is another popular coating, especially concerning biomedical applications, due to its biocompatible nature and the high stability conferred to the particles (Ryan et al., 2008; Fernández-López et al., 2009). Ginn et al. (2014) refer that in the years to come we will see an increase in the number of novel site-directed PEGylation chemistries and a shift in its application to a wider range of therapeutic molecules, including NPs for therapeutic and diagnostic purposes, becoming increasingly essential more studies with this coating. PEG coating improved the biopharmaceutical properties of drugs, increased stability and resistance to proteolytic inactivation, increased circulatory lives, and showed low toxicity (Ginn et al., 2014; Jain and Jain, 2008; Ryan et al., 2008). Moreover, it has been argued that PEG coating decreases AgNPs toxicity by reducing their cellular uptake (Brandenberger et al., 2010; Caballero-Díaz et al., 2013; Pang et al., 2016).

As liver is the most important organ for xenobiotic metabolism (Roberts et al., 2014), liver cell lines have been amply used in biomedical research involving the testing of drugs or other toxicants. The cytotoxicity of AgNPs towards liver cells has also been demonstrated in a few previous studies. Faedmaleki et al. (2014) showed that 20–40 nm AgNPs decreased HepG2 viability in a concentration-dependent manner. Also, Nowrouzi et al. (2010) studied the cytotoxicity of AgNPs on HepG2 and obtained IC50 value of 2.75–3.0 μg/mL for HepG2 after exposure to 5–10 nm AgNPs. Moreover, by determining changes in the activity of lactate dehydrogenase, alanine aminotransferase, aspartate aminotransferase, glutathione peroxidase, superoxide dismutase, lipid peroxidation and cytochrome c content, that same work provided evidence for the involvement of oxidative alterations upon exposure to low doses of AgNPs. Recently, Xin et al. (2015) also found dose-dependent cytotoxicity of AgNPs in HepG2 cells, which was attributed to the interplay of oxidative stress, DNA damage and mitochondrial injury. Consistently, Xue et al. (2016), suggested the cellular toxicological mechanism of AgNPs to be related with oxidative stress induced by the generation of ROS, leading to mitochondria injury and induction of apoptosis. However, there are few studies comparing the coating influence on AgNPs cytotoxicity to liver cells. Kennedy et al. (2014) studied the cytotoxicity potential of carbohydrate functionalization of ~54 nm AgNPs to HepG2 and neuronal-line Neuro 2A cells and found that particles functionalized with ethylene glycol, glucose and citrate coated nanoparticles show a similar toxicity, while galactose and mannose functionalized AgNPs were significantly less toxic to HepG2 cell line. Other studies comparing the influence of coating on AgNPs cytotoxicity have been addressed to other cell lines. Gliga et al. (2014) compared the cytotoxicity of uncoated, PVP- and citrate-coated AgNPs in bronchial BEAS-2B cells and found no coating-dependent differences in cytotoxicity. Caballero-Díaz et al. (2013) reported that pegylation of AgNPs reduced cellular uptake and reduced the toxicity in NIH/3T3 (mouse embryonic fibroblasts), compared to AgNPs coated with other polymers. The conflicting existing information addressed the need to deeply study surface coating AgNPs cytotoxicity, in order to be aware if or which nanoparticles (NPs) are more cytotoxic to the different cell lines.

In this study we aimed to compare the influence of coating (citrate *vs.* PEG) on the cytotoxicity of AgNPs in liver cells, using the human hepatoma cell line HepG2 as an *in vitro* model. HepG2 cells were exposed to citrate- and PEG-coated AgNPs of 30 nm diameter and the effects on cell viability, apoptosis induction, apoptotic expression genes, cell cycle profile and cyclins gene expression were assessed.

1. Material and methods

1.1. Chemicals

Sterile, purified and endotoxin-free AgNPs (Biopure AgNPs 1.0 mg/mL), with 30-nm diameter and a citrate or PEG surface, designated from now on as Cit30 and PEG30 NPs, were purchased from Nanocomposix Europe (Prague, Czech Republic). Citric acid ($C_6H_8O_7{\cdot}H_2O$) was purchased from Sigma Aldrich (St. Louis, Missouri, USA); PEG (MW 5 kDa) from Laysan Bio® (Arab, Alabama, USA) and silver nitrate reagent ($AgNO_3$) from Sigma Aldrich (St. Louis, MO, USA). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), antibiotics and phosphate buffer saline (PBS, pH 7.4) were purchased from Life Technologies (Carlsbad, CA, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA). RNase and propidium iodide (PI) were both from Sigma-Aldrich, St. Louis, MO-USA.

1.2. Physicochemical characterization of AgNPs

The morphology and size of AgNPs was assessed by scanning transmission electron microscopy (STEM) using a scanning electron microscope Hitachi SU-70 (Hitachi High-Technologies Europe GmbH, Germany) operating at 30 kV. Samples for STEM analysis were prepared by evaporating dilute suspensions of the nanoparticles on a copper grid coated with an amorphous carbon film. The hydrodynamic diameter and polydispersity index (PDI) of the nanoparticles were measured by dynamic light scattering (DLS) and the zeta potential was assessed by electrophoretic mobility, both measurements using a Zetasizer Nano ZS (Malvern Instruments, UK).

Silver quantification measurements were performed by inductively coupled plasma optical emission spectrometry (ICP-OES) in an Activa M Radial spectrometer (Horiba Jobin Yvon), employing a charge coupled device (CCD) array detector, with a wavelength range of 166–847 nm and radial plasma view. Samples were introduced into the ICP plasma

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