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In vitro investigations of gold nanocages: Toxicological profile in human keratinocyte cell line

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ABSTRACT

Gold nanocages (AuNCs) are comparatively novel nanostructures, as many of their characteristics are still to be exploited. The purpose of present study was to systematically investigate the toxicological effects of AuNCs on human keratinocyte cell line (HaCaT) utilizing Dark Field (DF)/Bright Field (BF) imaging and flow cytometry cell cycle techniques. We have applied surface modification, concentration, and incubation time of AuNCs as variables to investigate their effect on the cellular imaging and cell cycle response of HaCaT cells. The results indicate that the AuNCs interact with HaCaT cells in accordance to their surface charge and concentration. Cellular uptake is evident from DF images which lead to the cell cycle perturbations and apoptosis in HaCaT cells. AuNCs cause a prominent G2/M phase arrest after 24 h of incubation. To the best of our knowledge toxicological effects of AuNCs on cell cycle of HaCaT cell line *in vitro* are not reported previously.

1. Introduction

Hasty explosion of nanotechnology in the field of biology (Asare et al., 2012; AshaRani et al., 2009a; Ahamed et al., 2008; Ahamed et al., 2010; Arora et al., 2012; AshaRani et al., 2009b; Kruszewski et al., 2011; Schrand et al., 2008; Au et al., 2008; Chen et al., 2005a, 2007; Song et al., 2009; Anderson and Parrish, 1983; Yang et al., 2007; Ku and Wang, 2005; Ku et al., 2005; Greulich et al., 2011; Cho et al., 2009; Kang et al., 2010; Austin et al., 2011; Mackey et al., 2013; Xiong et al., 2005, 2007; Sun et al., 2003) has provoked the need to explore toxic effects of nanosized materials on human health specifically and on environment in general. This is only way for nanobiotechnology to grow conscientiously and sustainably (Asare et al., 2012; AshaRani et al., 2009a).

Once in the vicinity of cells, Nanoparticles (NPs) are uptaken in a variety of mechanisms, out of which endocytosis is very common. As a consequence of uptake of NPs cell signaling is activated, resulting in reactive oxygen species (ROS) generation, inflammation, cell cycle arrest and ultimately cell death (Ahamed et al., 2008, 2010; Arora et al., 2012). Silver nanoparticles (AgNPs) are well established for their induction of antiproliferation, diminished mitochondria function and stimulation of apoptosis and necrosis in model Human and mice cell lines (Arora et al., 2012; AshaRani et al., 2009b; Kruszewski et al., 2011). NPs evidently enter the nucleus and interact with the nuclear material directly or indirectly, affecting the DNA synthesis and perturbing cellular functions (AshaRani et al., 2009a; Schrand et al., 2008) which results in the formation of mutant or tumorigenic cells. Gold Nanocages (AuNCs) have been comprehensively studied over the past few years for their biological applications by Xia and co-workers (Au et al., 2008; Chen et al., 2005a, 2007; Song et al., 2009; Yang et al., 2007; Cho et al., 2009; Mackey et al., 2013; Xiong et al., 2005, 2007; Sun et al., 2003; Hu et al., 2015; Gao et al., 2014). This research emphasizes the effect of AuNCs on cell cycle response of HaCaT cell line, which is a less focused area. This study is significant as HaCaT cells are keratinocyte (skin) cells, so toxicological relevance here would be most relevant to skin surface exposure to nanoparticles. Since the concentrations used here (0.1 nM and 0.4 nM) are somewhat high relative to accidental exposure levels (e.g. water contamination) and slightly smaller than pharmacologically relevant-levels, which possibly suggests that these concentrations reflect what might be useful for skin surface imaging (e.g. SERS) possibly for melanoma screening, as an example.

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The extent of cell cycle perturbations is affected by certain factors like particle size, shape, surface modification, concentration, exposure time and also by the cell line type under examination (Schrand et al., 2008). Besides shape and size of NPs, uptake is highly dependent upon surface charges (Cho et al., 2009). Cytotoxicity induced by NPs can be measured in terms of apoptosis which is considered as a vital cellular event for homeostasis and for elimination of damaged or unfit cells (AshaRani et al., 2009b). In the present study we have examined the extent of apoptosis induced by different concentrations, surface modifications and incubation times for AuNCs in Human Keratinocyte cell line *in vitro*.

This study was embarked on to examine the potential toxicological effect of AuNCs after incubation with the HaCaT cell line which exhibits functional and morphological properties of normal epidermal keratinocytes (Ahamed et al., 2010), also deliberated to proliferate and differentiate and is taken as an appropriate model to study growth and toxicity in human dermis cells. (Sun et al., 2003). Gold nanocages are considered as biocompatible (Xiong et al., 2005, 2007; Sun et al., 2003; Hu et al., 2015; Gao et al., 2014; Fratoddi et al., 2014, 2015; Chen et al., 2005b) probably because of the gold, but the unreacted silver inside the hollow cavity can be a reason for the increase in apoptotic population and cell cycle perturbations. Silver and gold NPs have the ability to perturb the HaCaT cell cycle individually, which we have reported previously (Austin et al., 2011; Mackey et al., 2013) as well as in combination as shown in the present work.

2. Experimental

2.1. Synthesis of AuNCs

Gold nanocages (AuNCs) with 45 nm wall length and 5 nm wall thickness were prepared using silver nanocubes (AgNCs) as a template by galvanic replacement reaction (Xiong et al., 2005, 2007; Sun et al., 2003; Hu et al., 2015; Gao et al., 2014; Fratoddi et al., 2014, 2015; Chen et al., 2005b; Mahmoud et al., 2010; Siekkinen et al., 2006; Chithrani and Chan, 2007). The silver nanocubes were prepared by a previously reported method (Austin et al., 2011; Mackey et al., 2013; Xiong et al., 2005, 2007y; Sun et al., 2003; Hu et al., 2015). In this method 35 ml of ethylene glycol (EG) were heated at 150 °C for 1 h, followed by the addition of 0.38 g polyvinyl pyrrolidone (PVP) (molecular weight of 55 000 g) solution dissolved in 5 mL of ethylene glycol, with temperature at 150 °C. To the resulting solution 0.4 mL of 3 mM sodium sulfide dissolved in EG was added, immediately followed by the addition of 2 ml of 282 mM silver nitrate dissolved in EG. This solution should be injected slowly into the reaction mixture. Reaction took 10–15 min in completion with reduction of silver ions into AgNCs. For purification of AgNCs, the solution was diluted with a mixture of acetone and water and centrifuged at 14 000 rpm for 10 min. Then precipitated AgNCs were re-dispersed in 20 ml of water to make the gold nanocages. To prepare the 45 nm AuNCs with wall thickness of 5 nm, the solution of purified AgNCs was brought to boiling and a 10 mg/L hydrogen tetrachloroaurate aqueous solution was injected slowly until the absorption spectrum of the solution was red shifted to a stable wavelength of 727 nm characteristic of AuNCs (Mackey et al., 2013; Siekkinen et al., 2006) see Fig. 1. Extinction coefficient (ε) of Gold nanocages was calculated based on ICP measurements and was found to be 5.0×10^9. The stock solution of known concentration of AuNCs was obtained applying Eq. S1 (see supporting data). Then this stock solution was utilized to prepare PEG and peptide conjugated AuNCs in phosphate buffer solution (PBS) at pH 7.4. In the third step these conjugated AuNCs were further diluted in Dulbecco's modified Eagle's medium (DMEM, Mediatech) supplemented with 10% v/v fetal bovine serum (FBS, Mediatech) and 1% v/v antimycotic solution (Mediatech) to 0.1 and 0.4 nM, for treatment of HaCaT cells.

2.2. Conjugation of AuNCs

AuNCs were first conjugated with polyethylene glycol (PEG). Briefly, 1 mM aqueous solution of PEG was added to AuNCs at 10^4 M excess (see supporting information). The mixture was ultrasonicated for 2 h followed by centrifugation at 6000 rpm for 20 min and re-suspended in water. In this way AuNCs were coated with thiol-terminated polyethylene glycol (mPEG-SH 5000) through Au-S bond. This was done to prevent nonspecific adsorption of proteins to the particles in a physiological environment. In second step RGD (arginine-glycine-aspartic acid) a cell penetration peptide, also known as cancer specific targeting peptide and NLS (lysine-lysine-lysine-arginine-lysine) sequence peptide, known as nuclear localization signal peptides, were introduced into PEG-AuNCs solution to form two formulation i.e., NLS-AuNCs and RGD/NLS-AuNCs. Conjugation of peptides was achieved by addition of a 5 mM aqueous solution of NLS peptide at 1×10^4 M excess and 1 mM RGD peptide at 8×10^3 M excess of AuNCs respectively. Mixture was placed in dark for 24 h, after which the peptide conjugated nanoparticles were centrifuged at 6000 rpm for 15 min and re-dispersed in Phosphate Buffer Solution (PBS) (Mackey et al., 2013). The UV-visible spectra were recorded and a red shift was observed (see Fig. 1) as a consequence of PEG and peptide conjugation to AuNCs.

2.3. Cell culture

A nonmalignant Human epithelial cell line HaCat (human keratinocytes), were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Mediatech) supplemented with 4.5 g/L glucose and sodium pyruvate, 10% v/v fetal bovine serum (FBS) (Mediatech), and 1% antimycotic solution (Mediatech). Cell cultures were maintained at 37 °C in a 5% CO_2 humidified incubator.

2.4. DLS and TEM

Dynamic light scattering (DLS) measurements were made by means of commercial laser light scattering equipment (Brookhaven) consisting of a BI-200SM motor-driven goniometer and BI-9025AT digital auto correlator at scattering angle of 90°. A cylindrical 22 mWuni-phase Helium- Neon laser (637 nm) with a pinhole of 100 nm and BI-ISTW software was used. A JEOL 100C transmission electron microscope (TEM) was used to characterize gold nanocages synthesized.

2.5. Gold nanocages uptake

To determine the percentage of uptaken AuNCs by HaCaT cells, our previously reported spectroscopic method was used (Austin et al., 2011; Mackey et al., 2013). Shortly, the HaCaT cells were grown in 96-well tissue culture plates for 12 h, followed by the removal of growth media by the fresh AuNCs containing growth media. After a 24 h of incubation with AuNCs, this media was removed and placed in another 96-well plate. The optical density was analyzed on a Biotek Synergy H4 multimode plate reader at wavelengths of 727 nm i.e. plasmon absorption for the AuNCs. The difference between growth media initially added to the cell culture and after 24 h of incubation was recorded. This difference was then converted to a percentage of what was initially added to the cell culture and plotted against surface modification as shown in Fig. 2.