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Research Paper

Low cytotoxicity of anisotropic gold nanoparticles coated with lysine on peripheral blood mononuclear cells "*in vitro*"

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ABSTRACT

The aim of this study was to evaluate the cytotoxic effects of anisotropic (non spherical morphologies) gold nanoparticles coated with the amino acid Lysine (Lys) on peripheral blood mononuclear cells (PBMC) "*in vitro*". Gold (Au) nanoparticles tested in this study were synthesized by a seed-mediated growth using Lys as a structure and shape directing agent. Cytotoxic effects were evaluated by cell viability (resazurin assay), reactive oxygen species (ROS) induction (2′,7′-dichlorofluorescein diacetate assay), DNA damage (comet assay) and apoptosis/necrosis (Annexin V/propidium iodide assay) after PBMC were exposed to increasing concentrations (10, 25, 50, 100, and 250 μM) of AuNPs coated with Lys (AuNPs-Lys) at different exposure times (3, 6, 12, and 24 h). The results demonstrated that AuNPs-Lys exhibited low cytotoxicity towards PBMC, (high cell viability), with low levels of ROS, DNA damage and apoptosis/necrosis detected after treatment. These data suggest that AuNPs-Lys, might be viable for biomedical application subject to further investigations.

1. Introduction

Nanotechnology has emerged over the past decade as a major technological initiative (Fu et al., 2014; Hubbs et al., 2013; Medina et al., 2007; Radomska et al., 2016; Science and Council, 2000) and has led to the fabrication of novel materials, devices, and systems at the nanoscale size (nanoscale refers to matter with at least one dimension between 1 and 100 nm) (Hubbs et al., 2013; Medina et al., 2007; Science and Council, 2000). In this context, many applications in science and technology have been developed (*i.e.*, biomedical, food industry, nanoelectronics, sensors, energy, environment, aerospace, security, among others) (Science and Council, 2000). Regarding biomedical applications, nanomedicine (nanotechnology applied to medicine) has appeared as a new multidiscipline in order to improve disease detection, imaging, and drug delivery through the use of nanodevices (Heath, 2015; Mazaheri et al., 2015; Melita et al., 2015; Science and Council, 2000; Takedatsu et al., 2015).

Recently, metallic nanoparticles have attracted great scientific attention due to their diversity and wide-spread applications (Ahn et al., 2013; Luo et al., 2015; Mu and Sprando, 2010; Tourinho et al., 2012; Zhang et al., 2009). Among all metallic nanoparticles used currently, gold nanoparticles (AuNPs) are increasingly used in nanomedicine because they exhibit a combination of physical, chemical, optical and electronic properties unique for those applications (Dykman and Khlebtsov, 2012; Ghosh et al., 2008; Sperling et al., 2008). For example, AuNPs are largely employed in drug/gene delivery systems as contrast agents and nanosensors, in the imaging and diagnostics of diseases, the sensitization of cells and tissues to treatment regimens, as well as the monitoring and guidance of surgical procedures; preferentially administration of electromagnetic radiation to specific disease sites is also facilitated through this technology (Dykman and Khlebtsov, 2012; Ghosh et al., 2008; Khlebtsov and Dykman, 2011; Sperling et al., 2008). AuNP toxicity in mammalian cells has been investigated in the areas of membrane injury, reactive species oxygen generation, inflammatory responses, DNA damage and apoptosis induction (Chuang et al., 2013; Chueh et al., 2014; Khlebtsov and Dykman, 2011; Mironava et al., 2010; Pan et al., 2007). Moreover, most of these toxicity issues have been associated with the coating agents employed. Then, a major challenge in nanomedicine science is to design highly biocompatible nanomaterials for their use in biomedical applications.

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In this regard, it has been demonstrated that the surface functionalization of nanomaterials with biomolecules increases their biocompatibility and safety (Fraga et al., 2013; Kim et al., 2013). Hence, novel nanoparticles (including AuNPs) coated with proteins, peptides, amino acids, silica, polymer, PEG, among others, have been recommended as an alternative to developing biocompatible nanoparticles for nanomedicine applications (Kang and Ko, 2015; Liu et al., 2015). Indeed, the use of biopolymers or silica coatings can greatly reduce the toxic effects exhibited by AuNPs (Liu et al., 2015; Shi et al., 2007).

Therefore, the aim of this study was to evaluate the cytotoxic effects of novel anisotropic (non-spherical morphologies) AuNPs directly obtained and coated with the amino acid Lysine (AuNPs-Lys) on peripheral blood mononuclear cells (PBMC) "*in vitro*".

2. Materials and methods

2.1. Synthesis of Au-Lys nanoparticles

Gold nanoparticles with anisotropic structures (star-shape) were produced by a two-step synthesis. Firstly, spherical gold nanoparticles were prepared by adding 10 ml of double-distilled water (ddH_2O) to a glass scintillation vial and heated to boil. When liquid started to boil, 5 µl of $HAuCl_4$ (500 mM) were added and mixed vigorously. Then, after 2 min, 20 µl of sodium citrate (500 mM) were added. The stirring was stopped after 3 min when the solution acquired red color and cooled down to room temperature before continuing the next step. Second step, to form anisotropic particles coated with Lysine (AuNPs-Lys), was performed using 100 µl of citrate-gold seeds in 10 ml buffer Lysine (Lys) 25 mM, 10 µl of $NH_2OH{\cdot}HCl$ and 1 ml of HAuCl4 (2.5 mM), mixed with gentle stirring for 10 min, then incubated overnight at 37 °C. Nanoparticles were concentrated by centrifugation at 2000 rpm for 10–15 min. Size distribution in solution was determined by photon-correlation spectroscopy (dynamic light scattering) (ZetasizerNano ZS, Malvern).

2.2. Electron microscopy characterization

For transmission electronic microscopy (TEM), AuNPs-Lys samples were diluted in ddH_2O and loaded onto formvar-carbon copper grids 300 mesh (Electron Microscopy Sciences) and characterized with a high resolution-transmission electronic microscopy (HR-TEM) JEOL-2010F operated at 200 kV. For ultra-high resolution field emission scanning electron microscopy (UHR-FE-SEM) imaging, samples were washed with ddH_2O and centrifuged, then the pellet was re-suspended in a small volume of ddH_2O, 10 µl of nanoparticles were loaded onto 5 × 5 mm ultraflat silicon wafer chips (TedPella) and dried in a desiccator under vacuum. Electron microscopy imaging were carried out with an UHR FE-SEM HITACHI S5500 (HITACHI High Technologies) coupled with SE, BSE, Duo-BF/ADF STEM, and EDX detectors, using an accelerating voltage of 30 kV. Imaging processing with DigitalMicrograph (Gatan), QUARTZ (PCI) and ImageJ v.1.50f (NIH).

2.3. Cell culture and in vitro dosing with auNPs-Lys

PBMC were isolated from heparinized blood (six healthy volunteers; 20-30 years old, non-smokers, 3 males and 3 females) by Ficoll–Hypaque density-gradient centrifugation (Sigma-Aldrich, St. Louis MO, USA) as described previously (Orta-García et al., 2015). After, PBMC (1×10^6 cells ml^{-1}) were cultured in a 25-cm^2 cell culture flask in RPMI-1640 media (supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 50 U ml^{-1} penicillin, and 50 mg ml^{-1} streptomycin; Gibco-Invitrogen Corp., Carlsbad, CA, USA) at 37 °C in 5% CO_2 atmosphere. The study was approved by the bioethics committee of the School of Medicine, UASLP. Also, an informed signed consent was obtained from all volunteers.

For AuNPs-Lys dosing, PBMC were distributed on 24-well culture plates (Costar, Corning, NY, USA) at 1×10^6 cells ml^{-1}. AuNPs-Lys stock (25 mM) was diluted in sterile ultrapure water and added to culture plates to achieve 10, 25, 50, 100, and 250 µM final concentrations. The volume of AuNPs-Lys solution added did not exceed 0.5% of the culture media. Culture plates were incubated for 3, 6, 12, and 24 h.

2.4. Cell viability assay

Cell viability was investigated by seeding PBMC into 24-well plates at a cell density of 1×10^6 cells ml^{-1} per well and cultured for 24 h at 37 °C and 5% CO_2 atmosphere. Subsequently, the media culture was removed and the cells were washed once with cold phosphate-buffered saline solution (PBS) and replaced with freshly RPMI-1640 cell culture medium supplemented. Then, cells were exposed to increasing concentrations of AuNPs-Lys: 10, 25, 50, 100, and 250 µM and incubated for 3, 6, 12, and 24 h. Two hours before the spectrofluorometric reading to determine cell viability trough resazurin assay (Riss et al., 2013), cells were centrifuged and the supernatant was withdrawn and replaced by freshly prepared resazurin reagent (Sigma-Aldrich, St. Louis, MO, USA) diluted in RPMI-1640 cell culture medium supplemented to reach a final resazurin concentration of 30 µg ml^{-1} in a final volume of 500 µl per well. Plates were incubated immediately at 37 °C and 5% CO_2 atmosphere. Fluorescent measurements with an excitation wavelength $\lambda = 560$ nm and an emission wavelength $\lambda = 590$ nm were performed to determine the degree of metabolism of resazurin in a Spectrofluorometer Synergy H1 (BioTek Instruments Inc. Winooski, VT, USA). Untreated PBMC were used as negative control and cells treated with H_2O_2 (0.6%) were set as positive control (Sigma-Aldrich, St. Louis, MO, USA).

The cell viability was calculated with the following equation:

$$\text{Cell viability}(\%) = \frac{\text{sample fluorescence}}{\text{control fluorescence}} * 100\%$$

2.5. Quantification of intracellular reactive oxygen species (ROS)

PBMC were treated with increasing concentrations of AuNPs-Lys: 10, 25, 50, 100, and 250 µM and incubated for 24 h (according to cell viability assay). After treatment, PBMC were washed twice with PBS and re-suspended at 1×10^6 cells ml^{-1} in PBS. Immediately, 1 ml of 2′,7′-dichlorofluorescein diacetate (DCFH-DA; Sigma-Aldrich, St. Louis MO, USA) was added (20 µM). Then, PBMC were incubated at 37 °C during 30 min protected from light. The ROS quantification was performed using a BD FACS Calibur flow cytometer ($\lambda ex = 480$ nm/ $\lambda em = 530$ nm) (Becton-Dickinson, San Jose, CA, USA). A minimum of 10,000 cells were analyzed per sample and the results expressed as fluorescence mean intensity (FMI) (Perez-Maldonado et al., 2005).

2.6. Determination of DNA damage

After PBMC treatment (10, 25, 50, 100, and 250 µM, 24 h of exposure), single cell gel electrophoresis (comet assay) was performed as described previously in several works performed by our research group (Jasso-Pineda et al., 2015, 2012; Orta-García et al., 2015; Perez-Maldonado et al., 2011). Briefly, 50 µl of cell suspension were mixed with 500 µl of low melting point agarose (1%) and added on a slide precoated with a base layer of 1% regular agarose, a third layer of 100 µl of 1% low melting point agarose was made. After solidification of three agarose layers, slides were placed in cups that contained lysis solution [10 mM Tris-HCl, 2.5 M NaCl and 0.1 M EDTA, freshly prepared 1% triton X-100 and 10% DMSO (pH 10.0)] for 24 h at 4 °C. After lysis treatment, slides were then placed in the electrophoresis buffer (300 mM NaOH, 1.0 mM EDTA, pH > 13) for 20 min in dark at 0 °C. Then, electrophoresis was conducted in the alkaline buffer for 20 min at 0.8 V cm^{-1} and 300 mA, following by gently washing with 0.4 M Tris-