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Extensive evaluations of the cytotoxic effects of gold nanoparticles

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ABSTRACT

Background: Many in vitro studies have revealed that the interference of dye molecules in traditional nanoparticle cytotoxicity assays results in controversial conclusions. The aim of this study is to establish an extensive and systematic method for evaluating biological effects of gold nanoparticles in mammalian cell lines.
Methods: We establish the cell-impedance measurement system, a label-free, real-time cell monitoring platform that measures electrical impedance, displaying results as cell index values, in a variety of mammalian cell lines. Cytotoxic effects of gold nanoparticles are also evaluated with traditional in vitro assays.
Results: Among the six cell lines, gold nanoparticles induce a dose-dependent suppression of cell growth with different levels of severity and the suppressive effect of gold nanoparticles was indirectly associated with their sizes and cellular uptake. Mechanistic studies revealed that the action of gold nanoparticles is mediated by apoptosis induction or cell cycle delay, depending on cell type and cellular context. Although redox signaling is often linked to the toxicity of nanoparticles, in this study, we found that gold nanoparticle-mediated reactive oxygen species generation was not sustained to notably modulate proteins involved in antioxidative defense system.
Conclusion: The cell-impedance measurement system, a dye-free, real-time screening platform, provides a reliable analysis for monitoring gold nanoparticle cytotoxicity in a variety of mammalian cell lines. Furthermore, gold nanoparticles induce cellular signaling and several sets of gene expression to modulate cellular physical processes.
General significance: The systematic approach, such as cell-impedance measurement, analyzing the toxicology of nanomaterials offers convincing evidence of the cytotoxicity of gold nanomaterials.

1. Introduction

Engineered nanomaterials, defined as particles ranging from 1 to 100 nm in one or more dimensions, are increasingly being used in different fields ranging from electronics to photonics, chemistry, and biomedicine because of their unique physicochemical properties [1–4]. The widespread use of engineered nanomaterials in many common products increases the amount of nanomaterial in the environment and leads to increased occupational and public exposure. With this proliferation of nanomaterials comes an obligation to improve our understanding of the potential human health risks of nanomaterial exposure. In fact, many peer-reviewed reports have thoroughly described adverse effects of a variety of nanoparticles including, but not limited to, carbon nanotubes [5–9], fullerenes [10–12], nanosilver [13–15], and metal oxide nanoparticles [16–19]. Accumulating evidence now strongly supports the view that the reactivity and relatively small surface area per unit of mass increase the bioavailability and toxicity of nanoparticles compared to their bulk size counterparts [20–22]. However, uncertainties and conflicts in the literature regarding the toxicity of nanoparticles remain [3,5,22–29].

The inconsistency of literature reports may reflect experimental challenges associated with evaluating the toxicity of nanoparticles. In vitro cell-based assays are currently the most commonly used approach for screening nanoparticles for cytotoxicity owing to their relative simplicity, sensitivity, and cost-effectiveness compared to animal testing. There has been a proliferation of reports describing methods for measuring the cytotoxicity of nanoparticles. Among the approaches described are cell viability assays, which measure live versus dead cells in a sample and provide overall estimates of cellular responses to nanoparticles. Most such cell viability assays involve monitoring and quantifying differential metabolism of added dye molecules [30,31], including trypan blue [32], Coomassie blue [33], 3-(4,5-cimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) [13,16,33], 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) [34], or endogenous enzymatic activity, such as lactate dehydrogenase (LDH) [35,36], by living versus dead cells. Unfortunately, such cell viability assays encounter limitations and challenges when applied to evaluate the toxicity of nanoparticles,

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which interact with assay constituents such as enzymes or dyes in ways that potentially bias the results. Moreover, a lack of coordination in research programs at the early stages of work has led to an unfortunate degree of variation in experimental conditions that creates obstacles for reaching definitive conclusions. For example, carbon nanotubes have been found to interact with dye molecules that are used in several cytotoxicity assays, such as MTT, WST-1, neutral red, and Coomassie blue assays [37–39], leading to undependable outcomes. Moreover, the reliability of the LDH assay in evaluating the cytotoxicity of copper, silver, and titanium dioxide nanoparticles is questionable because of inactivation of the LDH protein by nanoparticles [36]. These colorimetric dye-based cytotoxicity assays, therefore, may overestimate or underestimate the toxicity of nanoparticles, depending on the assay, producing inconsistencies in the risk assessments of nanoparticles. Thus, it is important to establish a set of rapid and cost-effective screening platforms to establish the overall toxicity of nanoparticles and identify underlying mechanisms. However, to date, few studies have described label-free, real-time screening platforms for the systemic investigation of nanoparticle cytotoxicity in mammalian cells [40,41].

Among the aforementioned nanomaterials, gold nanoparticles have generated controversy for their potential toxic impact, despite incredible advances in their use for diagnostic and therapeutic purposes, including biosensor applications, targeted delivery of anticancer drugs, bioimaging of cells and tissues, and immunoassays [30,42–44]. Given the potential applications of gold nanoparticles in various fields of nanomedicine, it is imperative that we improve our understanding of their toxic effects on mammalian cells. The purpose of this study was to assess the cytotoxicity of gold nanoparticles using an application of a label-free, real-time screening platform that monitors cell viability by measuring changes in the electrical impedance at the electrode/cell interface. In addition to this real-time screening platform, several traditional cytotoxicity assays were employed to validate the cytotoxicity assessment of gold nanoparticles. Our results suggest that gold nanoparticles are cytotoxic toward numerous lines of mammalian cells, and the cellular responses to gold nanoparticles are dependent on the cellular context but not directly to the sizes and cellular uptake.

2. Materials and methods

2.1. Materials

Fetal bovine serum (FBS) and penicillin/streptomycin were obtained from GIBCO/BRL Life Technologies (Grand Island, NY, USA). 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCFDA) was from Molecular Probes Inc. (Eugene, OR, USA). Commercially available nanosize gold particles were obtained from Sigma Aldrich (St. Louis, MO, USA) and were evaluated by transmission electron microscopy. Anti-poly(ADP) ribose polymerase (PARP), anti-p53, anti-PCNA, anti-caspase 8, anti-phosphorylated ERK, anti-phosphorylated JNK, and anti-phosphorylated p38 antibodies were purchased from Cell Signaling Technology, Inc. (Beverly, MA, USA). The anti-MDM2, anti-ERK, anti-JNK, anti-p38, anti-c-Fos, anti-catalase, anti-thioredoxin reductase, anti-glutathione peroxidase-1/2, and anti-SOD antibodies were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA). The anti-Cyclin B1, anti-GADD45 and anti-Bax antibodies were from Abcam Inc. (Cambridge, MA). The anti-β-actin antibody was from Millipore Corp. (Temecula, CA, USA). Three different sized gold nanoparticles, aqueous solutions and other chemicals were purchased from the Sigma Chemical Company (St. Louis, MO, USA), unless otherwise specified.

2.2. Transmission electron microscopy

The structure of gold nanoparticles was examined using a transmission electronic microscope (JEM-2010; JEOL Ltd., Japan) and according to the procedure described previously [16].

2.3. Inductively coupled plasma mass spectrometry analysis

Control cells or cells exposed to different sizes and concentrations of gold nanoparticles were digested with aqua regia, and the final sample solutions were diluted in a fixed volume (10 mL). Each treatment was repeated for three times. The concentration of elemental gold was determined using an Agilent7500ce inducible coupled plasma mass spectrometer (ICP-MS) system (Agilent Technologies Inc., Japan).

2.4. Cell culture

AGS (human gastric adenocarcinoma cells), A549 (human lung adenocarcinoma epithelial), NIH3T3 (mouse embryonic fibroblast), PK-15 (porcine kidney), and Vero (African green monkey kidney) cells were grown in Dulbecco's Modified Eagle Medium (DMEM); MRC5 (human normal lung tissue) cells were grown in minimal essential medium (MEM). Culture media were supplemented with 10% FBS, 100 units/mL penicillin and 50 μg/mL streptomycin. Cells were maintained at 37 °C in a humidified atmosphere of 5% CO_2 in air, and the medium was replaced every 2–3 days.

2.5. Continuous cell monitoring with the xCELLigence System

For continuous monitoring of changes in cell growth, cells (5×10^3 cells/well) were seeded onto E-plates and incubated for 30 min at room temperature, after which plates were placed onto the RTCA station (xCELLigence Real-Time Cell Analysis System, Roche, Germany). Cells were grown overnight, and impedance was measured every hour prior to treatments as previously described [45]. Cell impedance is represented by the cell index (CI) = $(Z_i - Z_0)$ [Ohm] / 15 [Ohm], where Z_0 is background resistance and Z_i is the resistance at an individual time point. A normalized cell index was determined as the cell index at a certain time point (CI_{ti}) divided by the cell index at the normalization time point (CI_{nml_time}).

2.6. Cell viability assay

Cells (3×10^3) were seeded in 96-well culture plates and permitted to adhere overnight at 37 °C in medium containing 10% serum. Cells were then treated with different concentrations of gold nanoparticles for 72 h. At the end of treatment, cell viability was determined using a rapid, MTS-based colorimetric assay (CellTiter 96 cell proliferation assay kit; Promega, Madison, WI, USA) as described by the manufacturer. All experiments were performed at least in triplicate on three separate occasions. Data are presented as means ± SDs.

2.7. Trypan blue exclusion assay

Cells seeded at 10^4 cells/dish were cultured for the indicated times, and then trypsinized, collected by centrifugation, and washed with PBS. Cell pellets were suspended in 50 μL PBS and 50 μL of a 0.4% (w/v) trypan blue stain solution, and cell numbers were counted and recorded. Each data point represents the mean of three separate experiments (mean ± SD).

2.8. Colony-formation assay

Two hundred cells were seeded onto a 6-cm dish and incubated in culture medium with different concentrations of gold nanoparticles for 10 days to allow colony formation. After incubation, colonies were fixed in 1.25% glutaraldehyde at room temperature for 30 min, rinsed with distilled water and stained with a 0.05% methylene blue solution. The number of colonies was counted and recorded.