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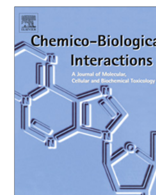

Cytotoxicity in the age of nano: The role of fourth period transition metal oxide nanoparticle physicochemical properties

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A B S T R A C T

A clear understanding of physicochemical factors governing nanoparticle toxicity is still in its infancy. We used a systematic approach to delineate physicochemical properties of nanoparticles that govern cytotoxicity. The cytotoxicity of fourth period metal oxide nanoparticles (NPs): TiO_2, Cr_2O_3, Mn_2O_3, Fe_2O_3, NiO, CuO, and ZnO increases with the atomic number of the transition metal oxide. This trend was not cell-type specific, as observed in non-transformed human lung cells (BEAS-2B) and human bronchoalveolar carcinoma-derived cells (A549). Addition of NPs to the cell culture medium did not significantly alter pH. Physiochemical properties were assessed to discover the determinants of cytotoxicity: (1) point-of-zero charge (PZC) (i.e., isoelectric point) described the surface charge of NPs in cytosolic and lysosomal compartments; (2) relative number of available binding sites on the NP surface quantified by X-ray photoelectron spectroscopy was used to estimate the probability of biomolecular interactions on the particle surface; (3) band-gap energy measurements to predict electron abstraction from NPs which might lead to oxidative stress and subsequent cell death; and (4) ion dissolution. Our results indicate that cytotoxicity is a function of particle surface charge, the relative number of available surface binding sites, and metal ion dissolution from NPs. These findings provide a physicochemical basis for both risk assessment and the design of safer nanomaterials.

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1. Introduction

There are currently more than 2800 nanoparticulate-based applications commercially available. It is estimated that by 2017, this field will represent a $48.9 billion market [1]. As engineered nanoparticles (NPs) currently occupy a significant portion of the market and are anticipated to proliferate commercially, there is an urgent need to study their potential impact on human health and the environment.

To date, there exists no epidemiological or clinical evidence demonstrating that inhalation of NPs leads to adverse health effects in humans [2]. However, toxicological studies using animal models and cell cultures suggest that NPs are more toxic and inflammogenic than larger particles of similar composition and of equal mass [3]. We have demonstrated intricate relationships between NPs, production of ROS and changes in intracellular Ca^{2+} concentrations $[Ca^{2+}]_{in}$. These studies suggest that NPs can trigger cell death by multiple pathways [4]. NPs increase $[Ca^{2+}]_{in}$. Moderation of this increase by nifedipine suggests that a portion of this increase reflects the influx of extracellular calcium. Membrane disruption (e.g., as indicated by lipid peroxidation and membrane depolarization) may also play a role in this influx [4,5]. NPs also disrupt store-operated calcium entry (SOCE) [6]. The increase in intracellular ROS may also have multiple sources. There exist synergistic relationships between intracellular $[Ca^{2+}]$ and OS as the increases in both can be reduced by an antioxidant. Finally, while $[Ca^{2+}]_{in}$ and ROS affect each other, they induce cell death by distinct pathways.

Structural defects on the NPs, which can act as electron-donor/acceptor groups, may alter the electronic configuration and contribute to the formation of reactive oxygen species (ROS) [7]. Particle dissolution has also been considered as a factor in NP-induced toxic responses [8,9]. Particle size and morphology are factors that also contribute to toxicity [10,11]. It remains unclear whether additional physicochemical properties of metal oxide

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NPs dictate the toxic responses. To elucidate these properties, we systematically examined an array of oxides of transition metals in the fourth period of the Periodic Table (Ti, Cr, Mn, Fe, Ni, Cu, Zn). These types of nanomaterials have been extensively used in catalysis [12], magnetocooling [13], optical and recording devices [14,15], purification of enzymes and other biological materials [16], water purification devices [17], magnetic field assisted radionuclide therapy [18], embolics [19–21], cosmetic and skin care products, and targeted drug delivery agents [22–27]. This series of NPs offers an opportunity to investigate the determinants of toxicity, which may lead to the design of safer nanomaterials. Toxicity can be investigated using *in vitro* and *in vivo* systems. Both systems provide different information for various scientific purposes and in many cases are complementary to each other. As there are numerous nanomaterials, it is improbable, though not impossible, to investigate each nanomaterials with *in vivo* systems. Therefore, *in vitro* systems provide an alternative to study nanotoxicity in that (1) it is cost efficient, (2) it provides information to prioritize animal testing, and (3) it informs computational toxicology in the context of quantitative structure–activity relationship (QSARS).

We hypothesize that toxicity is a function of multiple physicochemical properties of nanoparticles. We selected TiO_2, Cr_2O_3, Mn_2O_3, Fe_2O_3, NiO, CuO, and ZnO NPs from a single commercial source to minimize variability. In order to determine whether cytotoxic responses are cell-type specific, two human lung cells were studied. Cells were exposed to these NPs and cytotoxicity was measured. Isoelectric points (i.e., point-of-zero charge), number of available surface binding sites, and band-gap energies of the NPs were measured. The NPs were also subjected to kinetic experiments to determine the extent of metal ion dissolution. Our results indicate that certain physicochemical properties of metal oxide NPs strongly correlate with cytotoxicity.

2. Materials and methods

2.1. Nanoparticles, reagents, and instrumentation protocols

The nanoparticles, reagents, and instrumentation protocols used in the experiments are detailed in the Appendix A. Transmission electron microscopy (TEM), X-ray photoelectron spectroscopy (XPS), and band gap measurements were performed on the NPs. Characterizations of graphite furnace atomic absorption analysis (GFAA) and inductive coupled plasma-mass spectrometry (ICP–MS) of the aqueous solution supernatants exposed to the NPs. Correlations of observed physicochemical properties of the materials were correlated with cytotoxicity.

2.2. Cell culture and exposure of cells to NPs

Human bronchial epithelial cells (BEAS-2B) and human bronchoalveolar carcinoma-derived cells (A549) are *in vitro* models considered as ideal for both studying the prevention of human lung carcinoma development and nanotoxicity testing [28]. These cells were maintained using the same procedures described in our previous studies [4,5,29–31].

Cells were grown at 37 °C in a 5% CO_2 humidified environment. Upon reaching 85% confluence, the cells were seeded into 24 well plates and allowed to attach for 24 h. The cell densities used followed ATCC protocol recommendations, and were well within sensitivity and detection limits of the analytical instruments used. To reduce experimental variation and ensure accuracy, particles were dried in a desiccator before being weighed on an analytical balance. Particles were suspended in cell culture medium, vortexed vigorously, and then sonicated. A series of dilutions in cell culture medium were performed to achieve desired concentrations. The suspensions were immediately applied to cells to minimize agglomeration. Cells without NPs and reagent blanks were used as controls in each experiment.

2.3. Cytotoxicity assay and apoptosis

At the end of cell exposure to NP suspensions, the medium was discarded and the sulforhodamine B assay was used to determine cell viability relative to the control group [31]. Briefly, the cells were fixed with cold 10% trichloroacetic acid (TCA) for 1 h at 4 °C. The TCA solution was then discarded and the cells were washed three times with distilled water, followed by complete drying. Sulforhodamine B (0.2% in 1% acetic acid) was added to stain the cells for 30 min at room temperature. The staining solution was discarded and the cells were washed with 1% acetic acid three times to eliminate excess dye. After complete drying, the dye was dissolved in cold 10 mM Tris buffer (pH = 10.5). Aliquots (100 μL) of dye solution were transferred onto a 96-well plate, and absorbance was measured at 550 nm using a microplate reader (FLOURstar, BMG Labtechnologies, Durham, NC, USA).

Apoptotic cells were stained with annexin V–FITC and 7-aminoactinomycin D (7-AAD) followed by quantification using a Beckman Coulter Cell Lab Quanta SC System. Morphological examination of apoptotic cells was performed using the same dyes and observed with an Olympus IX 51 epifluorescence microscope.

2.4. Statistical analysis

For toxicity studies, three independent experiments were conducted, using triplicates for each treatment group. Data are expressed as mean ± standard deviation. The relationship between cytotoxicity and the physicochemical properties of nanoparticles were analyzed with Spearman's Rank Correlation Analysis.

3. Results

3.1. Size, morphology, and specific surface area

The approximate physical sizes (APS) of the seven commercially available transition metal oxide NPs ranged from 16 ± 5 nm (NiO) to 82 ± 31 nm (Mn_2O_3) (Table 1). The morphology of NPs observed with TEM was needle-like (TiO_2), spherical (Mn_2O_3, Fe_2O_3), or nearly spherical (Cr_2O_3, NiO, CuO, ZnO) (Fig. A1). The specific surface area (SSA) of NPs ranged from 8.71 m^2/g (Mn_2O_3) to 178.95 m^2/g (TiO_2). While TiO_2, Fe_2O_3, and CuO had similar sizes, they possessed distinctly different specific surface areas. This could be due to variations in surface porosity and discrepancy in morphology.

3.2. Influence of pH in cell culture medium on cell viability

Cytotoxicity of NPs may simply reflect changes in pH over time. To evaluate this possibility, NPs were added to the cell culture medium and pH was measured at 0, 6, 12, 18, and 24 h. Immediately after adding NPs to cells in medium, the pH became slightly elevated compared to the control cell in medium only. The pH fluctuated briefly, and then stabilized, except for NiO, which increased the pH between 12 and 24 h. Very little change in pH was observed at low NP concentrations (Table A1). As concentrations of NPs increase, pH variations increased with all NPs. However, the extent of pH fluctuations was 0.29 ± 0.14 and 0.31 ± 0.03 units with and without NPs, respectively. Cell morphology and size in the groups with NPs were similar to those in the control groups.

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