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Inorganic nanoparticles enhance the production of reactive oxygen species (ROS) during the autoxidation of L-3,4-dihydroxyphenylalanine (L-dopa)

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ABSTRACT

Public concerns over the toxicity of nanoparticles (NPs) are growing due to the rapid development of nanotechnology. An important mechanism of nanotoxicity is oxidative stress resulting from reactive oxygen species (ROS). In this study, the chemical production of ROS by inorganic NPs oxidizing the mammalian phenolic compound, L-3,4-dihydroxyphenylalanine (L-dopa) was evaluated using a ROS sensitive dye, 2′,7′-diclorodihydrofluorescin (DCFH). CeO_2, Fe_2O_3 and Fe^0 NPs enhanced ROS production during the autoxidation of L-dopa by more than four-fold in reactions that were dependent on O_2. This is the first report of chemical ROS production due to interaction of phenolic compounds with NPs. Mn_2O_3 oxidized DCFH in a reaction that did not require O_2 or L-dopa, suggesting a direct redox reaction between the Mn_2O_3 and the dye. CeO_2, Mn_2O_3 and to a lesser extent Fe^0 formed clear electron paramagnetic resonance (EPR) signature for hydroxyl radicals when incubated in aerobic aqueous suspensions with spin traps. The results indicate that NPs can generate ROS via chemical reactions with medium components and biomolecules susceptible to oxidation, such as L-dopa. NPs were reactive whereas micron-sized particles were not. The combined assay with L-dopa and DCFH is a method proposed to screen for chemical ROS production by NPs.

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1. Introduction

Growth in the nanotechnology sector is leading to increased production of nanoparticles (NPs). This has given rise to concerns about the potential risks associated with exposure of workers and the general public to NPs. Manufactured nanomaterials such as carbon-based, inorganic- and hybrid structures (quantum dots) have been widely used in various industrial applications (Nowack and Bucheli, 2007). Particularly, inorganic NPs (metal and metal oxide) have received increasing interest due to their extensive applications in medical (Au, Ag), cosmetic, sun screen (TiO_2, ZnO, Fe_2O_3) and industrial (CeO_2, SiO_2, Al_2O_3) applications (Ju-Nam and Lead, 2008; Ema et al., 2010). Due to their small size (<100 nm), NPs exhibit high specific surface areas that are far greater than their corresponding bulk counterparts. The small size often results in higher reactivity, which could enhance the adverse effects to cells with the potential to be toxic (Nel et al., 2006; Schrand et al., 2010).

Many studies have indicated the adverse and toxic effects of NPs to mammalian cells, bacteria or aquatic organisms. (Brunner et al., 2006; Baun et al., 2008; Park et al., 2008). Based on the current available toxicity studies, several mechanisms for NPs toxicity have been hypothesized. The mechanisms proposed are adsorptive surface and cell uptake (Limbach et al., 2007), dissolution of toxic ions (Brunner et al., 2006; Heinlaan et al., 2008), translocation to target organs, oxidation of cell compounds and generation of reactive oxygen species (ROS) (Xia et al., 2006; Park et al., 2008; Kim et al., 2010). Oxidative stress caused by ROS is considered to be an important mechanisms of NPs toxicity (Meng et al., 2009; Kovacic and Somanathan, 2010). The main ROS species include superoxide anion ($O_2^{\cdot-}$), hydroxyl ($OH^{\cdot}$) and hydrogen peroxide (H_2O_2), which are strong oxidants that react rapidly with most biological molecules and can damage cells and DNA *via* oxidative stress (Halliwell and Whiteman, 2004).

In biological systems, generation of ROS by NPs could be due to a direct chemical process involving interaction of NPs with O_2 and/or biomolecules. Alternatively, NPs could induce a biochemical process by promoting cellular enzymatic ROS production. The study of chemical ROS generation by NPs trough their interaction with biological media components could be a good indicator of their potential toxic effects (Limbach et al., 2007; Sauvain et al., 2008). Recently, an *in vitro* test was used as a nonspecific indicator of induced oxidative damage by measuring a decrease in antioxidant capacity of ferric-iron reducing ability of blood serum (FRAS) exposed to NPs (Rogers et al., 2008).

Phenolic compounds such as catechol and L-dopa are among components in biofluids highly susceptible to oxidation. Catechol is synthesized in plant cells and L-dopa is commonly found in

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mammalian cells. L-dopa is synthesized from the amino acid tyrosine and it exists as an intermediate in the synthesis of melanin and dopamine, which are implicated in Parkinson's disease (Serra et al., 2000; Misu et al., 2003; Stefanescu et al., 2009). The chemical generation of ROS via chemical oxidation of phenolic biomolecules by inorganic NPs is proposed here as a screening method for possible toxic effects of NPs to biological systems.

Fluorimetric and colorimetric ROS-indicators dyes such as, 2′,7′-dichlorodihydrofluorescin (DCFH) and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) have been used to study the generation of ROS (Heckert et al., 2008; Chen et al., 2010). DCFH is the dye most used to study both, the biochemical (cells) and chemical (cell-free) generation of ROS by NPs (Chen et al., 2010). The non-fluorescent ROS-indicator dye, DCFH, is oxidized to its highly fluorescent electron product 2′,7-dichlorofluorescein (DCF) by ROS. The objective of this study was to evaluate chemical ROS generation from the oxidation of phenolics by inorganic NPs. The proposed chemical reaction is the oxidation of catechols to semiquinone radicals coupled to the reduction of NPs. Subsequently, semiquinone is oxidized to quinone at the expense of O_2 becoming reduced to superoxide anion radical and ultimately other ROS species (Fig. 1). DCF formation from the ROS-indicator dye, DCFH, was used to monitor the ROS enhancement of phenol autoxidation by NPs.

2. Materials and methods

2.1. Nanomaterials

Mn_2O_3 (30–60 nm, 98% purity), Fe_2O_3 (20–40 nm, 99% purity), Fe^0 (40–60 nm, 99.9% purity), ZrO_2 (20–30 nm, 99% purity), and ZnO (10–30 nm, 99.8% purity) were acquired from SkySpring Inc (Houston, TX, USA). CeO_2 (20 nm, 99.9% purity) was purchased from MTI Co (Richmond, CA). Al_2O_3 (<50 nm, 99% purity), SiO_2 (10–20 nm, 99.5% purity), CeO_2 (50 nm, 99.95% purity) were obtained from Sigma–Aldrich (St. Louis, MO, USA). HfO_2 (100 nm, 99.9% purity) was acquired from American Elements Co. (Los Angeles, CA, USA). All nanomaterials were obtained as dry powders.

2.2. Chemicals

2′,7′-dichlorodihydrofluorescin diacetate (DCFH-DA), 2′,7′-dichlorofluorescein (DCF, 97% purity), 5,5-dimethyl-1-pyrroline N-oxide (DMPO) (⩾97% purity), 3,4-dihydroxyphenyl-L-alanine (L-dopa), 1,2-dihydroxybenzene (catechol, 99% purity), and CeO_2 micron sized (<5 μm, 99.9% purity), all were acquired from Sigma–Aldrich. 2′,7′-Diclorodihydrofluorescin (DCFH) was prepared from DCFH-DA according to a previously published protocol (Cathcart et al., 1983). Stock solutions of 2000 mg L^{-1} of L-dopa or catechol were prepared in a mixture of MilliQ (MQ) water:methanol (7:3 v/v) or in MQ water, respectively.

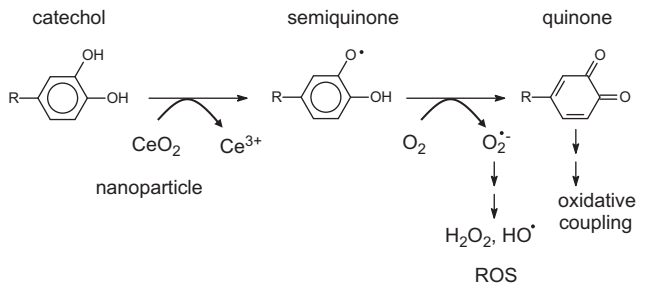

Fig. 1. Hypothesis of chemical reactions leading to the generation of ROS by NPs (CeO_2 as an example) by their interaction with catechol moieties.

2.3. Nanoparticle stock suspensions

Each NP was suspended in a 2000 mg L^{-1} stock suspension in MQ water and adjusted to a pH of 7.2 with a minimum volume of NaOH 0.1 N or HCl 0.1 N. All suspensions were sonicated for 5 min at 70% amplitude using a probe sonicator (Ultrasonic processor 130 W model, Sonics, Newtown, CT, USA). The hydrodynamic particle size and zeta potential of NPs dispersed in ROS assay media were also measured (details in Supplementary information).

2.4. Assay for reactive oxygen species formation

Chemical ROS generation by NPs through their interaction with phenolic biomolecules (catechol and L-dopa) was determined using the ROS-sensitive dye, DCFH, which is oxidized to its highly fluorescent DCF derivative by ROS (Chen et al., 2010). All assays were performed in 25 mM NaH_2PO_4 (pH 7.4), in a total volume of 4 mL containing 500 μM of catechol or L-dopa, 20 μM of DCFH and 200 mg L^{-1} of NPs (dosed from NP stock suspensions described above). Experiments were conducted under aerobic (in vials of 10 mL) or anaerobic (absence of O_2) conditions in order to test the effect of O_2 on the chemical ROS reaction. Anaerobic experiments were conducted on serological bottles of 10 mL, which were flushed with pure nitrogen gas as follows. After adding the NPs, the liquid was immediately flushed by 30 s and the bottles were rapidly capped with butyl rubber stoppers and sealed with aluminum caps. Then, the headspace was flushed with the same gas for 3 min to create anaerobic conditions.

All assays were carried out at 37 °C and masked from light by covering each vial with aluminum foil. Controls with L-dopa alone and NPs alone were run in parallel under same conditions (all in the presence of DCFH). Fluorescence readings were taken at different intervals of reaction time using a fluorescence spectrometer LS55 (Perkin Elmer, Llantrisant, UK) with excitation and emission wavelengths of 485 and 535 nm, respectively. Concentrations of DCF in samples were estimated by performing a calibration curve with a DCF standard. There was no measurable fluorescence due to the NP suspension in the absence of DCFH.

2.5. Replications

The experiments were run with triplicated replicates and in a few cases duplicates were utilized. The results in the graphs are averages of the replicates and the error bars indicate the standard deviation.

2.6. Analytical techniques

Electron paramagnetic resonance (EPR) spectroscopy measurements were conducted to detect the oxygen radicals in NPs suspension in water (1000 mg L^{-1}). The spin trapping agent used was 5,5-dimethyl-1-pyrroline N-oxide (DMPO, 0.02 M)), a common probe to detect $OH^{\cdot}$, which yields DMPO-OH as the spin adduct and has a quartet EPR signal (details in Supplementary information) (Lipovsky et al., 2009). The EPR measurements were performed on a Bruker ESP 300 X-band EPR spectrometer (Rheinstetten/Karlsruhe, Germany) under the following conditions: 9.65 GHz of frequency, microwave power of 20 mW, field modulation amplitude of 1 G, swipe time of 40 s and at room temperature.

In order to determine if Mn^{2+} was released in chemical ROS assay experiments with Mn_2O_3 NPs under aerobic and anaerobic conditions, the soluble manganese was monitored along the reaction time by inductively coupled plasma with optical emission spectroscopy (ICP-OES) in an Optima 2100 DV instrument (Perkin Elmer, Shelton, CT, USA) at a wavelength of 257.01 nm. Sample preparation indicated in Supplementary information.