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In vitro toxicological effects of zinc containing nanoparticles with different physico-chemical properties

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ABSTRACT

Nanomaterials (NM) exhibit novel physicochemical properties that determine their interaction with biological substrates and processes. Recent nano-technological advances are leading to wide usage of metallic nanoparticles (NPs) in various fields. However, the increasing use of NPs has led to their release into environment and the toxicity of NPs on human health has become a concern. Moreover, there are inadvertently generated metallic NPs which are formed during various human activities (e.g. metal processing and energy production). Unfortunately, there are still widespread controversies and ambiguities with respect to the toxic effects and mechanisms of metallic NPs, e.g. metal oxides including ZnO.

In this study, we generated zinc containing NMs, and studied them in vitro. Different nano-sized particles containing Zn were compared in in vitro study to elucidate the physicochemical characteristics (e.g. chemical composition, solubility, shape and size of the particles) that determine cellular toxicity. Zn induced toxicity in macrophage cell line (RAW 264.7) was detected, leading to the cell cycle disruption, cell death and excitation of release of inflammatory mediators. The solubility and the size of Zn compounds had a major role in the induced toxic responses. The soluble particles reduced the cell viability, whereas the less soluble NPs significantly increased inflammation. Moreover, uptake of large ZnO NPs inside the cells was likely to play a key role in the detected cell cycle arrest.

1. Introduction

Despite the extensive use of nanomaterials (NMs) today, there is still a limited understanding of nanoparticle (NP) mediated toxicity. Indeed, there are many uncertainties about the properties of these materials and how they may affect the biological systems (Nel et al., 2006). NMs can induce toxicity through their physical properties e.g. size, curvature, shape, and surface characteristics such as charge, functionalized groups, and free energy (Cedervall et al., 2007). When NMs are introduced in biological systems, NMs dynamically interact with all the encountered biomolecules and form the bio-corona that is also highly dependent on physical properties of NMs. Finally, NM surrounded by bio-corona will contact with the cells (Neagu et al., 2016).

Exposure to NMs is usually occurring in occupational settings but is also inevitable in environment. Zinc is an excellent example of NM, which occurs in both of those settings. A round 100,000 tons of zinc oxide (ZnO) produced annually for e.g. rubber and concrete applications, solar cells, sunscreens and emission gas treatment catalysts etc. (Colvin, 2003; Klingshirn, 2007). Moreover, Zn nanoparticles (NP) are formed and released in ambient air unintentionally in heavy industries (Al-Masri et al., 2006), in transport from brake linings (Hjortenkrans et al., 2007) and in combustion processes (Tissari et al., 2015).

To date, there are several studies that have linked ambient particulate matter (PM) toxicity to small particle size and large reactive surface area as well as individual chemical compositions of PM e.g. zinc (Gao et al., 2005; Jalava et al., 2015; Wu et al., 2013). Similarly to ambient PM (Schaumann et al., 2004) engineered NMs can cause pulmonary inflammation and symptomatic responses in the lung through increased inflammatory mediator production (Andujar et al., 2014). Moreover, exposure to many engineered metal NPs and unin-

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tentionally generated metal rich NPs produces reactive oxygen species (ROS) and oxidative stress in cells and animals (Tao et al., 2003; Zhu et al., 2008). Another property of NPs that induce toxic activities is the release of reactive ions when thermodynamics favor particle dissolution in a suspending medium or biological environment (Franklin et al., 2007). Although it is known that ZnO particles are toxic to mammalian cells in vitro and the human lung in vivo, the mechanisms of toxicity are not fully understood. For example, the contribution of sequential nano-bio-interface to dissolution and toxicity is still under debate (Gatti and Montanari, 2015).

In this study, we generated Zn containing NPs with varying chemical composition, solubility, particle shape and size. The composition and properties of the produced NPs were characterized and compared to $ZnCl_2$ as a reference compound. The particle shape, size and degree of agglomeration were studied with the transmission electron microscopy (TEM). The solubility of the particles in cell culture medium was measured with the atomic absorption spectrometry (AAS) and assessed using a Pitzer ion interaction model. Induced toxicity of generated NPs was analyzed using a RAW 264.7 mouse macrophage cell line. After a 24-h exposure period, the cellular metabolic activity (CMA), production of inflammation mediators, and cell cycle were determined in order to resolve which properties of the NPs most likely explain the detected toxic effects.

2. Experimental procedures

2.1. Materials for nanoparticle synthesis

The liquid precursor solutions that were used for the particle production are shown in Supplementary file 1. Zinc acetate dihydarate (Sigma-Aldrich, ≥ 99.0%) and K_2SO_4 (Riedel-de Haën, 99%) were used as received in the synthesis. The precursors were dissolved in ion-exchanged water in the molarities shown in Supplementary file 1. For the flame spray pyrolysis (FSP), Zn-acetate dihydrate ($C_4H_{10}O_6Zn{\cdot}2H_2O$) (Sigma-Aldrich, ≥ 99.0%) was dissolved in ethanol. Zinc chloride ($ZnCl_2$, Sigma-Aldrich, ≥ 97%) in 5.9 M water solution was used as an ionizing Zn compound reference in the studies.

2.2. Nanoparticle production

Fig. 1 shows the chemical vapor synthesis (CVS) used for generation of the particles (CVS-1, CVS-2 and CVS-3). The precursor solution was atomized using an ultrasonic nebulizer (RBI Pyrosol 7901, France). Dry nitrogen was used as a carrier gas at the flow rate of 1 L min^{-1} to transfer the produced droplets to the furnace through a pre-heater. The pre-heater was set to + 150 °C in order to remove the solvent from the droplets before the actual reaction zone in the high temperature furnace at 1400 °C. The flow was fully laminar and the residence time in the furnace was 5 s. The aerosol exiting the furnace was rapidly cooled with air at a flow rate of 35 L min^{-1} using a porous tube diluter (PRD). The simultaneous dilution and cooling quenched the reactions, prevented re-condensation of the solvent and decreased the wall deposition losses of the produced particles. Furthermore, FSP-1 (ZnO rods) were previously produced with flame spray pyrolysis as described in detail by Torvela et al., 2014.

2.3. Instrumentation and characterizations

2.3.1. Particle on-line and off-line analyses

The properties of the particles produced with CVS were analyzed directly from the gas phase using on-line analyses as follows. The particle size distribution was determined using a scanning mobility particle sizer (SMPS, DMA 3081 with CPC 3775, TSI Inc.) and number concentration was measured using a condensation particle counter (CPC 3775, TSI Inc.). The properties of FSP generated particles were measured with the fast mobility particle sizer (FMPS 3091, TSI Inc.) (Torvela et al., 2014). The concentrations determined with the FMPS and SMPS were corrected with the dilution ratios.

PM samples for toxicological and chemical analyses were collected with Dekati gravimetric impactor (DGI) on polytetrafluoroethylene (PTFE) filters (Millipore) (Ruusunen et al., 2011). The continuous sampling for the DGI took 30 min so that enough mass was collected for toxicological analysis. Several sets of filters from DGI stages below 1 μm were combined into one sample presenting PM_1 (particulate mass concentration with aerodynamic diameter below 1 μm) in each studied case.

PM_1 samples for element analysis were eluted with HF/HNO_3 acid mixture and analyzed with an inductively coupled plasma mass spectrometer (ICP-MS, Perkin Elmer Elan 6000). The samples for anion analysis were eluted with deionized water and analyzed with ion chromatograph (IC, Dionex DX-120) system.

2.3.2. X-ray diffraction

The phase and crystallinity of nanoparticles were determined by X-ray crystallography (XRD) (Bruker D8 Discover with Cu α radiation at 40 kV and 40 mA), at 2θ between 10 and 70°. The crystalline phases were analyzed using the reference data from International Center Diffraction Data (ICDD).Crystallite sizes were obtained using fundamental parameter approach fitting to the full diffractogram using Topas software.

2.3.3. Transmission electron microscopy

Electron microscopy samples were collected on holey carbon copper grid (S147-400 Holey Carbon Film 400 Mesh Cu, Agar Scientific) directly from the gas phase with an aspiration sampler (Lyyränen et al., 2009). Transmission electron microscopy (TEM) and Energy dispersion spectroscopy (EDS) analyses were carried out using a JEM 2100-F (JEOL) field emission TEM equipped with Si(Li)-type detector coupled with a EDS analysator system (NS7, Thermo Scientific). The microscope was operated at 200 kV.

2.4. Assessment of nanoparticle solubility

2.4.1. Equilibrium model

Theoretical solubility of sample particles was assessed with an equilibrium model for the samples CVS-1, CVS-3, FSP-1 and $ZnCl_2$. Unfortunately, CVS-2 could not be considered because no data for zinc succinate was available. Furthermore, the CVS-3 was considered as a mixture of $ZnSO_4$ and K_2SO_4. The calculations were done with the FactSage 6.2 software, Equilib module, which can be used for minimizing the Gibbs free energy (thermodynamic equilibrium) for multiphase multicomponent systems (Bale et al., 2002). The calculations included solid and aqueous phases. The aqueous solution was described using Pitzer ion interaction model available in the software. The considered temperatures were 25 °C, for which there is the most accurate data in the Pitzer model, and 37 °C, which was the cell medium temperature. In addition, varying sample particle dosages and the inorganic constituents in the Roswell Park Memorial Institute (RPMI) cell culture medium (($Ca(NO_3)_2$, $MgSO_4$, KCl, $NaHCO_3$, NaCl, $NaHPO_4$) were taken into account in the calculations (see Supplementary file 2).

2.4.2. Solubility measurements

Amount of soluble Zn was measured in RPMI cell culture medium containing 10% fetal bovine serum (FBS), 2 mM L-glutamine and 100 U mL^{-1} penicillin–streptomycin at three time points (30 min, 6 h and 24 h). The sample handling procedure was same as in the toxicological study set-up. For solubility study, PM dose of 150 μg mL^{-1} was selected. In designated time point ultrafiltration system (Amicon ultra centrifugal filter, NMWL 3 KDa) was used to separate dissolved zinc from the NPs. The soluble Zn was measured by AAS (Analytic Jena Zeenit 700) using air-acetylene flame. The solubi-