Toxicology Letters 225 (2014) 177–184

Contents lists available at ScienceDirect

Toxicology Letters

journal homepage: www.elsevier.com/locate/toxlet

Phosphate-enhanced cytotoxicity of zinc oxide nanoparticles and agglomerates

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HIGHLIGHTS

- NIH/3T3 cells were exposed to individualized and agglomerated ZnO nanoparticles.
- The cytotoxic response in growth media with and without phosphates was assessed.
- With phosphates present, zinc phosphate formation enhanced cell death.
- Agglomeration-dependent cell death only existed in the presence of phosphates.
- Nanomaterial interactions with growth media constituents can be easily overlooked.

ARTICLE INFO

Article history:
Received 29 October 2013
Received in revised form 5 December 2013
Accepted 6 December 2013
Available online 18 December 2013

Keywords:
Zinc phosphate toxicity
Zinc oxide toxicity
Nanohazard
Culture media byproduct
Aggregation

ABSTRACT

Zinc oxide (ZnO) nanoparticles (NPs) have been found to readily react with phosphate ions to form zinc phosphate ($Zn_3(PO_4)_2$) crystallites. Because phosphates are ubiquitous in physiological fluids as well as waste water streams, it is important to examine the potential effects that the formation of $Zn_3(PO_4)_2$ crystallites may have on cell viability. Thus, the cytotoxic response of NIH/3T3 fibroblast cells was assessed following 24 h of exposure to ZnO NPs suspended in media with and without the standard phosphate salt supplement. Both particle dosage and size have been shown to impact the cytotoxic effects of ZnO NPs, so doses ranging from 5 to 50 μg/mL were examined and agglomerate size effects were investigated by using the bioinert amphiphilic polymer polyvinylpyrrolidone (PVP) to generate water-soluble ZnO ranging from individually dispersed 4 nm NPs up to micron-sized agglomerates. Cell metabolic activity measures indicated that the presence of phosphate in the suspension media can led to significantly reduced cell viability at all agglomerate sizes and at lower ZnO dosages. In addition, a reduction in cell viability was observed when agglomerate size was decreased, but only in the phosphate-containing media. These metabolic activity results were reflected in separate measures of cell death via the lactate dehydrogenase assay. Our results suggest that, while higher doses of water-soluble ZnO NPs are cytotoxic, the presence of phosphates in the surrounding fluid can lead to significantly elevated levels of cell death at lower ZnO NP doses. Moreover, the extent of this death can potentially be modulated or offset by tuning the agglomerate size. These findings underscore the importance of understanding how nanoscale materials can interact with the components of surrounding fluids so that potential adverse effects of such interactions can be controlled.

Abbreviations: NP, nanoparticle; PBS, phosphate buffered saline; PVP, polyvinylpyrrolidone; PS, penicillin streptomycin; PSG, penicillin streptomycin L-glutamine; FBS, fetal bovine serum; $DMEM^{+phos}$, Dulbecco's modified Eagle's medium (DMEM) with phosphates; $DMEM^{-phos}$, DMEM without phosphates; XRD, X-ray diffraction; HRTEM, high-resolution transmission electron microscopy; AAS, atomic absorption spectrometry; DLS, dynamic light scattering; ICP-MS, inductively coupled plasma-mass spectrometry.

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http://dx.doi.org/10.1016/j.toxlet.2013.12.005

1. Introduction

The rapid expansion of nanotechnology research in recent years has led to an increased awareness of how advancements could potentially elicit both positive and negative consequences for the environment and human health. Our current level of exposure to nanomaterials, in the products we buy and industrial waste we produce, is expected to increase significantly in the near future. As such, researchers and public officials have started devoting more attention and resources toward understanding nanomaterial-related health hazards and how to establish the proper framework from which generalizations can be drawn that relate the danger of a nanoscale material to its physical properties (e.g., size, shape, composition, surface chemistry) (Barnard, 2006; Nel et al., 2006). Nanometer-sized ZnO falls under the classification of "potential nanohazard" due to its use in consumer products such as cosmetics and sunscreens (Gulson et al., 2010; Sadrieh et al., 2010), its introduction into the environment through wastewater and other processing/disposal pathways (Adams et al., 2006; Limbach et al., 2008), and the potential for inhalation (Brown, 1988).

The health and environmental safety of ZnO nanoparticles has thus far been assessed by examining its penetration into human skin (Gamer et al., 2006; Gulson et al., 2010; Nohynek et al., 2007; Sadrieh et al., 2010) and its toxicity in bacterial (Adams et al., 2006; Jin et al., 2009; Reddy et al., 2007; Sawai et al., 1995) and fungal strains (Sawai and Yoshikawa, 2004), various mammalian cell lines (Brunner et al., 2006; Deng et al., 2009; Gojova et al., 2007; Jeng and Swanson, 2006; Reddy et al., 2007; Xia et al., 2006, 2008; Yin et al., 2010), and in whole-animal models (Xia et al., 2011). These studies have primarily focused on the toxicological effects of particle size, ZnO dose dependence, and nanoparticle (NP) dissolution resulting in Zn^{2+} liberation into the growth media. Other work (Cho et al., 2011; Limbach et al., 2005) has looked more broadly at the role of NP agglomeration and associated sedimentation rates on the toxicological response specific to the cell culture environment. Importantly, ZnO has been demonstrated (Jung et al., 2009; Reed et al., 2012) to convert to zinc phosphate ($Zn_3(PO_4)_2$) crystallites in the presence of phosphate salts. As phosphates are ubiquitous in physiological fluids as well as wastewater streams, it is important to examine the potential effects that the formation of $Zn_3(PO_4)_2$ crystallites may have on cell viability with varying agglomerate size and dosage.

Toward this goal, the cytotoxicity of individual water-soluble ZnO NPs (with a nominal diameter of ~4 nm) and ZnO NP agglomerates (ranging from ~8 nm to several microns in size) in cell culture media with and without phosphates was examined. ZnO NPs in agglomerated states (Limbach et al., 2005) (as opposed to studying the toxicity of ZnO NPs with different nominal particle diameters (Adams et al., 2006; Deng et al., 2009)) were examined to find potential agglomerate size effects. To control agglomerate size, NPs were sterically stabilized (Lafuma et al., 1991) using polyvinylpyrrolidone (PVP), an amphiphilic polymer with a small molecular weight that is considered to be non-toxic in vivo (even at moderately high concentrations (Bergfeld et al., 1998; Robinson, 1990)) and is used for a variety of applications in areas such as medicine, pharmaceuticals, and food and cosmetics production. By adjusting the amount of soluble PVP available to physisorb and stabilize the ZnO, agglomerate size was adjusted from the micron scale down to individually dispersed NPs. Following 24 h of exposure to various ZnO dispersions in media with or without phosphates, cell viability was assessed by quantifying cell metabolic activity and measuring the release of cytosolic lactate dehydrogenase. These relatively short-term cell culture studies were selected to avoid biasing viability due to cells being unable to replenish their internal phosphate reserves in the phosphate-free media.

2. Materials and methods

2.1. Materials

Zinc acetate, potassium hydroxide, sodium chloride, methanol, hexane, isopropanol, 4-(2-hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), phosphate buffered saline (PBS; 1×, pH 7.4), and 8 kDa polyvinylpyrrolidone (PVP) were purchased from Sigma Aldrich and used without further purification. Penicillin streptomycin (PS) solution, penicillin streptomycin L-glutamine (PSG) solution, and DMEM growth media were obtained from Mediatech (Manassas, VA). Heat-deactivated fetal bovine serum (FBS) was acquired from Hyclone Laboratories (Logan, UT).

2.2. Media preparation

Prior to media preparation, heat-inactivated FBS was dialyzed against HEPES buffered saline (10 mM HEPES, 150 mM NaCl) with a 0.5–1.0 kDa membrane (Spectrum Laboratories) to remove phosphates present within the FBS. This dialyzed FBS was used for both media preparations (with and without phosphates), so that any loss of serum constituents would have an equal effect on both groups. DMEM with L-glutamine (cat# 10-013-CM) was supplemented with 10% dialyzed FBS and 1% PS and used as the "with-phosphates" medium (hereafter termed $DMEM^{+phos}$). DMEM without phosphates or L-glutamine (cat# 17-206-C1) was prepared with 10% dialyzed FBS and 1% PSG and used as the "without-phosphates" growth medium (hereafter termed $DMEM^{-phos}$). For preparation of ZnO NP dilutions, both fully supplemented media types were concentrated to 4× using a centrivap and then filtered using 0.22 μm syringe filters (Millipore). ICP-MS analyses found that the concentration of Zn^{2+} in both DMEM preparations was below the detectable limit of the instrument, and no phosphorous was detected in $DMEM^{-phos}$.

2.3. ZnO nanoparticle preparation

Colloidal ZnO NPs, ~4 nm in diameter, were synthesized by hydrolyzing zinc acetate dihydrate in a potassium hydroxide/methanol solution in the presence of PVP (an amphiphilic polymer) at a weight ratio of 3:5 (PVP:Zn^{2+}); for a more detailed description of the procedure, please refer elsewhere (Guo et al., 2000; Sun et al., 2007). Excess free ions were removed from the ZnO dispersion through repeated (i) destabilization of the NPs with hexane, (ii) removal of the supernatant, and then (iii) redispersion in methanol under sonication (Sun et al., 2007). The ZnO particles were then concentrated by evaporating methanol from the purified ZnO with a rotovap until a near gel-like state was achieved. Following the initial ZnO preparation, colloidal ZnO was further modified under ultrasonication at 25 °C through physisorption of PVP dissolved in deionized water (DI water) at three pre-determined weight ratios (PVP:ZnO = 300:1, 200:1, 100:1) based on surface area considerations (~265 m^2/g fully dispersed), yielding ZnO agglomerates varying from several microns in size down to individually dispersed NPs. The ZnO stock dispersions were then diluted with DI water and 4× media to yield a final media concentration of 1×, and once added to the cell culture wells, resulting ZnO dosages of 50, 40, 30, 20, 10, or 5 μg/mL.

2.4. ZnO nanoparticle characterization

ZnO NPs were characterized with a variety of methods to determine particle size, structure, and spectroscopic properties and to confirm that the PVP present during synthesis and ultrasonication had simply physisorbed to the NP surface. The crystalline structure of the colloidal ZnO was analyzed by X-ray diffraction (XRD; D8