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Solubility-driven toxicity of CuO nanoparticles to Caco2 cells and *Escherichia coli*: Effect of sonication energy and test environment

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ABSTRACT

Due to small size and high surface energy nanoparticles (NPs) tend to agglomerate and precipitate. To avoid/diminish that, sonication of NPs stock suspensions *prior* toxicity testing is often applied. Currently, there is no standardized particle sonication protocol available leading to inconsistent toxicity data, especially if toxicity is driven by NPs' dissolution that may be enhanced by sonication.

In this study we addressed the effect of sonication on hydrodynamic size (D_h), dissolution and toxicity of copper oxide (CuO) NPs to mammalian cell line Caco-2 *in vitro* and bacteria *Escherichia coli* in the respective test environments (cell culture MEM medium, bacterial LB medium and deionised (DI) water). NPs were suspended using no sonication, water bath and probe sonication with different energy intensities.

Increased sonication energy (i) decreased the D_h of CuO NPs in all three test environments; (ii) increased dissolution of NPs in MEM medium and their toxicity to Caco-2; (iii) increased dissolution of NPs in LB medium and their bioavailability to *E. coli*; and (iv) had no effect on dissolution and antibacterial effects of NPs in DI water. Thus, to reduce variations in dissolution and toxicity, we recommend sonication of NPs in DI water following the dilution into suitable test media.

1. Introduction

The safety evaluation of industrial chemicals (*e.g.*, nanoparticles (NPs)) is a key issue in EU chemical regulation REACH (registration, evaluation, authorisation and restriction of chemicals). However, while the information on toxicity of NPs is rapidly expanding, the amount of high quality data remains limited (Krug, 2014; Oomen et al., 2014) leading to the inconsistent toxicity values for the same type of NPs (Bondarenko et al., 2013a). One reason for that are the variations in methodological settings that can significantly influence the physico-chemical characteristics of NPs and hence, their toxicity. The latter is especially crucial if toxicity is driven by dissolution of NPs (for example, CuO, Ag, ZnO) that depends on a variety of factors *e.g.*, test medium composition, temperature and time (Kasemets et al., 2009). During the recent years it has become evident that it is necessary to systematically and accurately define and report physico-chemical characteristics (such as primary size, specific surface area, purity, crystalline structure) of NPs *prior* the test as well as in the test conditions (hydrodynamic size, zeta potential, dissolution) in order to interpret the results of the toxicity tests (Kahru and Ivask, 2013; Krug, 2014; Nel et al., 2013; Zhu et al., 2012). However, the preparation of NP dispersion and the influence of preparation method on the physico-chemical (such as aggregation/agglomeration and dissolution) and biological (toxicity) characteristics of NPs received considerably less attention. Currently, there is no common standardized protocol for the preparation of NP dispersions for the nanotoxicology studies (*e.g.*, solvent, additives, intensity of sonication).

Different approaches for the preparation of NP suspensions have been published over the last few years. To keep NPs in dispersion, the suspension of NPs is usually treated by vigorous stirring or ultrasound either using ultrasonication bath (providing low intensity energy input) or probe sonicator (higher intensity energy input). There are several studies showing that the particle dispergation and ultrasonication conditions can affect the properties of NPs in solution (dispersibility, hydrodynamic size, agglomeration, aggregation and dissolution) (Bihari et al., 2008; Jiang et al., 2009; Meißner et al., 2014; Taurozzi et al., 2012) and consequently, the toxicity (Cronholm et al., 2011; Magdolenova et al., 2012; Piret et al., 2014). Most of these previous studies focused on the effects of dispergation method on physico-chemical behaviour of TiO_2 NPs that are not dissolving. However, little is known about the effect of sonication procedure on the hydrodynamic size, dissolution and

Abbreviations: ATCC, American Type Culture Collection; D_h, hydrodynamic size; DI water, deionised water; DLS, dynamic light scattering; MEM, minimum essential medium; E_{spec}, specific energy; FBS, fetal bovine serum; LB, Luria-Bertani medium; MBC, minimal biocidal concentration; NEAA, non-essential amino acids; NPs, nanoparticles; OD, optical density; PBS, phosphate buffered saline; PDI, polydispersity index; REACH, regulation concerning the registration, evaluation, authorization and restriction of chemicals; ROS, reactive oxygen species; SSA, specific surface area; TXRF, total reflection X-ray fluorescence.

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subsequent toxicity of solubilisation-prone NPs such as CuO NPs. A few studies comparing the toxic effects of sonicated *vs* not sonicated CuO NPs (Cronholm et al., 2011; Midander et al., 2009) and sonicated *vs* stirred CuO NPs (Piret et al., 2014) inferred that CuO dispergation method may affect dissolution of NPs and hence, their toxicity. Indeed, previous studies have shown that dissolution is the most important factor in the toxicity of CuO NPs to various bacteria (Bondarenko et al., 2012; Juganson et al., 2015; Kaweeteerawat et al., 2015; Käkinen et al., 2011; Puzyn et al., 2011) as well to mammalian cells *in vitro* (Ivask et al., 2015; Karlsson et al., 2013, 2014; Zhang et al., 2012). This implies that variations in sonication protocol affecting the dissolution of NPs will most probably influence their cytotoxicity. As the dissolution of NPs depends on the surface properties such as surface reactivity, charge, composition and presence of surface defects (Midander et al., 2007a,b) that can be modulated during sonication process, we hypothesised that the CuO NP preparation method can significantly affect dissolution and toxicity of NPs to various cell types.

In the present study, we asked (i) to what extent the sonication protocol affects the toxicity of NPs in various test environments to different cell types and (ii) whether the sonication-induced changes in hydrodynamic size or/and dissolution contribute into this process. Two types of NPs, CuO from Sigma-Aldrich and CuO NPs from Intrinsiq Materials were dispersed using four different approaches i) no sonication, ii) sonication in water bath for 30-min or sonication with probe sonicator with specific energies (E_{spec}) iii) $5.3 \cdot 10^4$ kJ/m^3 and iv) Espec $= 6 \cdot 10^5$ kJ/m^3. Three different test environments were used to prepare the CuO dispersions and test their toxicity and bioavailability to bacteria *Escherichia coli* and mammalian cell line Caco-2 *in vitro*. Both, bacteria (unicellular prokaryotic organisms with rigid cell wall and unable to particle internalisation by endocytosis) and mammalian cells (eukaryotic cells capable to particle endocytosis) are widely used as models in (nano)toxicology studies. However, most of the studies focus either on mammalian or bacterial cells and do not enable the direct comparison of the toxicity of NMs to these different cell types. It has been previously suggested that the toxicity and mechanism of action of CuO NPs to these two cell types are remarkably different due to presence or lack of particle-internalisation capability (Bondarenko et al., 2013a). It is generally accepted that the main mechanism of toxicity of metal-based dissolution-prone NMs (such as CuO) to bacteria is mediated *via* dissolved metal ions (Ivask et al., 2010; Sotiriou and Pratsinis, 2010; Bondarenko et al., 2012; Xiu et al., 2012). In contrast, additional particle-specific effects such as *e.g.*, size, shape and agglomeration status may be involved in the toxicity of NMs to mammalian cells (Lankoff et al., 2012; Piret et al., 2012; Karlsson et al., 2014). Thus, we hypothesised that sonication procedure may have different effects on bacterial *vs* mammalian cells through the modulation of agglomeration status and dissolution of CuO NMs.

2. Material and methods

2.1. Chemicals and nanoparticles

Autoclaved deionised (DI) water (18 MΩ, Millipore) was used throughout the study. CuO NPs were purchased from Sigma-Aldrich (CAS Number 1317-38-0) and from Intrinsiq Materials. NaCl, tryptone and yeast extract were from LabM. Minimum Essential Medium (MEM) with GlutaMAX, sodium pyruvate, non-essential amino acids (NEAA) and streptomycin-penicillin were from Gibco, Life Technologies; Fetal Bovine Serum (FBS) was from Biological Industries.

Bacterial test medium LB was prepared by dissolving 10 g of tryptone, 5 g of yeast extract and 5 g of NaCl in 1 l of DI water (Sambrook et al., 1989), autoclaved (121 °C for 15 min) and stored at room temperature. Cell culture medium MEM contained 15% FBS, 1% NEAA, 1% sodium pyruvate, 100 μg/ml and 100 U/ml streptomycin-penicillin, respectively.

2.2. Preparation of nanoparticle stock suspensions

CuO NP stock suspensions (20 ml, 1 g Cu/l) were prepared using different dispersion methods (Fig. 1): no sonication, bath sonication (Branson 1510) and probe sonication (450 Ultrasonifier, Branson Ultrasonics Corporation) equipped with 3 mm microtip, either in DI water (pH = 5.8), LB (pH = 7) or MEM (pH = 7.2) media. Calorimetric determination of the delivered acoustic energy of probe sonicator was performed *prior* experiment to determine the specific energy E_{spec} (Taurozzi et al., 2011) as described in supplementary information (Fig. S1). $E_{spec} = 5.3 \cdot 10^4$ kJ/m^3 corresponded to sonication of 20 ml of NP suspension for 3 min at 10% of probe sonicator power and $E_{spec} = 6 \cdot 10^5$ kJ/m^3 to 13 min 4 s at 25% of power. Probe sonication was performed in an ice bath to avoid heating of NP suspensions. Not sonicated NP suspension was shortly vortexed (1–2 s) to mix NPs before diluting the NP suspensions. The stock suspensions were used immediately after preparation.

2.3. Physico-chemical characterization of CuO stock suspension

2.3.1. Hydrodynamic size, zeta potential and polydispersity of CuO NPs

Hydrodynamic size and polydispersity index (PDI) of CuO NPs were determined using dynamic light scattering (DLS) (Zetasizer Nano-ZS, Malvern Instruments, UK) at the concentration of 100 mg Cu/l at 0, 2 and 24 h after stock suspension preparation in DI water, LB or MEM. The measurements were carried out in triplicates using standard polypropylene plastic cuvettes of 1 cm path length. Zeta potential of CuO NPs in DI water was measured in triplicate using Zetasizer Nano-ZS and Disposable folder capillary cells.

2.3.2. Dissolution of CuO NPs

Time dependent (0, 2 and 24 h) dissolution of CuO NPs in different test environments (DI water, LB, MEM) was determined using total reflection X-ray fluorescence (dissolution, Picofox S2, Bruker Corporation). For this CuO NPs stock solutions were diluted to 100 mg Cu/l in the respective medium, centrifuged at 20,000*g* for 30 min (Centrifuge Sigma 3-16PK). To measure dissolution, CuO NP suspensions were centrifuged immediately after dilution (0 h dissolution) or after 2 h or 24 h of incubation at 30 °C followed by centrifugation at 20,000*g* for 30 min. After centrifugation supernatants were collected and analysed by TXRF by mixing 40 μl of supernatant with 40 μl of reference element (2 mg/l Ga) and pipetting the 3 μl of the mixture to quarts sample holder (Analyslide Petri Dish, Pall Corporation). Three independent experiments were performed.

2.3.3. Analysis of bioavailable copper

The quantification of bioavailable Cu was performed in LB medium using recombinant biosensor bacteria *E. coli* MC1061 (pSLcueR/pDNPcopAlux) in which bioluminescence is specifically induced by subtoxic concentrations of bioavailable Cu ions (Ivask et al., 2009) essentially as described by Bondarenko et al. (2013b). Bacteria were pre-grown overnight on a shaker (200 rpm, 30 °C) in 3 ml of LB medium supplemented with 100 μg/l of ampicillin and 100 μg/l of tetracycline to maintain the recombinant plasmids. 20 ml of fresh LB was inoculated with 1/50 diluted overnight culture, and bacteria were grown at 30 °C until exponential phase (OD_{600} of 0.6) and diluted with LB medium until $OD_{600} = 0.1$ (approximately 10^6 bacterial cells/ml).

100 μl of bacterial suspension was exposed to 100 μl of 0.01–30 mg Cu/l dilutions of $CuSO_4$ or CuO NPs in LB medium at 30 °C for 2 h. Dose–response curves of the Cu-biosensor were obtained by plotting the applied concentrations of Cu against the bioluminescence of Cu-biosensor (as fold induction) in respective samples. Fold induction was calculated by dividing the bioluminescence of Cu-biosensor in the sample to the background bioluminescence (0 mg Cu/l). Bioluminescence was measured using Orion II Luminometer (Berthold Detection Systems, Germany).