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An interlaboratory comparison of nanosilver characterisation and hazard identification: Harmonising techniques for high quality data

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ABSTRACT

Within the FP7 EU project NanoValid a consortium of six partners jointly investigated the hazard of silver nanoparticles (AgNPs) paying special attention to methodical aspects that are important for providing high-quality ecotoxicity data. Laboratories were supplied with the same original stock dispersion of AgNPs. All partners applied a harmonised procedure for storage and preparation of toxicity test suspensions. Altogether ten different toxicity assays with a range of environmentally relevant test species from different trophic levels were conducted in parallel to AgNP characterisation in the respective test media. The paper presents a comprehensive dataset of toxicity values and AgNP characteristics like hydrodynamic sizes of AgNP agglomerates and the share (%) of Ag^+-species (the concentration of Ag^+-species in relation to the total measured concentration of Ag). The studied AgNP preparation (20.4 ± 6.8 nm primary size, mean total Ag concentration 41.14 mg/L, 46–68% of soluble Ag^+-species in stock, 123.8 ± 12.2 nm mean z-average value in dH_2O) showed extreme toxicity to crustaceans *Daphnia magna*, algae *Pseudokirchneriella subcapitata* and zebrafish *Danio rerio* embryos (EC50 < 0.01 mg total Ag/L), was very toxic in the in vitro assay with rainbow trout *Oncorhynchus mykiss* gut cells (EC50: 0.01–1 mg total Ag/L); toxic to bacteria *Vibrio fischeri*, protozoa *Tetrahymena thermophila* (EC50: 1–10 mg total Ag/L) and harmful to marine crustaceans *Artemia franciscana* (EC50: 10–100 mg total Ag/L). Along with AgNPs, also the toxicity of $AgNO_3$ was analyzed. The toxicity data revealed the same hazard ranking for AgNPs and $AgNO_3$ (i.e. the EC50 values were in the same order of magnitude) proving the importance of soluble Ag^+-species analysis for predicting the hazard of AgNPs. The study clearly points to the need for harmonised procedures for the characterisation of NMs. Harmonised procedures should consider: (i) measuring the AgNP properties like hydrodynamic size and metal ions species in each toxicity test medium at a range of concentrations, and (ii) including soluble metal salt control both in toxicity testing as well as in Ag^+-species measurements. The present study is among the first nanomaterial interlaboratory comparison studies with the aim to improve the hazard identification testing protocols.

1. Introduction

Hazard identification, i.e. the identification of the effects of concern, is an important step in assessing nanomaterial risk and is required under multiple regulatory frameworks worldwide (Hristozov et al., 2014). The first nanoecotoxicological studies emerged almost 10 years ago and ever since the field has considerably proliferated (Kahru and Dubourguier, 2010; Kahru and Ivask, 2013). For example, a search in the database Science Direct made in March 2009 yielded only 17 articles on keywords "silver nanoparticles and ecotoxicity" (Kahru and Dubourguier, 2010) while the same search done in July 2015 already revealed 268 records. According to Kahru and Ivask (2013), silver nanoparticles (AgNPs) are within the top five nanomaterials studied for their (eco)toxicological properties when considering nanomaterials listed in the OECD Sponsorship Programme for the Testing of Manufactured

Abbreviations: UL, University of Ljubljana, Slovenia; NICPB, National Institute of Chemical Physics and Biophysics, Estonia; CCMB, The Centre for Cellular & Molecular Biology, India; Eawag, Swiss Federal Institute of Aquatic Science and Technology, Switzerland; UFZ, The Helmholtz Centre for Environmental Research, Germany; FHG-IKTS, Fraunhofer Institute for Ceramic Technologies and Systems, Germany.

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Nanomaterials (OECD, 2010). Moreover, given that applications utilising antimicrobial properties of AgNPs are likely to further increase, e.g., due to the applications related to providing safe drinking water in large regions of the world such as India (Sarma, 2011) and Africa (Simonis and Basson, 2011), the risk assessment of AgNPs must have a priority.

A number of comprehensive reviews have already been published with an attempt to draw general conclusions on the environmental hazard of AgNPs (Fabrega et al., 2011; Bondarenko et al., 2013; Chernousova and Epple, 2013; Ivask et al., 2013, 2014; Sharma et al., 2014; Baker et al., 2014). The obtained toxicity values vary considerably. For example, a 275-fold variation in the toxicity values was observed for different mammalian cells in vitro (25 values), 500-fold for different strains of bacteria (46 different median EC_{50}, LC_{50}or Minimal inhibitory values) and 40-fold difference for *Daphnia magna* (13 different 48 h EC_{50} values) (data taken from Bondarenko et al., 2013; Ribeiro et al., 2014). One reason for this could be different types of AgNPs in terms of size, supplier and stabiliser/coating. Furthermore, dispersion procedures and exposure conditions varied between the studies.

The current view of the risk assessors is that although the information on ecotoxicity of various types of nanomaterials (NMs) is rapidly expanding, a limited number of high quality data is available for univocal hazard and risk assessment of NMs (Jackson et al., 2013; Oomen et al., 2014). There is an ongoing debate which criteria define high quality data and unification of these criteria is still needed (Krug, 2014). Several attempts have been made to define the criteria concerning physico-chemical characterisation (Mills et al., 2014; Kühnel et al., 2014) and toxicity testing (Kühnel et al., 2014). The latter has been done by the DaNa project (Data and knowledge on nanomaterials - processing of socially relevant scientific facts; www.nanoobjects.info) which suggested the co-called Literature Criteria Checklist (Kühnel et al., 2014). The term quality toxicity data in the present paper means that the toxicity study has considered (i) the basic rules for toxicity study (relevant exposure concentrations, reference controls, impurities…), (ii) specific NMs properties (interferences with the assay, suitable dispersion agents,…), and (iii) sufficient and competent NM physico-chemical characterisation data (Hristozov et al., 2014; Jackson et al., 2013; Oomen et al., 2014; Kühnel and Nickel, 2014; Bondarenko et al., 2013; Krug, 2014).

To increase the quality of hazard identification data a number of international initiatives has been undertaken to harmonise, and standardise the toxicity testing protocols. Among these are: the OECD Working party on Manufactured Nanomaterials (Kühnel and Nickel, 2014), ISO technical Committee 229, and the NanoSafety Cluster Working group 10 (Oomen et al., 2014). The EU FP7 large-scale integrated project NanoValid (www.nanovalid.eu) aims to develop a set of reliable reference methods for hazard identification and exposure assessment of engineered NMs. Within this scope, a consortium of six NanoValid partners jointly investigated the hazard of AgNPs, paying special attention to methodical aspects that are important for providing high-quality ecotoxicity data as defined in the chapter above. To diminish the variability caused by different batches of AgNPs and different storage and preparation of suspensions for toxicity tests, the partners were supplied with the same original stock dispersion of AgNPs, and a harmonised procedure for handling of AgNPs was applied. For bioassays, organisms from different environments and trophic levels were chosen. In addition to the toxicity tests, the partners were also responsible for in-house characterisation of the hydrodynamic size of AgNP agglomerates and the percentage (%) of Ag^+ and Ag^+-test medium ligand complexes in comparison to total Ag prior to separation (hereafter referred to as the share of Ag^+-species) in the respective test media.

The aim of this paper is to deliver the experience and recommendations from FP7 NanoValid consortium to improve the hazard identification of nanomaterials. We focused on the specific challenges associated with the characterisation of AgNP dispersion in toxicity test media in parallel to the toxicity testing using a wide array of environmentally relevant test species. In particular, we addressed the importance of harmonised procedures for AgNP characterisation during the course of experiments.

2. Materials and methods

2.1. Case study set-up

Six NanoValid partner research institutions participated in the study: University of Ljubljana (UL, Slovenia), National Institute of Chemical Physics and Biophysics (NICPB, Estonia), The Centre for Cellular & Molecular Biology (CCMB, India), Swiss Federal Institute of Aquatic Science and Technology (Eawag, Switzerland), Helmholtz Centre for Environmental Research (UFZ, Germany), and Fraunhofer Institute for Ceramic Technologies and Systems (FHG-IKTS, Germany). The experimental set-up is schematically presented in Fig. 1. The partners were provided with the same stock dispersion of polyvinylpyrrolidone (PVP) stabilised AgNPs (see Section 3.1 for characteristics).

All partners used the same procedure for the storage and preparation of test suspensions. First, the original stock was vortexed and then diluted to final test concentrations without prior sonication. Always freshly prepared dispersions were used for the bioassays. Each partner performed the characterisation of AgNPs (hydrodynamic diameter and the share of Ag^+-species) in their respective test media as well as in dH_2O using different concentrations of AgNPs. Analyses of the share of Ag^+-species and toxicity tests were done at different time periods after the receipt of the stock dispersion. Details on the time of analyses were carefully recorded and are presented in Fig. 1.

2.2. Toxicity tests

The following organisms from different taxonomic groups and a fish cell line were chosen: the rainbow trout (*Oncorhynchus mykiss*) intestinal cells (RTgutGC) in vitro, the naturally luminescent marine bacterium *Vibrio fischeri*, protozoa *Tetrahymena thermophila*, freshwater green alga *Pseudokirchneriella subcapitata*, freshwater crustacean *D. magna*, marine crustacean *Artemia franciscana*, and freshwater zebrafish *Danio rerio* (different early life stages). To investigate different toxicity of NPs for zebrafish due to potential time changes in NPs (aging), the tests with zebrafish were done by the same partner at two different time points: May 2013 and August 2014. All test media compositions are described in Supplementary information (Table S1). In addition, in all toxicity tests the $AgNO_3$ was used as an ionic control for AgNP.

2.2.1. Rainbow trout intestinal cells in vitro

The assay was performed by Eawag, Switzerland. Rainbow trout intestinal cells (RTgutGC cells) were cultured as described previously (Kawano et al., 2011). For cytotoxicity investigation, 150,000 cells were seeded per well of a 24 well plate (polystyrene) in 1 mL Leibovitz L-15 medium (L-15 supplemented with 5% FBS and 1% gentamycin) and grown for 48 h at 19 °C. Prior to incubation with AgNPs, the cells were washed twice with 1 mL exposure medium (L-15/ex, Schirmer et al., 1997) and then incubated with 1 mL L-15/ex containing the indicated concentrations of AgNPs for 24 h.

For determination of cytotoxicity, a combined assay involving three endpoints (metabolic activity, lysosomal and membrane integrity) was used (Schirmer et al., 1998). After incubation with AgNPs, the medium was removed and the cells washed twice with 1 mL phosphate buffered saline (PBS). The cells were then incubated with 400 μL PBS containing 5% (v/v) Alamar Blue and 4 μM 5-Carboxyfluorescein Diacetate, Acetoxymethyl Ester (CFDA-AM). After 30 min incubation, fluorescence (λ_{ex} = 530 nm λ_{em} = 595 nm for Alamar Blue, λ_{ex} = 493 nm λ_{em} = 541 nm for CFDA-AM) was quantified using a multiwell plate reader (Infinite M200, Tecan, Maennedorf, Switzerland). Cells were subsequently washed once with 1 mL PBS and then incubated in 400 μL PBS containing 1% (v/v) Neutral Red solution (final concentration of Neutral