Comparison of the toxicity of metallic nanoparticles and corresponding ionic and bulk forms using alternative *in vitro* assays with invertebrate cells

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Abstract

The use of nanomaterials has displayed a wide development in the last few decades which has raised concerns about the release of these materials into the environment and their potential threat for human and ecosystem health. Consequently, tools for environmental risk assessment of nanomaterials are needed, including *in vitro* techniques (Handy et al., 2008). The aim of the present work was first, to compare the cytotoxicity of different metallic nanoparticles (NPs) and corresponding ionic and bulk forms applying simple cell viability tests in invertebrate cells. The second objective was to compare the mechanisms of toxic action of CuO, Ag and CdS NPs using an array of functional tests covering the main cellular processes. For this, hemocytes and gill cells of a marine bivalve mollusc, the mussel *Mytilus galloprovincialis* were selected as immune and epithelial cell models, respectively. Mussels are used worldwide as bioindicators and sentinels of environmental pollution (Cajaraville et al., 2000). Due to their filter-feeding activity and well-developed endo-lysosomal system, mussels have been considered an important target for NP toxicity in the marine environment and thus, they represent a key species to evaluate NP toxicity (Canesi et al., 2012).

Nanoparticles of CuO, ZnO, TiO_2, SiO_2, Ag, Au and CdS were studied. Selected nanoparticles varied in size, crystal structure, mode of synthesis and presence of additives (Table 1). Nanoparticles were characterized by TEM for particle size and by DLS for particle size distribution and aggregation. Dissolution of NPs was also assessed. NP exposures (24 h) were performed in parallel with their respective bulk and ionic forms. Internalization of NPs was studied by confocal microscopy and TEM when possible. A two-tier strategy was developed to test NPs toxicity. In the step 1, NPs cytotoxicity was screened at a wide range of concentrations using the neutral red and thiazolyl blue tetrazolium bromide (MTT) assays. The cytotoxicity of different additives was also tested. LC50 values were calculated (Table 1) and the three most toxic NPs were selected for the step 2. In the step 2, mechanisms of action of NPs were evaluated at sublethal concentrations (below LC25) through the following functional tests: production of reactive oxygen species (ROS), catalase (CAT) activity, DNA damage by Comet assay, lysosomal acid phosphatase (AcP) activity, multixenobiotic resistance (MXR) transport activity, Na-K-ATPase activity (only in gill cells) and phagocytic activity and damage to actin cytoskeleton (only in hemocytes).

Uptake and accumulation of some NPs such as CdS quantum dots (QDs) occurred in the endo-lysosomal system of hemocytes whereas SiO_2 NPs were not internalized (Fig. 1). Based on the cell viability assays, NP forms were less toxic than ionic forms but more toxic than the bulk forms. Ag, CdS and CuO NPs (selected for the step 2) were the most toxic NPs tested and SiO_2 NPs the less toxic (Table 1). Size, mode of synthesis and the presence of additives influenced NPs toxicity. In the mechanistic tests, ionic forms were generally more toxic than bulk and NP forms. Common mechanisms of action of the three NPs tested were ROS production and oxidative stress, DNA damage (Fig. 2) and activation of lysosomal activity and MXR transport activity in the two cell types. CuO and Ag NPs decreased Na-K-ATPase activity in gill cells and affected the actin cytoskeleton in hemocytes. Effects on hemocyte phagocytic activity were particle specific in exposures to CdS (Fig. 3) and Ag. In conclusion, cell-mediated immunity and gill cell function represent significant targets for NPs toxicity in mussels. The two-tier strategy developed and the selected *in vitro* assays could provide relevant tools in environmental risk assessment of NPs.

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Table 1. Summary table of the selected NPs and the LC50 values (in mg metal/L) obtained in hemocytes, based on MTT assay.

NPs	Additives	Mode of synthesis	Size (nm)	Crystal structure	LC50 values (mg metal/L) Hemocytes MTT
Ag	Maltose	Wet chemistry	20	nd	5.8
			40		8.43
			90		9.5
	No	unknown[(a)]	20		22.7
			80		21.7
CuO		plasma	100	Tenorite	9.37
CdS	Glutathione	Wet chemistry	5	nd	10.4
ZnO	Ecodis P-90	Milling	20 – 70		13.4
			500x260x10-100		39.4
TiO_2	No	Wet chemistry	10	Rutile	25.5
			40		34.5
			60		37.6
		Plasma	100	55 % Rutile / 45 % Anatase	30.8
	DSLS[(b)]	Milling	60	Rutile	19.8
	No	unknown[(a)]	21[(c)]	10 % Rutile / 70 % Anatase - 20 % other materials	54.9
Au	Na-citrate	Wet chemistry	5	nd	76.3
			15		81.6
			40		83.4
SiO_2	Arginine	Wet chemistry	15		> 100
			30		> 100
			70		> 100

nd: no data; [(a)] Commercial NPs; [(b)] DSLS: Disodium laureth sulfoccinate; [(c)] According to the data available in the manufacture's web page (http://www.aerosil.com/product/aerosil/ja/effects/photocatalyst/pages/default.aspx); [(d)] According with Deiana et al. (2013); [(e)] According with Griffitt et al. (2008);

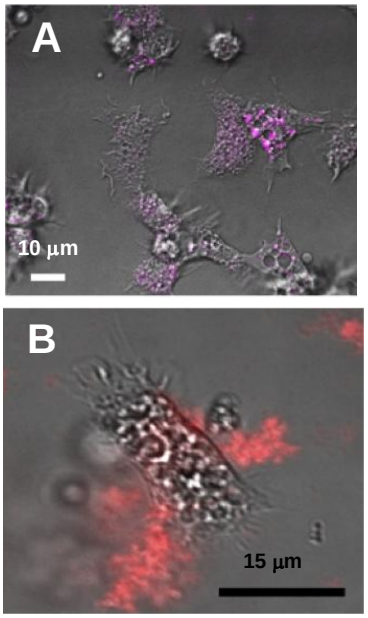

Figure 1. Fluorescent CdS QDs and SiO_2 NPs (hemocytes). (A) Intracellular localization of CdS QDs in endolysomal system. (B) SiO_2 NPs are not taken by mussel hemocytes. Taken from Katsumiti et al. (2014)

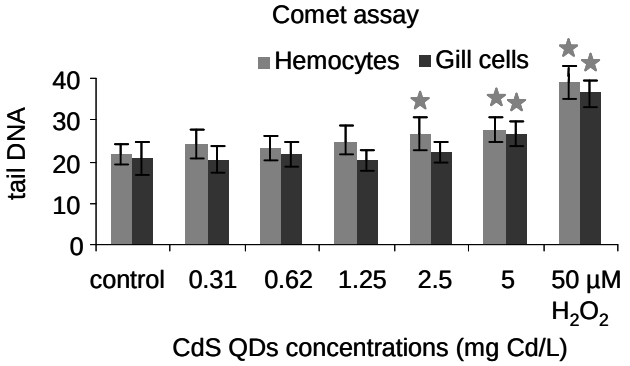

Figure 2. DNA damage in hemocytes and gill cells exposed to CdS QDs. Taken from Katsumiti et al. (2014)

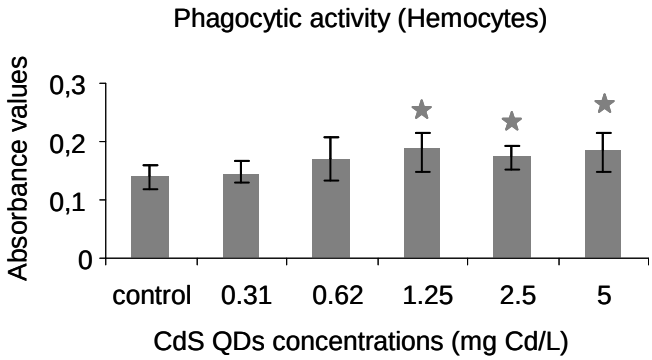

Figure 3. Phagocytic activity in hemocytes exposed to CdS QDs. Taken from Katsumiti et al. (2014)