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Immunocytotoxicity, cytogenotoxicity and genotoxicity of cadmium-based quantum dots in the marine mussel *Mytilus galloprovincialis*

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ABSTRACT

There is an increased use of Quantum Dot (QDs) in biological and biomedical applications, but little is known about their marine ecotoxicology. So, the aim of this study was to investigate the possible immunocytotoxic, cytogenotoxic and genotoxic effects of cadmium telluride QDs (CdTe QDs) on the marine mussel *Mytilus galloprovincialis*. Mussels were exposed to 10 μg L^{-1} of CdTe QDs or to soluble Cd [$Cd(NO_3)_2$] for 14 days and Cd accumulation, immunocytotoxicity [hemocyte density, cell viability, lysosomal membrane stability (LMS), differential cell counts (DCC)], cytogenotoxicity (micronucleus test and nuclear abnormalities assay) and genotoxicity (comet assay) were analyzed. Results show that *in vivo* exposure to QDs, Cd is accumulated in mussel soft tissues and hemolymph and induce immunotoxic effects mediated by a decrease in LMS, changes in DCC, as well as genotoxicity (DNA damage). However, QDs do not induce significant changes in hemocytes density, cell viability and cytogenetic parameters in opposition to Cd^{2+}. Soluble Cd is the most cytotoxic and cytogenotoxic form on *Mytilus* hemocytes due to a higher accumulation of Cd in tissues. Results indicate that immunotoxicity and genotoxicity of CdTe QDs and Cd^{2+} are mediated by different modes of action and show that *Mytilus* hemocytes are important targets for *in vivo* QDs toxicity.

1. Introduction

The widespread production and use of engineered nanoparticles (ENPs) will likely increase their release into the aquatic environment, as well as their interaction, bioaccumulation or transfer in aquatic food webs (Jackson et al., 2012; Farrell and Nelson, 2013; Ma and Lin, 2013). An example of ENPs are Quantum Dots (QDs), which are semiconductor metalloid-crystal structures with a nanometer diameter (2–100 nm) and contain a metalloid crystalline core (usually Cd-based) coated with a shell or ligands. A great capacity to recognize specific cellular targets, strong fluorescence at narrow and size-tunable wavelengths, resistance to photobleaching, electronic and catalytic properties, makes QDs one of the most exploited ENPs not only in nanomedicine but also in pharmacy, biology and electronics (Michalet et al., 2005; Rizvi et al., 2010; Zhang et al., 2012). So far, few studies exist on the ecotoxicological effects of these ENPs in marine organisms, focusing mainly in algae (Morelli et al., 2012), mussels (Gagné et al., 2008; Peyrot et al., 2009; Bruneau et al., 2013; Katsumiti et al., 2014; Munari et al., 2014), crustaceans (Jackson et al., 2012; Feswick et al., 2013) and fish (Tang et al., 2013; Zhang et al., 2013).

The ecotoxicology of QDs has been mainly related to two main mechanisms, the release of Cd ions from the ENPs core and/or the generation of free radicals or reactive oxygen species (ROS) (Gagné et al., 2008; Peyrot et al., 2009; Morelli et al., 2012; Tang et al., 2013). Cd is a non-essential metal classified as a priority substance in the field of water policy by European Water Framework Directive (Directive, 2008/105/EC), as human carcinogen by the International Agency for Research on Cancer (IARC, 1993) and the US Environmental Protection Agency (EPA, 1999) and its carcinogenic and genotoxic effects towards aquatic organisms well studied (Emmanouil et al., 2007; Vincent-Hubert et al., 2011; Chandurvelan et al., 2013). Given the capacity of Cd-based QDs to induce oxidative stress and Cd^{2+} release, the immunocytotoxic, cytogenotoxic and

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genotoxic effects of QDs in aquatic species need to be thoroughly investigated.

Bivalve molluscs, mainly the mussel *Mytilus* spp., are good bioindicators to assess the toxicity and environmental risk of nanoparticles (NPs) in aquatic ecosystems (Canesi et al., 2012; Ciacci et al., 2012; Montes et al., 2012). The immune system of bivalves is also a sensitive target of NPs toxicity (Canesi and Procházková, 2014). *Mytilus* spp. lack adaptive immunity, but the blood cells (hemocytes) are responsible for cell-mediated immunity *via* phagocytosis, production of reactive oxygen species (ROS), reactive nitrogen species (RNS), cytotoxic reactions, secretion of antimicrobial peptides and probably wound healing (Canesi et al., 2010, 2012). *Mytilus* hemocytes classified as granular hemocytes (eosinophilic) and agranular hemocytes (hyalinocytes and basophils) possess high, low and no phagocytic capability, respectively (Le Foll et al., 2010). Particle composition, size, shape, aggregation state and concentration are keys elements for determining the cytotoxicity, immunoactivity and immunoefficiency of QDs to bivalve species, in combination with exposure routes (saltwater, freshwater, sediment), differences within and among species and time of exposure (Gagné et al., 2008; Peyrot et al., 2009; Bruneau et al., 2013; Katsumiti et al., 2014). Furthermore, it was recently demonstrated that different QDs induce significant effects on hemocytes immune parameters of the marine mussels *Mytilus edulis* and *Mytilus galloprovincialis* and the freshwater mussel *Elliptio complanata* (Gagné et al., 2008; Peyrot et al., 2009; Bruneau et al., 2013; Katsumiti et al., 2014). However, the mechanism of *in vivo* toxicity of ENPs on different types of hemocytes is still unclear.

In terms of biomarkers of cytogenotoxicity, the micronucleus (MN) and nuclear abnormalities assay has been applied to measure chromosomal damage in mussels (Burgeot et al., 1996; Vincent-Hubert et al., 2011; Bolognesi and Fenech, 2012; Canesi et al., 2014). The comet assay is a sensitive technique for the detection of DNA strand breaks induced by NPs in bivalves hemocytes and provides a marker for genotoxic effects (Al-Subiai et al., 2012; Gomes et al., 2013). Therefore, the combination of different genotoxicity and cytogenotoxicity tests (comet assay, MN test and nuclear abnormalities assay) allows a more realistic analysis of the immunogenotoxic effects of pollutants in bivalve molluscs.

Accordingly, the aim of this work was to characterize the CdTe QDs behavior in aquatic systems and analyze their possible immunocytotoxic, cytogenotoxic and genotoxic effects on *M. galloprovincialis* hemocytes after short-term exposure (14 days). Immunocytotoxicity of QDs was evaluated by measuring several functional parameters, as hemocyte density, cell viability, lysosomal membrane stability (LMS) and differential cell counts (DCC) of circulating hemocytes, and cytogenotoxic effects analyzed by the MN test, nuclear abnormalities and comet assay. Furthermore, the immunotoxic effects of QDs were compared with those of their soluble counterpart Cd^{2+} after *in vivo* exposure to an environmentally realistic Cd concentration.

2. Materials and methods

2.1. QD characterization

2.1.1. Stock solution

Powder of orange CdTe QDs (2–7 nm diameter, emission wavelength at 590 ± 5 nm) was purchased from PlasmaChem GmbH (Berlin, CAS# 1306-25-8), and according to the supplier they consist of a CdTe core coated by carboxyl groups (-COOH) to prevent aggregation. A QD stock solution was made using Milli-Q water (100 mg L^{-1}), sonicated for 30 min (Ultrasonic bath VWR International, 230 V, 200 W, 45 KHz frequency) and kept in constant shaking. Soluble cadmium stock solution (Cd^{2+}) was prepared identically but not sonicated using cadmium nitrate ($Cd(NO_3)_2 \cdot 4H_2O$) (Merck).

2.1.2. Morphology, size, surface charge and aggregation kinetics

Particle shape and size was characterized using Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS), and zeta potential determined by Electrophoretic Light Scattering (ELS). For TEM analysis, a drop of the stock solution was deposited onto a 300 mesh copper grid coated with a carbon layer and the excess solution was removed by tissue paper and allowed to dry at room temperature. The images were obtained in a JEOL (JEM-1011) microscope, using image analysis software (Soft Imaging System). The hydrodynamic diameter (d_h) of QDs was determined by DLS using a ZetaSizer Nano (ZS 90, Malvern, Inc.) analyzer. A He–Ne laser (633 nm wavelength) was used as a light source and the intensity of scattered light was measured at 90°. In these measurements, 12 mm square disposable polystyrene cells (DTS0012, Malvern, Inc.) were used. Zeta potential (ζ-potential) was determined using the same equipment in a disposable polycarbonate capillary cell (DTS1061, Malvern Inc.) at 25 °C. The aggregation kinetics of QDs under environmentally relevant conditions of salinity and pH were determined by time-resolved DLS measurements. Each measurement was performed over a time period of 12 h. The polydispersity index (PDI) was also determined during a cycle of 12 h by DLS. For these analysis, CdTe QDs (40 mg L^{-1}) were suspended in two aqueous media, Milli-Q water (18 MΩ/cm) and natural seawater ($S = 36.3$), over a wide range of pH (1.7–12), sonicated for 15 min and the pH changed using HNO_3 or NaOH.

2.1.3. Sedimentation rate (SR)

The SR of CdTe QDs (40 mg L^{-1}) was measured by the change in turbidity with time (0–24 h), as described in Sousa and Teixeira (2013). The SR is related to the normalized nanoparticle turbidity C/C_0, where C is the turbidity at time t and C_0 the initial turbidity at time 0. The SR is $\delta(C/C_0)/\delta t$, estimated from the initial 5% decrease in normalized particle turbidity which occur within the first hour for the fast sedimentation (fast SR) conditions and within 12–24 h for slow sedimentation (slow SR) conditions (Keller et al., 2010).

2.1.4. Spectral properties

QD optical properties were investigated in Milli-Q water at pH 8.0 by means of UV–Vis absorption spectra using a spectrophotometer (Jasco V-650) controlled with Jasco's Spectra Manager™ software and fluorescence spectra by Infinite 200 PRO multimode reader (TECAN) equipped with Tecan i-Control™ software, using the excitation and emission spectra of 530 and 545 nm, respectively.

2.2. Exposure experiments

Mussels *M. galloprovincialis* Lam. (60.1 ± 5.1 mm shell length) were collected from Ria Formosa Lagoon (South of Portugal) and acclimated for 7 days in static tanks containing natural seawater ($S = 36.3$) at 16 °C and constant aeration. After acclimation, fifty mussels were placed in 30 L tanks filled with 25 L of seawater (2.0 mussels/L) and exposed to 10 μgCd L^{-1} of CdTe QDs and to their soluble counterpart Cd^{2+} jointly with a control group kept in clean seawater in a triplicate design (3 tanks per treatment). Exposure experiments were conducted in static conditions under 12 h:12 h light/dark cycles and abiotic parameters in seawater were analysed daily by measuring salinity, temperature, pH and oxygen saturation (36.3 ± 0.07, 16.6 ± 1.2 °C, 7.9 ± 0.1, 103.0 ± 1.3%, respectively). The animals were collected at the beginning of the experiment and after 3, 7 and 14 days of exposure. Due to the tendency of QDs to agglomerate in seawater, water was changed daily with redosing of