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In vitro exposure of haemocytes of the clam *Ruditapes philippinarum* to titanium dioxide (TiO_2) nanoparticles: Nanoparticle characterisation, effects on phagocytic activity and internalisation of nanoparticles into haemocytes

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ABSTRACT

The continuous growth of nanotechnology and nano-industries, the considerable increase of products containing nanoparticles (NPs) and the potential release of NPs in aquatic environments suggest a need to study NP effects on aquatic organisms. In this context, *in vitro* assays are commonly used for evaluating or predicting the negative effects of chemicals and for understanding their mechanisms of action. In this study, a physico-chemical characterisation of titanium dioxide NPs (*n*-TiO_2) was performed, and an *in vitro* approach was used to investigate the effects of *n*-TiO_2 on haemocytes of the clam *Ruditapes philippinarum*. In particular, the effects on haemocyte phagocytic activity were evaluated in two different experiments (with and without pre-treatment of haemocytes) by exposing cells to P25 *n*-TiO_2 (0, 1 and 10 μg/mL). In addition, the capability of *n*-TiO_2 to interact with clam haemocytes was evaluated with a transmission electron microscope (TEM). In this study, *n*-TiO_2 particles showed a mean diameter of approximately 21 nm, and both anatase (70%) and rutile (30%) phases were revealed. In both experiments, *n*-TiO_2 significantly decreased the phagocytic index compared with the control, suggesting that NPs are able to interfere with cell functions. The results of the TEM analysis support this hypothesis. Indeed, we observed that TiO_2 NPs interact with cell membranes and enter haemocyte cytoplasm and vacuoles after 60 min of exposure. To the best of our knowledge, this is the first study demonstrating the internalisation of TiO_2 NPs into *R. philippinarum* haemocytes. The present study can contribute to the understanding of the mechanisms of action of TiO_2 NPs in bivalve molluscs, at least at the haemocyte level.

1. Introduction

Nanotechnology is one of the fastest growing and most promising technologies but may present a variety of hazards for environmental and human health (Moore, 2006). The nanotechnology market, as a unified market, was first quantified in 2001 by the National Science Foundation, which predicted its value to be 1 trillion dollars by 2015, but the value of the market has increased steadily over time (Nel et al., 2006). In particular, metal nanoparticles (NPs) represent the highest-volume component of total metal oxide production (Kumar, 2006). However, due to the relative novelty of this technology, information about the potential risks that NPs can pose to non-target organisms are scarce. NPs are widely used in many consumer products and in a variety of disciplines, including medicine, cosmetics, renewable energy, electronic devices and environmental remediation. They show unique physico-chemical properties, such as large surface area, charge and shape, that differ from those of their respective bulk materials (Handy et al., 2008). These features may result in i) direct generation of reactive oxygen species (ROS), ii) a high affinity for organic and metallic pollutants, and iii) an ability to penetrate cells (Al-Subiai et al., 2012). The development of nanotechnologies has

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introduced remarkable amounts of manufactured NPs into the environment, including aquatic ecosystems. NPs can reach marine coastal areas from various sources and by various routes, which can affect their chemical nature, fate, behaviour and toxicity (Matranga and Corsi, 2012; Rana and Kalaichelvan, 2013).

Among NPs, titanium dioxide (TiO_2) is widely used because of its distinctive physico-chemical properties (photocatalysis, inertness, opacity, resistance to fading). *n*-TiO_2 commonly occurs in many consumer products (sunscreens, cosmetic, medicine, paints, textiles, plastics, paper and wood preservatives), industrial products, and photocatalytic processes (Couleau et al., 2012; Jacobash et al., 2014; Frenzilli et al., 2014). A review published by the United States Environmental Protection Agency (USEPA) estimated the annual production of *n*-TiO_2 to be 2000 metric tons in 2005, with 65% of this production used in products such as cosmetics and sunscreen lotions (USEPA, 2009). Jovanović (2014) reported that from 1916 to 2011, an estimated total of 165,050,000 metric tonnes of TiO_2 pigment were produced worldwide. The predicted levels of *n*-TiO_2 in the aquatic environment are on the order of a few μg/L (Menard et al., 2011).

From an ecotoxicological perspective, it has been demonstrated that the immune system of aquatic organisms is a sensitive target for NPs, which can induce changes in the immune responses of exposed cells/animals (Ciacci et al., 2012; Couleau et al., 2012; Jovanović and Palić, 2012; Barmo et al., 2013). Like other invertebrates, bivalves rely on an innate, non-lymphoid immune system involving both cellular and humoural components (Baracco et al., 1999; Wootton et al., 2003). Haemocytes are primarily involved in defence against pathogens but are also responsible for other physiological process, including wound and shell repair, shell production, digestion and transport of nutrients, and excretion (Matozzo et al., 2007; Donaghy et al., 2009). In molluscs, phagocytosis is one of the most important mechanisms for eliminating nonself materials (Takahashi and Muroga, 2008).

It is well known that both biotic and abiotic factors can strongly affect haemocyte parameters in bivalves. However, most previous studies (both *in vitro* and *in vivo*) have been focused on the evaluation of contaminant effects on bivalve haemocyte functionality (Pipe and Coles, 1995; Galloway and Depledge, 2001; Matozzo, 2014). Regarding NPs, recent studies have demonstrated that this distinctive class of contaminants can affect various biological responses at the cellular, subcellular and molecular levels (Canesi et al., 2012; Matranga and Corsi, 2012; Baker et al., 2014). In particular, *n*-TiO_2 (photoinducible, redox active and a potential generator of ROS) has been shown to induce immunostimulation or immune suppression in molluscs (Ciacci et al., 2012; Couleau et al., 2012; Barmo et al., 2013; Grimaldi et al., 2013; Wang et al., 2014).

Despite these findings, to the best of our knowledge only one study has investigated the negative effects of NPs in the clam *Ruditapes philippinarum* (Garćia-Negrete et al., 2013). To provide further information concerning NP toxicity in this clam species and in bivalves in general, an *in vitro* approach was used to evaluate the effects of *n*-TiO_2 on *R. philippinarum* haemocytes. In *R. philippinarum*, four cell types have previously been identified, namely, haemoblasts, serous cells, and two types of immunocytes (granulocytes and hyalinocytes) (Cima et al., 2000). In this study, *n*-TiO_2 features (size, shape and particle size distribution) were determined and NPs effects on phagocytic activity were evaluated in two experiments. In the former, haemocytes were first exposed to *n*-TiO_2 and then incubated with yeast cells; in the latter, haemocytes were incubated with a yeast suspension containing *n*-TiO_2. The aim of this experimental design was to investigate whether pre-treatment of haemocytes was able to induce more marked effects on clam haemocytes. In addition, to evaluate possible interactions between haemocytes and NPs, an electron microscope analysis of haemocytes was performed.

2. Materials and methods

2.1. Nanoparticle characterisation

Nanosised titanium dioxide P25 (declared size of 21 nm and ≥99.5% purity) was purchased from Sigma–Aldrich (Milano, Italy). *n*-TiO_2 particles were characterised by a combination of analytical techniques. The mean average diameter and shape of the primary particles were determined with a TEM (FEI Tecnai G12) operated at 100 kV. Digital images were taken with a TVIPS F114 camera, and the size of the particles was measured by IMAQ Vision (National Instrument, USA).

X-ray diffraction (XRD) characterisation was performed with a Bruker D8 Advance diffractometer. The analyses were performed in Bragg-Brentano configuration at 30 kV and 30 mA. The mean crystallite size was evaluated using the Sherrer equation.

The surface areas and porosities of TiO_2 NPs were characterised by nitrogen adsorption and desorption analysis at 77.35 K with an autosorb computer controlled surface analyser (AUTOSORB-1, Quantachrome). The surface areas were calculated with Brunauer-Emmett-Teller (BET) theory.

The particle size distribution was measured by laser diffraction (Malvern Mastersizer Hydro 2000, Malvern Instruments, UK). The NPs were dispersed using a small amount of dispersant medium (distilled water) and sonicated for 10 min before analysis. The dispersion was poured into the Hydro 2000 dispersion unit (Malvern, UK) until the obscuration was in range. The analysis was performed in triplicate. Particle size distribution was then defined using the particle refractive index values of water and titanium dioxide (1.330 and 2.741, respectively). The particle size distribution was evaluated as $d(0.5)$ and SPAN. The latter is an index of particle size polydispersity and is expressed by the following equation: $\text{Span} = d(0.9) - d(0.1)/d(0.5)$, where $d(0.9)$, $d(0.1)$, and $d(0.5)$ are the diameters at 90%, 10% and 50% cumulative volume, respectively, of the particles.

2.2. Clams

Specimens of *R. philippinarum* were collected from a reference site that was located inside a licensed area for clam culture in the southern basin of the Lagoon of Venice (Italy) and were acclimatised in the laboratory for 7 days before the beginning of the experiments. Clams were maintained in large aquaria containing a sandy bottom and aerated seawater (salinity of 35 ± 1 psu, temperature of 17 ± 0.5 °C) and were fed with microalgae (*Isochrysis galbana*) daily.

2.3. Haemolymph collection and haemocyte cultures

For eah experiment (see below), pools of haemolymph (from 5 clams each) were used. Haemolymph was collected from the adductor muscles with a plastic syringe, stored on ice, and added to an equal volume of 0.38% sodium citrate in 0.45 μm filtered sea water (FSW), pH 7.5, to prevent clotting. Haemolymph was centrifuged at 800 × *g* for 10 min. Haemocytes were resuspended in FSW to prepare short-term cell cultures.

Short-term haemocyte cultures were prepared according to Ballarin et al. (1994). Sixty microlitres of haemocyte suspension were placed in the centre of culture chambers made by a Teflon ring (15 mm internal diameter and 1 mm thick) smeared with petroleum jelly, glued to a siliconised glass slide, and covered with a coverslip. Chambers were kept upside down for 30 min at room