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Effects of emerging contaminants on neurotransmission and biotransformation in marine organisms — An *in vitro* approach

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ABSTRACT

The effects of gold (ionic form and nanoparticles — AuNPs) and pharmaceuticals (carbamazepine and fluoxetine) on enzymes involved in neurotransmission (acetylcholinesterase — AChE) and biotransformation (glutathione *S*-transferases — GST) were assessed by their incubation with *Mytilus galloprovincialis*' hemolymph and subcellular fraction of gills, respectively. AuNPs did not alter enzymatic activities unlike ionic gold that inhibited AChE and GST activities at 2.5 and 0.42 mg·L^{-1}, respectively. Carbamazepine inhibited AChE activity at 500 mg·L^{-1} and fluoxetine at 1000 mg·L^{-1}. GST was inhibited by carbamazepine at 250 mg·L^{-1} and by fluoxetine at 125 mg·L^{-1}. Increased AChE activity was found in simultaneous exposures to fluoxetine and bovine serum albumin coated AuNPs (BSA-AuNPs). Concerning GST, in the simultaneous exposures, AuNPs revealed protective effects against carbamazepine (citrate and polyvinylpyrrolidone coated) and fluoxetine (citrate and BSA coated) induced inhibition. However, BSA-AuNPs increased the inhibition caused by carbamazepine. AuNPs demonstrated ability to interfere with other chemicals toxicity justifying further studies.

1. Introduction

Estuarine and coastal areas are under strong anthropogenic pressure in several regions around the world, being contaminated by complex mixtures of contaminants that include, among other classes of contaminants, persistent organic pollutants (Doong et al., 2008), metals (Doong et al., 2008; Oliveira et al., 2010) and pharmaceuticals (Buchberger, 2007). Nanoparticles (NPs) are among the classes of environmental contaminants currently considered as contaminants of emerging concern (Sauve and Desrosiers, 2014), due to their increased use in several areas of human activity (*e.g.* electronics, cosmetics and biomedicine), their size dependent characteristic and the poor knowledge on their impact into the environment. It is expected that NPs will eventually end up in waterways, where they may exert pernicious effects to aquatic biota (Moore, 2006; Baun et al., 2008; Canesi et al., 2010). Despite the increased production of NPs-containing materials and the constant discovery of new applications, the knowledge on the biological effects of NPs exposure is still limited, particularly in terms of effects to saltwater organisms, likely due to their reported instability in high ionic strength media (such as saltwater). However, the use of stabilizing coatings and the presence, in natural waters, of a variety of substances that may act as dispersants and NPs stabilizers (Klaine et al., 2008) may increase the stability of these particles in high ionic strength environments such as estuaries.

Gold nanoparticles (AuNPs) are among the most widely used NPs in several fields of research from energy to biomedical applications (Lapresta-Fernández et al., 2012). Citrate layer, which is able to stabilize NPs by electrostatic repulsion (Manson et al., 2011), may easily be replaced by ligands binding stronger to the particle surface. In biomedical applications, AuNPs have been used coated with a variety of biomolecules such as proteins, DNA and carbohydrate moieties to increase their stability and applications (Housni et al., 2008). In the presence of proteins, AuNPs tend to form a protein corona (Pino et al., 2014). Bovine serum albumin (BSA) is a commonly studied protein that has a great importance in nanomedicine (Thi Ha Lien et al., 2010), presenting a high amino acid sequence similarity with human serum. It is biocompatible, water soluble and has the potential to increase the stability of AuNPs. Polyvinylpyrrolidone (PVP) is another capping/reducing/nucleating agent increasingly used in the coating of AuNPs due to its non-toxic, high stabilizing ability and outstanding solubility in various polar solvents (Behera and Ram, 2014).

With the increased use of AuNPs, it is expected that their levels in the environment increase, having potential to become a significant persistent nanomaterial input to environmental systems (Hull et al., 2011). However, the available information on the levels of AuNPs in the aquatic environment is very limited (García-Negrete et al., 2013). Predictions originating from their use in consumer products estimate levels between 0.1 and 1.43 μg·L^{-1} in water (Boxall et al., 2007). However, the properties (*e.g.* size and surface characteristics) of these particles after environmental release are hard to predict due to several environmental factors and, for example, passage through wastewater treatment plants,

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which has been shown to increase the stabilization of NPs such as cerium oxide (Limbach et al., 2005).

The investigation of the interactions between AuNPs (alone or combined with other compounds) and biological systems is of significant interest. Until recently, the available information on AuNPs ranked them as non-toxic and biocompatible (Lapresta-Fernández et al., 2012). However, recent studies have highlighted their possible toxicity (*e.g.* oxidative stress, cytotoxicity, genotoxicity and protein modifications), raising important concerns about their potential impact on human health and aquatic ecosystems (Farkas et al., 2010; Paino et al., 2012; García-Cambero et al., 2013), and supporting the need of increased research on their environmental impact alone or combined with other contaminants.

Pharmaceuticals are another group of emerging contaminants of concern detected in surface waters, ground waters and drinking water (Fent et al., 2006; Gonzalez-Rey et al., 2014) as a result of their high consumption, inefficiency of wastewater treatment plants processes (Buchberger, 2007) and, in some cases, low degradation rates in the environment. Carbamazepine (antiepileptic) and fluoxetine (antidepressant) are two human pharmaceuticals, with a low degradation rate in the environment (Clara et al., 2004; Redshaw et al., 2008; Metcalfe et al., 2010; Oliveira et al., 2014) that have shown, *in vivo*, the ability to alter biochemical and behavioral endpoints in aquatic (Gonzalez-Rey et al., 2014, Almeida et al., 2014) and soil (Oliveira et al., 2014) organisms. However, despite the increasing number of studies on the effects of these compounds to non-target organisms (Martin-Diaz et al., 2009; Nassef et al., 2010; Mesquita et al., 2011; Malarvizhi et al., 2012; Franzellitti et al., 2014; Hazelton et al., 2014; Oliveira et al., 2014), few studies have focused on the mixture of these emergent compounds with other environmental contaminants of concern such as NPs.

Exposing cellular suspensions to contaminants and assessing specific enzymatic activities has proven a useful methodology to assess the susceptibility of aquatic organisms to contaminants (Viarengo et al., 1997; Oliveira et al., 2004, 2005). Thus, this study aimed (1) to test the effects of AuNPs, carbamazepine and fluoxetine on neurotransmission and biotransformation enzymes, by studying their *in vitro* effects on acetylcholinesterase (AChE) and glutathione *S*-transferases (GST) activities, respectively; (2) to test if the coating of AuNPs would induce different effects; and (3) to assess the effects of combined exposure to pharmaceuticals and AuNPs.

2. Material and methods

2.1. Chemicals

Fluoxetine HCl was purchased from TCI (Japan). Carbamazepine, gold(III) chloride trihydrate, bovine serum albumin (BSA) and polyvinylpyrrolidone (PVP) were acquired from Sigma-Aldrich (Germany). All other chemicals were of analytical grade obtained from Sigma-Aldrich (Germany), Bio-Rad (Germany), Merck (Germany) and Panreac (Spain).

2.2. Nanoparticle preparation and characterization

AuNPs of 7 nm were prepared by citric acid reduction of gold(III) chloride trihydrate (Shiba, 2013). The resulting citrate coated gold nanoparticles (cAuNPs) were coated with BSA or PVP by incubating them for 30 min with a freshly prepared BSA (0.15 $\mu g \cdot \mu L^{-1}$) or PVP (0.25 $mg \cdot mL^{-1}$) in a ratio of 2:1 (AuNPs:BSA and AuNPs:PVP). After the incubation period, AuNPs were centrifuged and the pellet resuspended in ultrapure water. Characterization of cAuNPs, BSA-AuNPs and PVP-AuNPs was performed immediately after synthesis, coating and exposure to reaction buffer, by UV–Vis spectrophotometry (Cintra 303, GBC Scientific) and dynamic light scattering (DLS), assessing hydrodynamic size and zeta potential (ZP) (Zetasizer Nano ZS, Malvern). AuNPs were also characterized by transmission electron microscopy (Hitachi, H9000 NAR). NP concentrations were determined according to Liu et al. (2007).

2.3. Test organisms

Mussels (*Mytilus galloprovincialis*) specimens with 5.1 ± 0.4 cm (mean ± standard deviation) were caught in a reference beach (40°32′57.91″N; 8°46′29.41″W) in Aveiro (Portugal). Animals were transported to the laboratory in water from sampling site and acclimated to laboratorial conditions for two weeks in 80 L aquaria containing aerated and filtered (Eheim filters) artificial saltwater (35 $g \cdot L^{-1}$) in a natural photoperiod and temperature controlled (20 ± 1 °C) room. Animals were not fed during the acclimatization period.

2.4. Preparation of biological material for in vitro assays

After acclimatization period, the gills and hemolymph of nine mussels were sampled. Gills were homogenized (1:10 $wt.v^{-1}$) in phosphate buffer (0.1 M, pH 6.5) and centrifuged (Eppendorf 5810R) for 20 min at 12 0000 g (4 °C) to obtain a post mitochondrial supernatant fraction (PMS). The protein contents of the hemolymph and the PMS of gills were determined by the Bradford method (Bradford, 1976) adapted to microplate reader (Thermo Scientific Multiskan Spectrum). The protein content was ascertained to 0.7 $mg \cdot mL^{-1}$ by diluting samples in the 0.1 M phosphate buffer, pH 7.2 for AChE and 6.5 for GST activity assays. Samples were frozen in liquid nitrogen and stored at −80 °C until the *in vitro* assays were performed.

2.5. Validation of the in vitro assay

The unique properties of NPs (*e.g.* large surface area, optical and catalytic properties) may potentiate interference with various compounds of enzymatic assays (Oh et al., 2014; Šinko et al., 2014) producing artifacts if proper controls are not performed. The study of AChE inhibition by metals such as Ag, Cu, Cd, Ni, Hg and Zn using Ellman's assay may be biased by their reaction with acetylthiocholine as well as with 5-thio-2-nitrobenzoic acid ion formed in the reaction between 5,5′-dithiobis-(2-nitrobenzoic acid) – DTNB – and thiocholine, a product of substrate (*i.e.*, acetylthiocholine) hydrolysis by AChE, decreasing the measured absorbance (Frasco et al., 2005; Vrček and Šinko, 2013). Available studies (Oh et al., 2014) suggest a greater probability of NPs interference for concentrations equal or higher than 10 $mg \cdot L^{-1}$, as reported for lactate dehydrogenase assays in cell viability assessments. Vrček and Šinko (2013) reported that upon addition of Ag NPs (maximum concentration tested 4.9 $mg \cdot L^{-1}$) to a reaction mixture containing human recombinant acetylcholinesterase and acetylthiocholine a rapid increase of the absorbance resulting in an absorbance plateau was found, with very low increase in absorbance over time. In the current study, to screen for potential artifacts due to AuNPs absorbance or arising from their catalytic properties, in addition to the normal controls (without AuNPs), two additional controls, a 'AuNPs-only' and a 'AuNPs with assay reagents', were performed for the three AuNPs coatings. Addition of Au (ionic and AuNPs) to the reaction mixture did not lead to significant alterations of absorbance and the assessed slopes displayed correlation coefficients above 0.95 with no plateau found throughout the period of the enzymatic assays, an indication that no artifacts are expected for these NPs, at the tested concentrations.

2.6. Exposure conditions

Serial dilutions of AuNPs (cAuNPs, BSA-AuNPs and PVP-AuNPs) were performed, in ultrapure water, from a 10 $mg \cdot L^{-1}$ stock, to test effects in a concentration range from 54 $ng \cdot L^{-1}$ to 2.5 $mg \cdot L^{-1}$. To assess the effects of pharmaceuticals, stock solutions of carbamazepine and fluoxetine (16 $g \cdot L^{-1}$), were respectively prepared in ethanol and ultra-pure water