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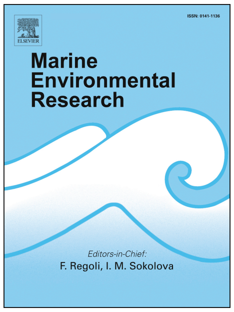

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Neutral red retention time assay in determination of toxicity of nanoparticles

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Abstract

The neutral red retention time (NRRT) assay is useful for detecting decreased lysosomal membrane stability in haemocytes sampled from bivalves, a phenomenon often associated with exposure to environmental pollutants including nanomaterials. Bivalves are popular sentinel species in ecotoxicology and use of NRRT in study of species in the genus Mytilus is widespread in environmental monitoring. The NRRT assay has been used as an *in vivo* test for toxicity of carbon nanoparticles (Moore MN, Readman JAJ, Readman JW, Lowe DM, Frickers PE, Beesley A. 2009. Lysosomal cytotoxicity of carbon nanoparticles in cells of the molluscan immune system: An *in vivo* study. *Nanotoxicology*. 3 (1), 40-45). We here report application of this assay adapted to a microtitre plate format to a panel of metal and metal oxide nanoparticles (2ppm). This showed that copper, chromium and cobalt nanoparticles are toxic by this criterion while gold and titanium nanoparticles are not. As the former three nanoparticles are often reported to be cytotoxic while the latter two are thought to be non-cytotoxic, these data support use of NRRT as a general *in vitro* assay in nanotoxicology.

1. Introduction

The unusual properties of nanomaterials provide them with several possible routes to toxicity in biological systems. Their small size sometimes enables them to cross important biobarriers e.g. skin, blood-brain, intestine, maternal-foetus (Tedesco and Sheehan, 2010; Elsaesser and Howard, 2012; Jiang et al., 2014). Their very large surface area to volume ratio enables a greater proportion of atoms to be displayed on the particle surface compared to corresponding macromaterials (Nel et al., 2009; Nel et al., 2013). Moreover, specific functional groups on nanoparticle surfaces may facilitate biospecific interactions allowing a range of possible biological effects (Hoet et al., 2004; Moore, 2006; Klaper et al., 2014). Nanomaterials can also translocate within the human body into other systems such as circulatory and lymphatic vessels (Gwinn and Vallyathan, 2006; Buzea et al., 2007; Elsaesser and Howard, 2012). Thus, nanoparticles have significant potential to cause adverse health effects in humans and other organisms upon prolonged exposure.

Because of increasing commercial production and use of nanomaterials, issues of their accumulation and fate in the environment and their possible effects on ecosystems arise (Moore, 2006; Tedesco and Sheehan, 2010; Ivask et al., 2014). The majority of human habitation worldwide is within 100km of coastlines and the aquatic environment collects domestic, agricultural, shipping and industrial runoffs from these coastal zones. This makes aquatic ecosystems particularly at risk to potential toxicity of nanomaterials of anthropogenic origin. Invertebrates are key elements of the aquatic food chain and mussels are amongst the most abundant of these (Baun et al., 2008). As filter-feeders, mussels are exquisitely selective in the particle size-range which they ingest (Defossez and Hawkins, 1997; Ward and Kach, 2009) and can bioconcentrate metals and organic pollutants within their tissues. This has led to their widespread study in ecotoxicology (Moore, 1985; Widdows and Donkin, 1992) and filter-feeders have been suggested as especially attractive targets for probing the environmental fate of nanomaterials (Moore, 2006; Ward and Kach, 2009; Canesi et al., 2012).

Lysosomes are important subcellular organelles that contain many hydrolytic enzymes, carry out protein degradation and detoxify some foreign compounds. At the cellular level, lysosomal digestion pathways include phagocytosis, endocytosis and autophagy. The lysosomal membrane protects the cytosol, and therefore the rest of the cell, from leakage of degradative enzymes. However, malfunctioning of lysosomes and their accumulation of toxic pollutants have been linked to lysosomal storage diseases and result in lysosomal injury and oxidative damage, in some cases leading to cell death (Moore et al., 2007). The neutral red retention time (NRRT) assay takes advantage of this phenomenon by measuring decreased time of retention of a dye, neutral red (ACS no. 553-24-2), within phagocytic haemocytes of a range of aquatic organisms including mussels, crustaceans and fish (Regoli, 1992; Tedesco et al, 2008; Lowe et al 1995; Svendsen et al, 2004). In the popular sentinel species, *Mytilus edulis*, hemocytes are essential immune system components (Rickwood and Galloway, 2004). NRTT has been reported as a useful indicator of the organism’s overall health status because animals exposed to pollutants often have compromised lysosomal stability (Moore et al., 2009; Borenfreund and Puerner 1985; Piola et al., 2013). A spectrophotometric version of the assay was developed by Babich and Borenfreund (1990) and a microscopic slide observation method was developed by Moore et al., (2009). This assay takes advantage of the tendency of haemocytes to take up nanoparticles most probably by either phagocytosis or macro-endocytosis and involves exposing haemocytes to nanoparticles on a microscope slide (Moore et al., 2009). In this short report, we have adapted this methodology to a microtitre plate format enabling high-throughput screening of large numbers of replicates, doses and nanoparticles simultaneously (Fig. 1). As proof of principle, we have assessed a panel of metal and metal oxide nanoparticles with this assay.

2. Materials and Methods

2.1. Mytilus edulis *sampling*

M. edulis individuals (4-6cm shell-length) were collected from an intertidal site in Cork Harbour, Ireland (location: 51.49°N, 8 18°W; Lyons et al., 2003). All Animals were acclimated in tanks for a week with a 12 h light/dark cycle at a temperature of 15°C and 34–36‰ salinity, fed and with regular changing of water.

2.2.Nanoparticle suspension preparation

Metal or metal oxide nanoparticles (copper oxide, titanium dioxide, gold, chromium oxide and cobalt oxide) of nominal sizes <50nm were purchased from Sigma-Aldrich (Dorset, UK). Nanopowders (10mg) were suspended in 10 ml of 20 mM citric acid adjusted to pH 7, and sonicated for 1h using a tip sonicator. A stepped microtip was used and the total power transferred to the suspension was 2.4W (determined by the calorimetric method). Ultrasound was applied as 15s pulses with 15s breaks between them (Taurozzi et al., 2010). The suspensions were left at $60^{o}C$ overnight and were then filtered using a 220nm pore size cellulose acetate filter (Millipore, Watford UK).

2.3.Exposure of haemolymph to nanoparticles

Haemolymph samples were freshly extracted for NRRT assay as described by Moore et al. (2009). In the present work, haemolymph from each of five animals was extracted from adductor muscle using a 20 gauge hypodermic needle fitted on a 1 ml syringe containing 100µl tris buffered saline buffer, which was pooled to provide a total volume of 2 ml haemolymph solution. Three biologically independent replicates were used (i.e. haemolymph was taken from 3x5 individual animals). Samples were constantly vortexed to resuspend the haemolymph and prevent aggregation. Haemolymph was then evenly aliquoted (500 µL) followed by exposure to nanoparticles at a final concentration of 2 ppm for 1 h at ambient temperature (20°C). Tubes were gently shaken every 5 min to optimise exposure. The above procedure was applied to a panel of metal or metal oxide nanoparticles and a control sample was treated identically but without the presence of nanoparticle.

2.4.Neutral red retention time (NRRT) assay

Following nanoparticle exposure, 100 µl haemolymph from all six treatment groups was loaded into individual wells of a 96-well microtitre plate (Sarstedt, Wexford Ireland). This was performed with three independent biological replicates. Fifty µl stock neutral red dye solution (200 µM) was then added. Four plates were used in parallel for time-points 15, 30, 60 and 90 min. All plates were placed in the dark allowing 15, 30, 60 or 90 min, respectively, for dye uptake. Dye and medium were quickly removed from the plates after incubation and washed with 150 µL fixative solution (1% formaldehyde, 1% calcium chloride) for 2 min. Plates were then rapidly drained, followed by addition of 200µl extraction buffer (1% acetic acid and 50% ethanol) and left in the dark for 20 min at room temperature. Absorbance of extracted dye was measured using a microplate reader (Elx808iu Ultra Microplate Reader, Bio-Tek Instrument Inc., Potton UK) at a wavelength of 570 nm.

3. Results and Discussion

3.1. Neutral red retention time assay of metal oxide nanoparticles

Haemolymph from *M. edulis* was exposed to a panel of metal or metal oxide nanoparticles at a final concentration of 2ppm (Fig. 1). Lysosomal membrane stability was tested by measuring NRRT at four different time points; 15, 30, 60 and 90 min. Results were analysed and statistically compared to the control group using a one-way anova test with confidence limit of 95% (Figure 2). Lysosomal membrane stability showed a significant decrease ($p<0.05$) upon exposure to copper, cobalt and chromium nanoparticles at all time-points tested, indicating toxic effects on lysosomes of these nanomaterials. However, no significant effects were observed on exposure of titanium or gold nanoparticles, suggesting they are less toxic by the criterion of this *in vitro* assay.

3.2.Toxicity of metal or metal oxide nanoparticles

The particles selected for this study have previously been reported to display a range of toxicity in biological systems. Titanium dioxide nanoparticles (which are widely used commercially as a component of sunscreens) are generally regarded as less toxic to aquatic species (Federici et al, 2007). However, it should be noted that, in mice, NO and tumour necrosis factor alpha production were elicited after exposure to titanium dioxide nanoparticles (<10nm). This finding suggested that both damage to the cell structure and macrophage dysfunction may occur, leading to reduction in both non-specific and specific immune responses in some individual animals (Liu et al 2010). Copper oxide and chromium oxide nanoparticles are notorious for their toxic effects, and have been implicated in toxicity to non-target organisms (Ivask et al, 2014), reduction of immune status (Zha et al 2009), damage to animal tissues (Chen et al, 2006; Griffitt et al, 2007), and induction of reactive oxygen species (Fahmy and Cormier, 2009; Horie et al 2011). Cobalt oxide nanoparticles readily enter cultured human cells where they are found to have a negative effect on cell viability (Papis et al., 2009). They have been reported to induce primary DNA damage in a concentration-dependent manner. Various redox enzyme activities were decreased after treatment with cobalt nanoparticles, suggesting potential toxic risk and inhibition of antioxidant capacity (Jiang et al, 2012).

3.3.Potential for high-throughput assay

The assay format reported here includes minimisation of biological variation in haemocyte populations by pooling haemolymph across five individual animals. Moreover, three independent replicates gave essentially identical results and allowed reproducible discrimination across the nanoparticle panel studied. Use of 96-well microtitre plates makes possible high-throughput analysis of large numbers of samples, replicates and concentrations within the time-scale suggested by Moore et al.

(2009). This could facilitate rapid quantitative analysis of novel engineered nanoparticles. An especially attractive feature of this assay format is that it mimics the kinds of strategies that many nanoparticles most probably employ in nature to gain entry to cells such as phagocytosis or macro-endocytosis. This is an ancient and long-established property of eukaryote cells (Elsaesser and Howard, 2012).

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Figure legends

Figure 1 Schematic overview of NRTT assay.

Figure 2 Neutral red retention time (NRRT) assay in response to a panel of nanoparticles. Neutral red dye extracted from exposed haemocytes was measured spectrophotometrically at 570nm in a plate reader (*$p < 0.05$ versus control values).

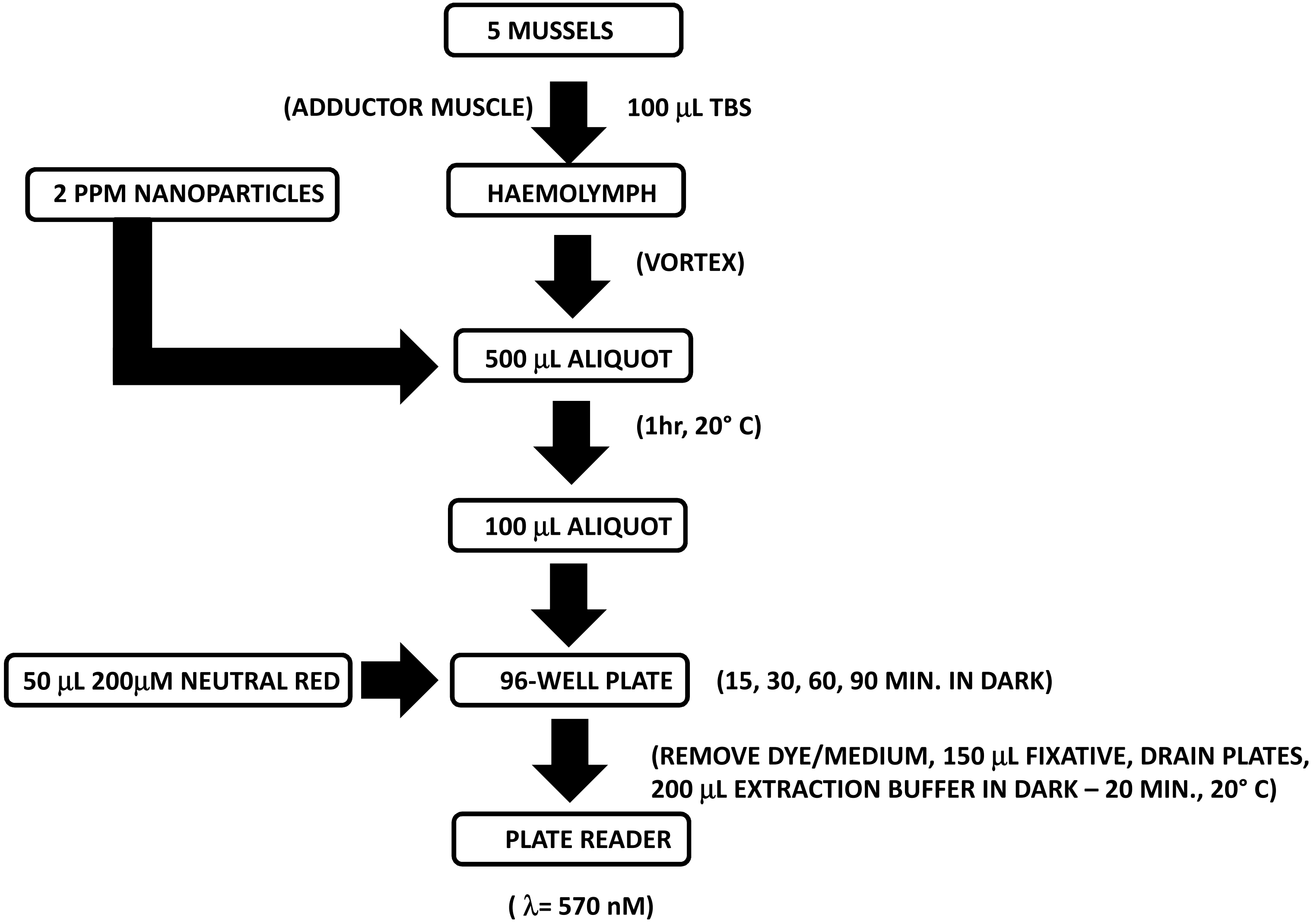
5 MUSSELS
(ADDUCTOR MUSCLE)
100 μL TBS
2 PPM NANOPARTICLES
HAEMOLYMPH
(VORTEX)
500 μL ALIQUOT
(1hr, 20° C)
100 μL ALIQUOT
50 μL 200μM NEUTRAL RED
96-WELL PLATE
(15, 30, 60, 90 MIN. IN DARK)
(REMOVE DYE/MEDIUM, 150 μL FIXATIVE, DRAIN PLATES, 200 μL EXTRACTION BUFFER IN DARK – 20 MIN., 20° C)
PLATE READER
(λ= 570 nM)

Fig. 1

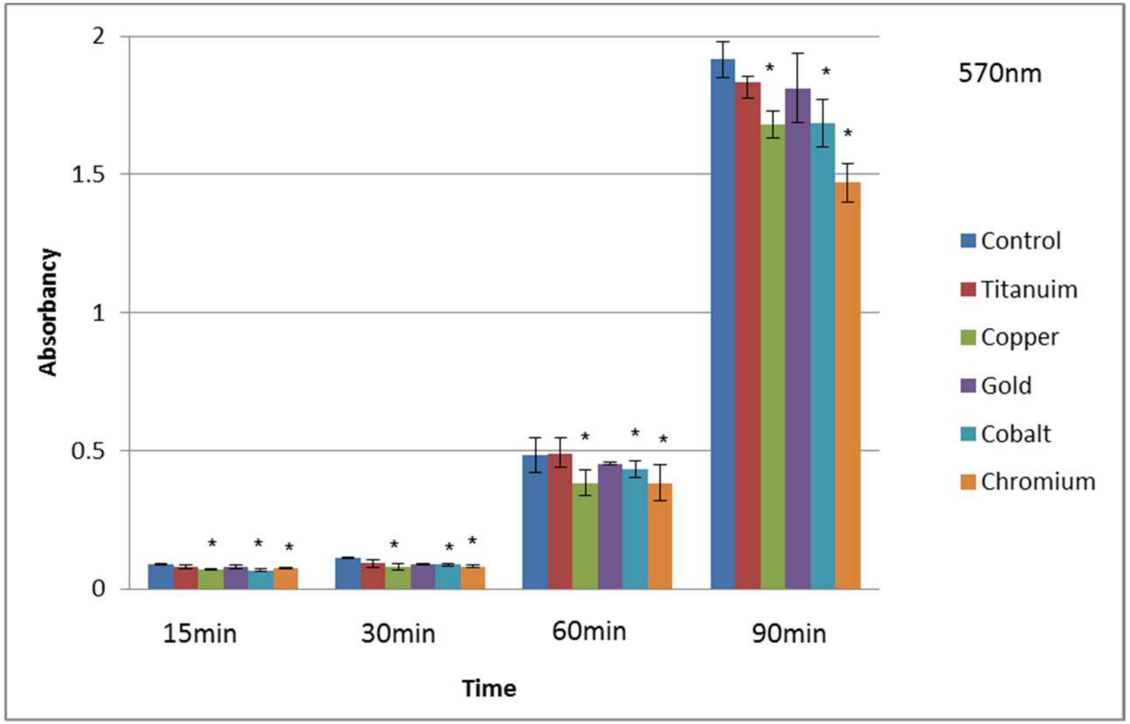

Figure 1

Figure 2

- Neutral red retention time assay used haemolymph of five pooled mussels.
- Assay was miniaturised for reading in a plate reader, facilitating many samples and replicates.
- Copper, chromium and cobalt nanoparticles were toxic while gold and titanium were not.