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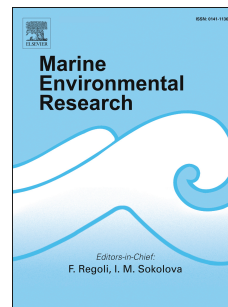
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1 **Neutral red retention time assay in determination of toxicity of nanoparticles**

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20 *Keywords: Mytilus, metal oxide, lysosome, membrane stability, neutral red, NRRT*

21

22 1

23 **Abstract**

24 The neutral red retention time (NRRT) assay is useful for detecting decreased
25 lysosomal membrane stability in haemocytes sampled from bivalves, a phenomenon
26 often associated with exposure to environmental pollutants including nanomaterials.
27 Bivalves are popular sentinel species in ecotoxicology and use of NRRT in study of
28 species in the genus *Mytilus* is widespread in environmental monitoring. The NRRT
29 assay has been used as an *in vivo* test for toxicity of carbon nanoparticles (Moore MN,
30 Readman JAJ, Readman JW, Lowe DM, Frickers PE, Beesley A. 2009. Lysosomal
31 cytotoxicity of carbon nanoparticles in cells of the molluscan immune system: An *in*
32 *vivo* study. *Nanotoxicology*. 3 (1), 40-45). We here report application of this assay
33 adapted to a microtitre plate format to a panel of metal and metal oxide nanoparticles
34 (2ppm). This showed that copper, chromium and cobalt nanoparticles are toxic by this
35 criterion while gold and titanium nanoparticles are not. As the former three
36 nanoparticles are often reported to be cytotoxic while the latter two are thought to be
37 non-cytotoxic, these data support use of NRRT as a general *in vitro* assay in
38 nanotoxicology.

39

40 **1. Introduction**

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

53 Because of increasing commercial production and use of nanomaterials, issues
54 of their accumulation and fate in the environment and their possible effects on
55 ecosystems arise (Moore, 2006; Tedesco and Sheehan, 2010; Ivask et al., 2014). The
56 majority of human habitation worldwide is within 100km of coastlines and the aquatic
57 environment collects domestic, agricultural, shipping and industrial runoffs from
58 these coastal zones. This makes aquatic ecosystems particularly at risk to potential
59 toxicity of nanomaterials of anthropogenic origin. Invertebrates are key elements of
60 the aquatic food chain and mussels are amongst the most abundant of these (Baun et

61 al., 2008). As filter-feeders, mussels are exquisitely selective in the particle size-
62 range which they ingest (Defosse and Hawkins, 1997; Ward and Kach, 2009) and
63 can bioconcentrate metals and organic pollutants within their tissues. This has led to
64 their widespread study in ecotoxicology (Moore, 1985; Widdows and Donkin, 1992)
65 and filter-feeders have been suggested as especially attractive targets for probing the
66 environmental fate of nanomaterials (Moore, 2006; Ward and Kach, 2009; Canesi et
67 al., 2012).

68 Lysosomes are important subcellular organelles that contain many hydrolytic
69 enzymes, carry out protein degradation and detoxify some foreign compounds. At the
70 cellular level, lysosomal digestion pathways include phagocytosis, endocytosis and
71 autophagy. The lysosomal membrane protects the cytosol, and therefore the rest of the
72 cell, from leakage of degradative enzymes. However, malfunctioning of lysosomes
73 and their accumulation of toxic pollutants have been linked to lysosomal storage
74 diseases and result in lysosomal injury and oxidative damage, in some cases leading
75 to cell death (Moore et al., 2007). The neutral red retention time (NRRT) assay takes
76 advantage of this phenomenon by measuring decreased time of retention of a dye,
77 neutral red (ACS no. 553-24-2), within phagocytic haemocytes of a range of aquatic
78 organisms including mussels, crustaceans and fish (Regoli, 1992; Tedesco et al, 2008;
79 Lowe et al 1995; Svendsen et al, 2004). In the popular sentinel species, *Mytilus edulis*,
80 hemocytes are essential immune system components (Rickwood and Galloway,
81 2004). NRTT has been reported as a useful indicator of the organism's overall health

82 status because animals exposed to pollutants often have compromised lysosomal
83 stability (Moore et al., 2009; Borenfreund and Puerner 1985; Piola et al., 2013). A
84 spectrophotometric version of the assay was developed by Babich and Borenfreund
85 (1990) and a microscopic slide observation method was developed by Moore et al.,
86 (2009). This assay takes advantage of the tendency of haemocytes to take up
87 nanoparticles most probably by either phagocytosis or macro-endocytosis and
88 involves exposing haemocytes to nanoparticles on a microscope slide (Moore et al.,
89 2009). In this short report, we have adapted this methodology to a microtitre plate
90 format enabling high-throughput screening of large numbers of replicates, doses and
91 nanoparticles simultaneously (Fig. 1). As proof of principle, we have assessed a panel
92 of metal and metal oxide nanoparticles with this assay.

93

94 2. Materials and Methods

95 2.1. *Mytilus edulis* sampling

96 *M. edulis* individuals (4-6cm shell-length) were collected from an intertidal site in
97 Cork Harbour, Ireland (location: 51.49°N, 8 18°W; Lyons et al., 2003). All Animals
98 were acclimated in tanks for a week with a 12 h light/dark cycle at a temperature of
99 15°C and 34–36‰ salinity, fed and with regular changing of water.

100

101 2.2. Nanoparticle suspension preparation

102 Metal or metal oxide nanoparticles (copper oxide, titanium dioxide, gold,
103 chromium oxide and cobalt oxide) of nominal sizes <50nm were purchased from
104 Sigma-Aldrich (Dorset, UK). Nanopowders (10mg) were suspended in 10 ml of 20
105 mM citric acid adjusted to pH 7, and sonicated for 1h using a tip sonicator. A stepped
106 microtip was used and the total power transferred to the suspension was 2.4W
107 (determined by the calorimetric method). Ultrasound was applied as 15s pulses with
108 15s breaks between them (Taurozzi et al., 2010). The suspensions were left at 60°C
109 overnight and were then filtered using a 220nm pore size cellulose acetate filter
110 (Millipore, Watford UK).

111

112 2.3. Exposure of haemolymph to nanoparticles

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

125

126 *2.4. Neutral red retention time (NRRT) assay*

127 Following nanoparticle exposure, 100 µl haemolymph from all six treatment
128 groups was loaded into individual wells of a 96-well microtitre plate (Sarstedt,
129 Wexford Ireland). This was performed with three independent biological replicates.
130 Fifty µl stock neutral red dye solution (200 µM) was then added. Four plates were
131 used in parallel for time-points 15, 30, 60 and 90 min. All plates were placed in the
132 dark allowing 15, 30, 60 or 90 min, respectively, for dye uptake. Dye and medium
133 were quickly removed from the plates after incubation and washed with 150 µL

134 fixative solution (1% formaldehyde, 1% calcium chloride) for 2 min. Plates were then
135 rapidly drained, followed by addition of 200µl extraction buffer (1% acetic acid and
136 50% ethanol) and left in the dark for 20 min at room temperature. Absorbance of
137 extracted dye was measured using a microplate reader (Elx808iu Ultra Microplate
138 Reader, Bio-Tek Instrument Inc., Potton UK) at a wavelength of 570 nm.

139

140

141 3. Results and Discussion

142 3.1. Neutral red retention time assay of metal oxide nanoparticles

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

153

154 3.2. Toxicity of metal or metal oxide nanoparticles

155 The particles selected for this study have previously been reported to display a
156 range of toxicity in biological systems. Titanium dioxide nanoparticles (which are
157 widely used commercially as a component of sunscreens) are generally regarded as
158 less toxic to aquatic species (Federici et al, 2007). However, it should be noted that, in
159 mice, NO and tumour necrosis factor alpha production were elicited after exposure to
160 titanium dioxide nanoparticles (<10nm). This finding suggested that both damage to

161 the cell structure and macrophage dysfunction may occur, leading to reduction in both
162 non-specific and specific immune responses in some individual animals (Liu et al
163 2010). Copper oxide and chromium oxide nanoparticles are notorious for their toxic
164 effects, and have been implicated in toxicity to non-target organisms (Ivask et al,
165 2014), reduction of immune status (Zha et al 2009), damage to animal tissues (Chen et
166 al, 2006; Griffitt et al, 2007), and induction of reactive oxygen species (Fahmy and
167 Cormier, 2009; Horie et al 2011). Cobalt oxide nanoparticles readily enter cultured
168 human cells where they are found to have a negative effect on cell viability (Papis et
169 al., 2009). They have been reported to induce primary DNA damage in a
170 concentration-dependent manner. Various redox enzyme activities were decreased
171 after treatment with cobalt nanoparticles, suggesting potential toxic risk and inhibition
172 of antioxidant capacity (Jiang et al, 2012).

173

174 *3.3.Potential for high-throughput assay*

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

181 (2009). This could facilitate rapid quantitative analysis of novel engineered
182 nanoparticles. An especially attractive feature of this assay format is that it mimics the
183 kinds of strategies that many nanoparticles most probably employ in nature to gain
184 entry to cells such as phagocytosis or macro-endocytosis. This is an ancient and long-
185 established property of eukaryote cells (Elsaesser and Howard, 2012).

186

187 **Acknowledgement**

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190

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- 287
- 288

289 **Figure legends**

290 **Figure 1** Schematic overview of NRTT assay.

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

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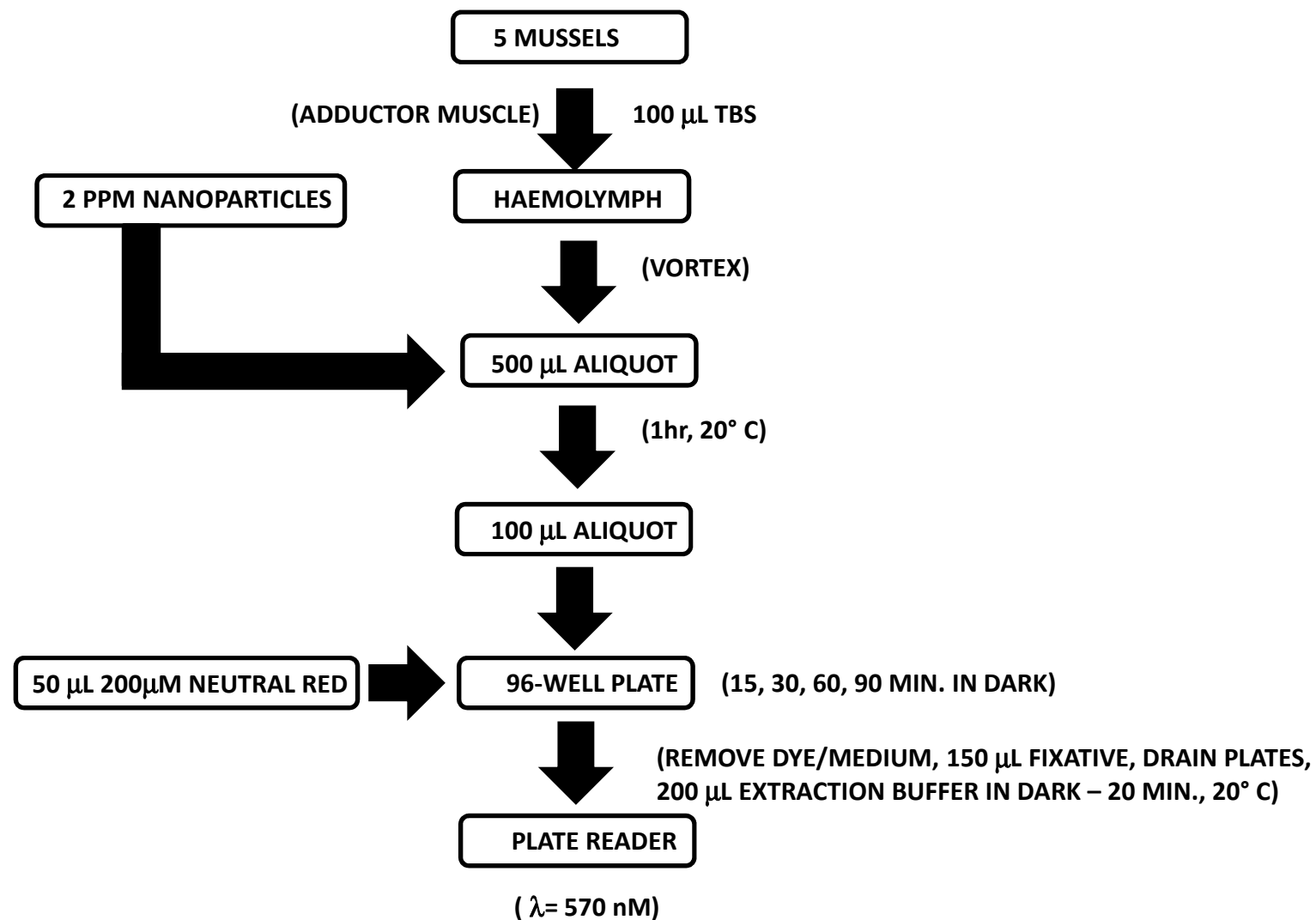


Fig. 1

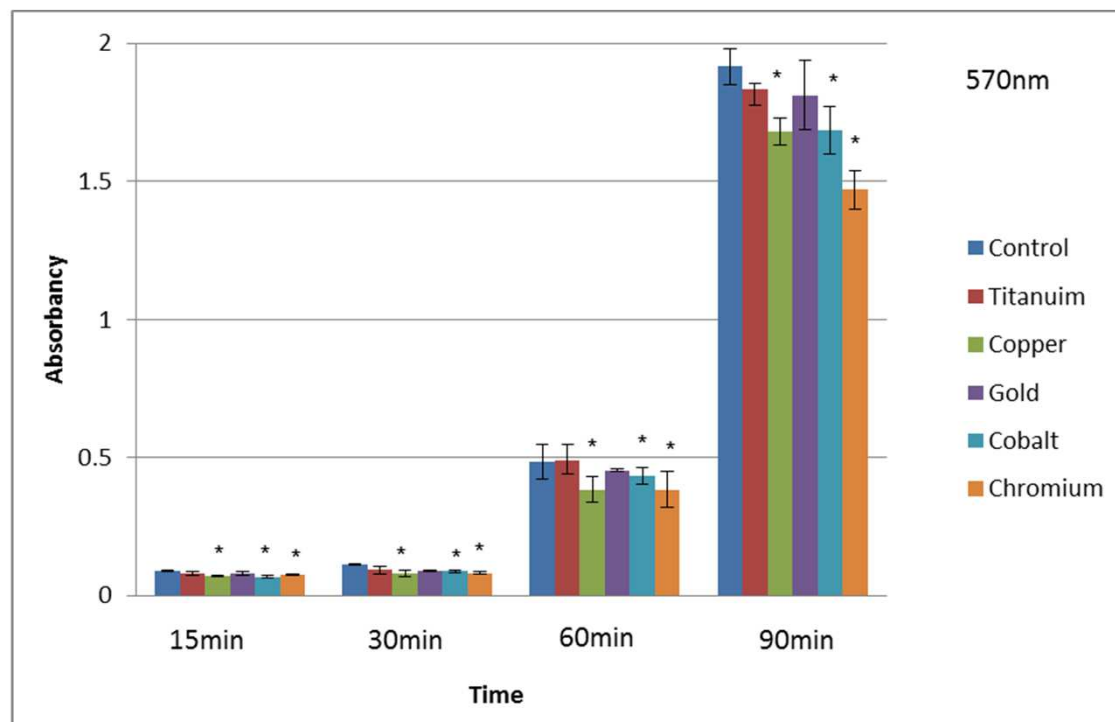


Figure 1

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.

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