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Sensory systems and ionocytes are targets for silver nanoparticle effects in fish

Olivia J. Osborne¹, Katsuki Mukaigasa², Hitomi Nakajima², Bjorn Stolpe³, Isabella Romer³, Uzoma Philips³, Iseult Lynch³, Sulayman Mourabit¹, Shigehisa Hirose⁴, Jamie R. Lead³, Makoto Kobayashi², Tetsuhiro Kudoh^{1}, Charles R. Tyler^{1*}.*

¹Biosciences, College of Life and Environmental Sciences, University of Exeter

²Molecular and Developmental Biology, Faculty of Medicine, University of Tsukuba

³School of Geography, Earth and Environmental Sciences, University of Birmingham

⁴Department of Biological Sciences, Tokyo Institute of Technology, Yokohama, Japan

*Correspondence

Corresponding author information:

Tetsuhiro Kudoh

Email: T.Kudoh@exeter.ac.uk

Telephone: +1392 723473

Biosciences, College of Life and Environmental Sciences, University of Exeter

Charles R. Tyler

Email: C.R.Tyler@exeter.ac.uk

Telephone: +1392 724450

Biosciences, College of Life and Environmental Sciences, University of Exeter

Abstract

Some nanoparticles (NPs) may induce adverse health effects in exposed organisms, but to date the evidence for this in wildlife is very limited. Silver nanoparticles (AgNPs) can be toxic to aquatic organisms, including fish, at concentrations relevant for some environmental exposures. We applied whole mount *in-situ* hybridisation (*WISH*) in zebrafish embryos and larvae for a suite of genes involved with detoxifying processes and oxidative stress, including metallothionein (*mt2*), glutathione S-transferase pi (*gstp*), glutathione S-transferase mu (*gstm1*), heme oxygenase (*hmox1*) and ferritin heavy chain 1 (*fth1*) to identify potential target tissues and effect mechanisms of AgNPs compared with a bulk counterpart and ionic silver (AgNO₃). AgNPs caused upregulation in the expression of *mt2*, *gstp* and *gstm1* and down regulation of expression of both *hmox1* and *fth1* and there were both life stage and tissue specific responses. Responding tissues included olfactory bulbs, lateral line neuromasts, and ionocytes in the skin with the potential for effects on olfaction, behaviour, and maintenance of ion balance. Silver ions induced similar gene responses and affected the same target tissues as AgNPs. AgNPs invoked levels of target gene responses more similar to silver treatments compared with coated AgNPs indicating the responses seen were due to released silver ions. In the *Nrf2* zebrafish mutant, expression of *mt2* (24 hpf) and *gstp* (3 dpf) were either non-detectable or were at lower levels compared with wild type zebrafish for exposures to AgNPs, indicating that these gene responses are controlled through the Nrf2-Keap pathway.

Keywords: Silver nanoparticles, *Danio rerio*, oxidative stress, target tissues, Nrf2 pathway

Introduction

Given the rapid expansion in global markets in nanotechnology, an increasing number of NPs will enter aquatic systems. There is concern internationally that some NPs may induce adverse health effects in exposed organisms, but to date the evidence for this in wildlife is very limited. More than 30% of nano products in the marketplace contain silver nanoparticles (AgNPs) (Wijnhoven et al., 2009) and current global use is approximately 10, 000 tonnes per year (Piccino et al., 2012). AgNPs are of particular concern to wildlife as most will be discharged via wastewater treatment works into surface waters where dissolution will release silver ions that are known to be toxic to many aquatic organisms (Hogstrand and Wood, 2009). The fate of most Ag NPs in wastewater treatment works (WWTWs), however, is in the sewage sludge as Ag₂S and they are transformed to similar chemical forms as their bulk counterparts (Ma et al., 2014).

Studies on the acute toxicity for AgNPs in zebrafish embryos and in subsequent early life stages have established adverse effect concentrations ranging between 50 µg/l and 500 µg/l, with strong indications for greater effects of AgNPs compared with bulk counterparts (Osborne et al., 2013, Asharani et al., 2008). Developmental effects of AgNP exposure in zebrafish include stunted growth, a reduced yolk sac mass and distortions in tail development, albeit these effects occur for high exposure concentrations (between 5,000 µg/l and 25,000 µg/l). These effects on embryogenesis have also been reported in other fish, including the medaka (*Oryzias latipes*) (Kashiwada et al., 2012). Very recently it has been demonstrated that silver nano particles can affect neural development genes and metal sensitive metallothioneins in zebrafish embryos (Xin et al., 2015). Reported effects of AgNPs are believed to result from silver ions dissociating from AgNPs, but there is limited evidence also for direct effects of the AgNPs themselves (Beer et al., 2012, van Aerle et al., 2013). In adult zebrafish, exposure to AgNPs has been shown to alter gene expression in biological pathways associated with DNA damage and repair (Griffit et al., 2013).

Heavy metals, including silver, induce oxidative stress (Ercal et al., 2001) in a wide range of organisms spanning algae (Pinto et al., 2003) to fish (Sanchez et al., 2005) and usually they do so via generation of Reactive Oxygen Species (ROS) resulting in lipid peroxidation. AgNPs have been shown to induce oxidative stress *in vitro* through an increase in the production of ROS (Foldbjerg et al., 2009). In their detoxification in the body, metals usually bind to thiol-containing compounds such as metallothionein (MT) and glutathione (GSH).

Standard approaches for testing the effects of NPs do not inform on material partitioning within the body or the target organs affected in an integrated manner. Whole mount *in-situ* hybridisation (*WISH*) as applied to zebrafish embryos and early life stages potentially offers a highly integrative, systems-wide approach to assess the toxicity of NPs through identifying target sites in the body where they effect gene expression. This approach in turn may help to predict their potential health effects (Nakajima et al., 2011b). Application of the technique of *in situ* hybridisation to assess for effects of toxicants on target genes, however, requires understanding on the ontogeny of the expression of the target genes, and this is known for very few genes of toxicological relevance.

In this study, we applied *WISH* in zebrafish embryos and early life stages exposed to AgNPs, a bulk counterpart and silver ions, for a suite of genes known to respond to metals (toxicity, transport and storage), oxidative stress and other markers of cellular stress. The genes selected were metallothionein (*mt2*), glutathione S-transferase pi (*gstp*), glutathione S-transferase mu (*gstm1*), heme oxygenase (*hmox1*) and ferritin heavy chain 1 (*fth1*). *Mt2* is involved with transport and storage of heavy metals (Andrews, 2000) and has been shown previously to be responsive to AgNPs in zebrafish embryos (Osborne et al., 2013). Glutathione S-transferases are a major group of detoxification enzymes that catalyze the nucleophilic addition of the tripeptide GSH to many xenobiotics and endogenous electrophiles. Glutathione transferase genes (*gstp*, *gstm1*) *i.e.* GSH are known to be response to toxic metals such as silver (Srinikath et al., 2013). *Gstm1* functions in the detoxification of electrophilic compounds and *gstp* plays roles in xenobiotic metabolism and

oxidative stress (Garner and Di Giulio, 2012). The *gstm1* gene encodes for the carcinogen detoxification enzyme glutathione S-transferase M1. Heme oxygenase (encoded by *hmox1*) is an enzyme that catalyzes the degradation of heme which in turn produces iron and protects against oxidative stress (Ponka et al., 1998). To identify the most appropriate life stages for studies on the effects of silver materials (AgNPs, bulk and AgNO₃ controls) for each of the chosen gene targets we initially undertook detailed studies to establish their ontogeny and tissue expression profiles in unexposed animals for up to 12 days post fertilization (dpf).

Nrf2 is an important transcription factor in the response to oxidative stress (Kobayashi and Yamamoto, 2005) and we further combined the use of *in situ* hybridisation in an Nrf2 mutant zebrafish (Mukaigasa et al., 2012) to investigate the role of this transcription factor in the toxicology responses to the silver materials tested.

Materials and Methods

Nanoparticle source and characterization

Citrate-covered Ag 10-nm nanoparticles (measured diameter of 9.9 ± 3.1 nm; AgNPCi; Römer et al., 2011) and larger sized citrate-covered Ag particles (measured diameter of 160 nm AgBCi) were acquired from the University of Birmingham. Uncoated Ag 35 nm nanoparticle (measured diameter 114 ± 65 nm; AgNP) and uncoated Ag bulk (measured diameter of 137 ± 62.0 nm; AgB) were acquired from Nanostructured and Amorphous Materials Inc. Houston, USA.

Detailed information on the characteristics of the particles derived from Nanostructured and Amorphous Materials are provided in (Scown et al., 2010) and (Osborne et al., 2013). A series of techniques were applied to quantify and characterize AgNPCi and AgBCi particles in the zebrafish culture medium, including Transmission Electron Microscopy (TEM), Dynamic Light Scattering (DLS) and Zeta Potential. (For more details concerning methodology of characterisation see supplementary material supplementary text S1). Knowing that AgNPs will undergo dissolution over

time we measured Ag^+ in the embryo culture medium over the exposure period using the GFAAS and confirmed the GFAAS measurements of Ag^+ using ICP-MS.

Fish source, culture and husbandry

Wild type Indian Calcutta (WIK) strain embryos were obtained from the Max Planck Institute, Tubingen, Germany and maintained at the University of Exeter (see supplementary material supplementary text S2 for details on fish maintenance). Nrf2 mutant zebrafish were supplied from the University of Tsukuba, Japan (Mukaigasa et al., 2012).

Embryo handling and Ag exposures

Embryos for gene expression ontogeny analysis and silver material exposures were collected from breeding colonies and transferred into a Petri dish and washed twice with embryo culture water. For all gene expression analyses and exposure studies, 15 to 20 embryos were studied for each gene/exposure (collected at the 1-2 cell stage, 1-1.5 hours post fertilisation (hpf)) per treatment well (5 ml) and each exposure study was replicated at least 3 times. The materials underwent sonication prior to use in the exposures using a Cole and Palmer Ultrasonicator Processor at full amplification and pulsing for two, 10-second bursts. This short term sonication is not known to cause release of the citrate coating of the citrate coated materials. The required amount of silver material was then introduced into the embryo culture medium (0.60 mg marine salts [Tropic marin] per 1 litre of deionised water, with the addition of 10 μl of methylene blue) containing the embryos. The embryos for both gene expression ontogeny analyses and ecotoxicology studies were incubated at $28 \pm 1^\circ\text{C}$ and the embryos were then fixed with 4% PFA at the appropriate collection time. The expression ontogeny analyses were conducted in embryos from fertilisation up to 12 dpf.

Ag material exposures

Based on the ontogeny of expression of the individual target genes in control animals (see results section) *in situ* expression analysis for the silver materials was adopted for the different target genes at specific life stages (see results section). The Ag dosing regimen adopted for assessing target gene responses using *WISH* analysis was between 500 µg/l and 1,000 µg/l based on previous findings for sublethal effect concentrations (Osborne et al., 2013). Citrate-coated AgNPs were included as most NPs adopted for industry applications are coated with a stabiliser (typically providing either electrostatic or steric stabilisation) to reduce aggregation (Christian et al., 2008). Silver ions (AgNO₃) (Perkin Elmer) were used as an ionic control at 20 µg/l, calculated to provide an approximate dissolution equivalence (1-2%) for the dosing of AgNPCi, based on previous dissolution experiments with these materials, exposure medium and exposure periods (Osborne et al., 2013). Table S1 provides a summary of the exposure time periods for each gene and time point for the *WISH* measurements carried out for each gene studied. *WISH* responses of the *gstp* gene were also analysed with non-coated AgNP and AgB particles for a dosing series of 0 µg/l, 5 µg/l, 50 µg/l, 500 µg/l, 5,000 µg/l and 25,000 µg/l and for *mt2* with AgNP encompassing exposure concentrations spanning 0 µg/l to 32 µg/l for sensitivity analysis. To investigate the role of the *nrf2* transcription factor in the toxicology responses to the silver materials tested, *Nrf2* mutant zebrafish embryos were exposed to AgNPCi (500 µg/l) and AgNO₃ (20 µg/l) at the 1-2 cell stage for a period of 24 h for *mt2* and 3 days for *gstp*. As for the studies on wild type zebrafish, all exposures for *Nrf2* mutants were repeated 3 times.

Synthesis of gene probes: mt2, gstm1, gstp, hmox1 and fth1, Whole mount in-situ hybridization, and Antibody staining of skin cells: vHATPase and NaKATPase.

The methodologies for gene probe synthesis, whole mount *in situ* hybridisation and antibody staining of skin cells are provided in the supplementary material text (S3, S4 and S5, respectively).

Results

Particle Characterization

A summary of the physicochemical properties of the AgNPCi and AgBCi particles is provided in the supplementary material (supplementary material Table S2 and Figure S1). DLS data illustrated that AgNPCi had a mean diameter of 20 nm and AgBCi a mean diameter of 160 nm when dispersed in de-ionised water (supplementary material, Table S2). In contrast, AgNPCi in the embryo culture medium had a mean diameter of 92.25 ± 1.8 nm and AgBCi had a mean diameter of 2456.2 ± 1287.5 nm (supplementary material, Table S2). When dispersed in the embryo medium the measured sizes as determined *via* TEM were 52^{93}_{22} nm for AgNPCi and 145^{509}_{114} nm for AgBCi. Zeta potential for AgNPCi was -47.78 ± 4.25 and for AgBCi was -35.97 ± 6.2 mV. In the embryo culture medium, the zeta potential for AgNPCi was -18.38 ± 1.6 and -21.54 ± 3.9 mV for AgBCi (supplementary material, Table S2). TEM micrographs illustrated that for AgNPCi individual nanoparticles were distinguishable in the embryo exposure medium but there was also evidence of aggregation. Micrographs of bulk particles showed they occurred as large aggregate in the embryo culture medium. It is possible that some of the aggregation seen in the TEM images for both the nano and bulk materials occurred as a consequence of the collection and fixing for the TEM processing (Bozzola and Russell, 1999).

Fate and behaviour of AgNPCi and AgBCi at different exposure concentrations over the period of embryo cultures

Agglomeration in embryo culture medium

DLS size measurements show increasing hydrodynamic diameter and PDI (see supplementary material, Table S2) for the AgNPCi in zebrafish embryo culture medium over the duration of the culture period for all particle concentrations.

Zeta potential and solution pH over time

Zeta potential measurements were carried out at all time-points for all concentrations with both AgNPCi and AgBCi (supplementary material Table S2 and Table S3), with pH also determined at each point. For all concentrations of AgBCi and the lower 3 concentrations of AgNPCi a similar trend was observed, with the zeta potential becoming more negative over the time course observed. The pH of the solutions did not vary significantly during this time, remaining close to pH 7, ruling out changes in the degree of dissolution of the citrate. The data suggest that as the particles lose silver ions and decrease in size, the relative proportion of citrate capping at the surface increases. At the highest concentration of AgNPCi the zeta potential follows the same trend up to 24 hours and then became less negative over time.

Dissolution studies

AgNPCi and AgBCi showed similar dissolution/ion release trend at the highest particle concentration, and this concentration had the highest cumulative release of ions at all time points (supplementary material Tables S4/S5 and Figure S5). The dissolution rates for the different concentrations of AgBCi were: 5 µg/L = 0.0079 µg/L/hour; 50 µg/L = 0.0145 µg/L/hour; 500 µg/L = 0.0602 µg/L/hour; and 5000 µg/L = 0.0811 µg/L /hour. The dissolution rates for the different concentrations of AgNPCi were: 5 µg/L = 0.0547 µg/L/hour; 50 µg/L = 0.2746 µg/L/hour; 500 µg/L/hour = 0.1769 µg/L/hour; 5000 µg/L = 0.0574 µg/L/hour. Previous studies have shown that particle size, coating and aggregation impact on ion release kinetics and that at constant total mass the larger the size of the particle, the slower the release of ionic species, since the dissolution rate is a function of surface area and smaller particles have much higher surface area per unit mass than larger particles (Zhang et al., 2011, Mitrano et al., 2014). The data presented here are in good agreement with literature data. See supplementary material supplementary text S6 for further details.

Developmental life stages adopted for assessing exposure responses to silver materials

Based on the ontogeny of expression of the different genes (see supplementary material Figure S2 and Figure S7 for detailed ontogeny of target genes in zebrafish during early life, 0-12 dpf and a stylised cartoon representing these expression profiles), appropriate life stages were chosen for exposure studies on silver materials. These stages allowed for analysis assessing possible stimulation and/or suppression of target gene expression. *Mt2*: 24 hpf and 4 dpf, *gstp*: 24 hpf, 48 hpf and 4 dpf, *hmox1* and *fth1*: 24 hpf and 5 dpf, *gstm1*: 24 hpf, 4 dpf and 5 dpf.

Quantifying Ag effects

An overview of threshold responses for the different target genes and responsive genes showing the fold change (FC) difference to that compared with controls is shown in Table I.

Effects of silver materials on gene expression

mt2

At 24 hpf, in non-exposed embryos 57% showed a detectable expression of *mt2* in the extended yolk sac region and this was consistently (80% or more of the embryos) elevated for exposures to 500 µg AgNPCi/l (4.6-fold), 500 µg AgBCi/l (4.7-fold) and 20 µg AgNO₃/l (2.7-fold) (Figure 1 Ai-Av) (Table I). *Mt2* showed an enhanced level of expression in the extended yolk sac region for exposures to AgNPs as low as 4 µg/l (the lowest concentration tested; Figure 2 Bii). At 4 dpf (Figure 2 Ci-Cii) a 24-hour exposure to AgNPCi resulted minimally in a 2-fold higher level of *mt2* expression (Table I) across various regions of the larval body including head, jaw and yolk sac (and consistently so, in 92% of the embryos).

gstp

At 24 hpf *gstp* expression was enhanced in the extended yolk sac by between 1.5 and 1.7-fold above controls (Table I) for exposures to 500 µg AgNPCi/l, 500 µg AgBCi/l and 20 µg AgNO₃/l, and in a consistent manner (more than 90% of the embryos responding in the same manner) (Figure 2 Ai-Aiv). For exposures to non-coated silver materials, at 48 hpf (Fig.2 Bi-Cv) AgNP activated *gstp*

expression in regions of the head and pectoral fin in a concentration related manner (75% of embryos showing this response). Levels of *gstp* expression for embryos exposed to AgNP were 2.1-fold greater in the head, and 1.4-fold greater in the pectoral fin compared with controls. Exposure to AgB induced a 1.5-fold higher expression in the head and 1.3-fold higher expression in the pectoral fin compared with controls (Table I). For ionic silver, there were 1.5-fold and 1.6-fold higher levels of expression in the head and pectoral fin, respectively (Figure 2 Ci) (Table I). Antibody staining of ionocytes with vHATPase (shown in green Figure 2 Dii/Dv) NakATPase (shown in red, Figure 2 Di/Dv) showed that focal areas of *gstp* expression on the yolk sac, extended yolk sac and some other body regions of zebrafish larvae were co-localised with vHATPase and NakATPase skin cells. In 4 dpf larvae, (Figure 2 Ei-Eii) 24-h exposure to AgNPCi (1 000 µg/l) resulted in a 2-fold higher expression of *gstp* (Table I) across various parts of the body of the larvae (and in 100% of the embryos examined; Figure 2 Ei-Eii).

gstm1

At 24 hpf *gstm1* was expressed in controls (57% of the embryos) at detectable (albeit low) levels in the extended yolk sac region. Exposure to 500 µg AgNPCi/l, 500 µg AgBCi/l and 20 µg AgNO₃/l induced enhanced expression between 1.6 and 1.8-fold (Table I) higher in the extended yolk sac region. AgNPCi and AgBCi induced between 1.2 and 1.8-fold higher expression in the head region (occurring in over 60% of embryos). The same pattern occurred for exposures to AgNO₃ but with higher fold inductions (4.8-fold in the head for 40% of the embryos; Figure 3 Ai-Aiv, Table I). At 4 dpf larvae showed a consistently (70% of the embryos) enhanced expression of *gstm1* for exposures to 1 000 µg AgNPCi/l, 1 000 µg AgBCi/l and 20 µg AgNO₃/l with focal activity in regions of the head (between a 1.9 and 2.1 fold increase) and yolk sac (between 1.2-1.7-fold increase) (Table I). There was no detectable expression of *gstm1* in control embryos (Figure 3 Bi-Biv). Exposure of 5 dpf larvae

to AgNPCi (1 000 µg/l) for 6 h induced expression of *gstm1* in both the head region (by a 3.1-fold) and in the yolk sac (2.3-fold, Figure 3 Ci-Cii, Table I) in all of the larvae.

hmox1

At 24 hpf there was no effect of any of the silver treatments (AgNPCi, AgBCi nor AgNO₃) on the expression of *hmox1* in the yolk sac region or the lens region of the eye (Figure 4 Ai-Aiv). At 5 dpf, *hmox1* expression was downregulated in all of the larvae in the head region (0.5 of controls and yolk sac (0.7 of controls) (Figure 4 Bi-Bii, Table I).

fth1

At 24 hpf, *fth1* expression occurred mainly in the head and in almost all (97%) of control embryos (Figure 4 Ci-Civ). There was also some more minor expression seen on the skin. There were no obvious effects of exposure to AgNPCi, AgBCi nor AgNO₃ on the expression pattern or intensity for *fth1* compared to controls. However, at 5 dpf a 6 h exposure to AgNPCi (1 000 µg/l) resulted in a suppressive effect (in all larvae) on the expression of *fth1* in both the head and yolk sac region (expression was between 0.4 and 0.5 of controls; Figure 4 Di-Dii, Table I).

Effects of AgNP on expression of mt2 and gstp in the nrf2 mutant

We examined expression of *mt2* and *gstp*, in an Nrf2 mutant zebrafish to investigate their regulatory mechanism. In wild type (WIK) zebrafish at 24 hpf *mt2* was expressed in the extended yolk sac region in response to AgNPCi and AgNO₃ (Figure 5 Ai and Aiii) but in an Nrf2 mutant the same exposures induced no detectable expression in the extended yolk sac region in the majority of embryos (over 70%; Figure 5 Aii and Aiv). There was variable, but comparatively minor expression by up to 0.7 of *mt2* in the remaining Nrf2 mutant embryos compared with wild type fish. In wild type zebrafish (at 3 dpf), exposure to AgNPCi and AgNO₃ induced *gstp* expression in the olfactory region, pectoral fin, cloaca, and neuromasts of the head region (Figure 5 Bi and Biii). In contrast with this, in the Nrf2 mutant, *gstp* expression was not observed in either the olfactory region or the cloaca in the AgNPCi treatment group in any embryo (Figure 5 Bii). For exposure to 20 µg AgNO₃/l (Figure 5 Biv),

there was expression of *gstp* in the neuromasts and olfactory region in the Nrf2 mutant, albeit at a lower level compared with the wild type zebrafish, and no detectable *gstp* expression in the cloaca.

Discussion

Adopting *WISH* as a technique we identified target tissues for exposure to AgNPs, a bulk counterpart and silver ions via responses in genes associated with metal handling, detoxification and oxidative stress (Valko et al., 2006). We provide evidence that key sensory systems and ionocytes are targets for these materials in fish exposed *via* the water and that the toxicological responses to the silver materials tested are due principally to silver ions. Furthermore, we show that the *nrf2* pathway is involved in the toxicological response to AgNPs.

Whole organism gene expression ontogeny

Detailed expression ontogeny analysis on zebrafish embryos and early life stages (15 to 20 individuals x 12 stages x 5 markers x triplicate analyses) identified the potential receptive tissues and life stages for toxicological effects of silver materials (Supplementary material, Figure S2). In unexposed embryos, we found that *mt2* was weakly expressed in the extended yolk sac (at 24 hpf), as we have shown previously (Osborne et al., 2013). Low level expression of *gstp* also occurred in the yolk sac and in head, pectoral fin, jaw, olfactory and gill and this probably serves to provide constitutive protection against oxidative damage in the developing embryo/larvae. Similarly, the expression of *gstm1* (at 24 hpf) in the yolk sac seen in control embryos may serve to help protect against electrophilic compounds during early life development. Overall, the oxidative stress genes *gstp* and *gstm1* shared similar tissue expression patterns.

Hmox1 and *fth1* that both play roles in maintaining cellular iron and in the control of the porphyrin metabolic pathway, showed similar patterns of expression in non-exposed embryos and larvae, but the patterns differed from the genes associated with oxidative stress. Expression of *hmox1* and *fth1* in the head, yolk sac and liver is consistent with the roles of iron in processes including growth and

immunity. The finding that liver was a major tissue expressing *fth1* in 6 dpf fish is consistent with this tissue containing the highest levels of ferritin (Neves et al., 2009). The variable expression of *hmox1* and *fth1* between different tissues and within tissues over time is reflective of the variable requirement for metals such as iron in development and growth.

For all genes studied, the yolk sac was a major site of activity which may relate to its role in processing of metabolites generally (Chen et al., 2004). Based on the patterns of expression in control embryos and larvae, we selected certain zebrafish early life stages - between 24 hpf and 5 dpf - that allowed for effective assessment of silver material exposures on expression of these target genes. The expression of the target genes detected at 3-4 hpf in the blastoderm probably represented maternally derived mRNA.

Fate and behaviour of silver materials in the embryo culture medium

Ion release kinetics of AgNPs is dependent on several environmental factors such as pH, temperature, dissolved oxygen, ionic strength, as well as the physicochemical properties of the AgNPs (*e.g.* surface coating, shape, size and method of synthesis ; Badawy et al., 2010; Dobias et al., 2013; Loza et al., 2014; Zhang et al., 2011, Kittler et al., 2010, Ma et al., 2011). The DLS data indicated rapid agglomeration of the AgNPCi and AgBCi particles occurred at all concentrations studied in the zebrafish embryo culture medium. The agglomeration was more pronounced at the lower particle concentrations, while for the higher AgNPCi particle concentrations (500 and 5000 µg/L) agglomerates remained < 200nm in size for at least 72 hours of the exposure. AgNPCi particles at 5ppb concentration likely dissolve completely in the media. The higher concentrations reach equilibrium after 5 hours, which was retained for around 80 hours, beyond which secondary processes (*e.g.* precipitation, aggregation, settling) may start occurring. The equilibrium concentration of Ag⁺ available in solution was between 15-25 ppb (µg/L) for the 50, 500 and 5,000 µg/L AgNPCi over the first 72 hours, and below 10 ppm (µg/L) for all but the highest concentration of AgBCi.

Genes and tissues responsive to silver materials

There were concentration dependent responses to all the silver materials for *mt2* (Figure 1), which were seen principally in the region of the extended yolk sac (Figure 1 Ai-Aiv). *Mt2* responses were detected down to an exposure concentration of 4 µg/l for AgNP. From predicted modelling studies, these levels are environmentally relevant (Gottschalk et al., 2009) (Mueller and Nowack, 2008). In oyster embryos, Ag has been shown to induce *mt2* at a concentration of only 0.16 µg/l, as detected *via* real time quantitative PCR analyses, which is a more sensitive detection method than *in situ* hybridisation (Ringwood et al., 2010). In adult fish, responsive tissues to Ag reported in previous studies include the liver and gills (Hogstrand et al., 1996).

Gstp expression was induced by Ag materials in a variety of tissues, consistent with findings from previous studies showing oxidative stress in fish exposed to AgNPs (Choi et al., 2009, Foldbjerg et al., 2009). Induction of this gene was also seen in olfactory bulbs, an established tissue target for heavy metals and potentially affecting olfaction (Gobba, 2006). A key finding was that *gstp* activation co-regionalised with other parts of the sensory system, including neuromasts (Figure 2 Biii). Neuromasts are receptors comprised of groups of hair cells usually found in the lateral line and head region of the zebrafish that detect water movement (Trump et al., 2008). Located in close proximity with the surrounding environment they function in sensing and in orientation more generally. These sensory receptors are essential for various behaviours, social interactions, prey detection and predator avoidance (Froehlicher et al., 2009). It is known that heavy metals can alter vision, taste and olfaction, orientation and auditory functions (Kasumyan and DÖving, 2003) and copper can trigger the cell death and loss of neuromasts (Linto et al., 2006). Neuromasts however do have the capacity for re-generation. Our findings indicate that exposure to silver particles could impact on the neuromasts and other associated sensory functions in the zebrafish and they support a recent study showing effects of TiO₂NPs on the hair cell (*i.e.* neuromasts, He et al., 2014) in zebrafish. Another

recent study has shown some metal based NPs can affect the behaviour in early life stages of zebrafish, and these affects were a consequence of disruptions of sensory cells of the lateral line (McNeil et al., 2014).

We also observed enhanced *gstp* expression co-regionalised with ionocytes (skin cells) specifically ion transferring NaKATPase channels (Lin et al., 2008) in zebrafish larvae (Figure 2 Di-Dv). The distribution of ionocytes we identified in zebrafish larvae using antibody staining aligned with that reported previously for ionocyte localisation in zebrafish (Esaki et al., 2009), with wide distribution over the skin and yolk sac (Hiroi et al., 1998). In adult fish, ionocytes are principally located in the gill area and are involved in the molecular transfer and exchange of ions (Dymowska et al., 2012). In trout gills, exposure to silver can inhibit basolateral membrane Na^+/K^+ ATPase activity in ionophores (Wood et al., 1999). Responses in *gstp* expression in zebrafish larvae indicate that silver might affect ionic regulation in the skin. In gill ionophores, Ag ion affects on the Na^+/K^+ pumps reduces active N^+ and Cl^- uptake, which can consequently lead to an imbalance of Na^+/Cl^- ions in the blood plasma and in extreme cases, even death (Hogstrand and Wood, 2009).

Gstp induction also occurred in response to Ag in regions of the head, yolk sac, skin and the pectoral fin (48 hpf). Recently, *gstp* upregulation was shown to occur during the regeneration of caudal fin, indicating a role in repair in response to tissue damage (Timme-Laragy et al., 2012). *Gstm1* is upregulated in the presence of large number of xenobiotics (Higgins and Hayes, 2011) indicating a general role in the prevention of xenobiotic induced oxidative stress. Our studies on the zebrafish embryo support a wider body of literature showing Ag NPs cause oxidative stress in various cell systems (Foldbjerg et al., 2009). In human liver cells, it has been reported that PVP coated AgNPs can generate ROS in only 30 minutes and this effect is maintained for 12 hours (Piao et al., 2011). In this study, we also showed a clear inductive response for *gstm1* after a 6-hour exposure to AgNPCi.

We found a reduced expression of both *hmx1* (Figure 4 Bi-Bii) and *fth1* (Figure 4 Di-Dii) in the head region and yolk sac (Table I) after a 6-hour exposure to AgNPs in 5 dpf zebrafish. Oxidative stress (occurring through ROS) and inflammatory responses have been shown also to have suppressive effects on *hmx1* in mice, for example in chronic inflammatory illness (Poss and Tonegawa, 1997). Intracellular excess iron causes oxidative stress by generating Fe^{2+} and in turn a hydroxyl radical in the Fenton reaction (Harrison and Arosio, 1996). It is still uncertain whether ferritin causes more oxidative stress by releasing Fe^{2+} as part of its cytoprotective role of oxidative stress (Arosio et al., 2009). It has been suggested that this is why *fth1* down-regulation or over expression can be seen during oxidative stress (Orino and Watanabe, 2008). In our study we observed suppressed effect of *fth1* (Figure 4 Di-Dii).

The toxicity of AgNP is via silver ion

Increasing evidence suggests that the toxicity of silver nanoparticles is a direct effect of dissociating silver ions (Kittler et al., 2010, Osborne et al., 2013) and our data for the expression of the various genes studied would support this.

In the first instance at 24 hpf, *gstp* and *mt2* induction in the yolk sac region was common across all silver treatments (Figure 2 Ai-Aiv) for equivalent estimated availability of silver ions. Secondly, AgNP induced stronger *gstp* responses compared with the bulk counterpart for the same affected tissues (Figure 2 Bi-Cv) and this too is consistent with the response being due to silver ions, as dissolution tends to be faster for nanoparticles compared with their bulk counterparts (Choi et al., 2008). Furthermore, we found coating of particles in citrate reduced toxicity, and coating of particles reduces the level (rate) of dissolution and thus bioavailability of silver ions (Marambio-Jones and Hoek, 2010). Also, there were no differences between the tissues affected for *gstm1* induction for the nano and bulk silver material exposures (4 dpf) *i.e.* the response patterns were the same.

Nrf2 plays a key role in the toxicological response to silver

A study on *nrf2* reported expression in the nose, liver and gill of fish (Nakajima et al., 2011a). In mammals, it is established that *nrf2* plays a major role in mediating the oxidative stress response in cells (Theodore et al., 2008) and activation of this transcriptional response is triggered in the presence of reactive oxygen species (ROS) (Motohashi and Yamamoto, 2004) (Knörr-Wittmann et al., 2005). Studies with mice have indicated that the metallothionein gene contains an antioxidant response element (ARE) (Ohtsuji et al., 2008) to which Nrf2 binds. Here, we established that *mt2* and *gstp* were both induced by Ag in wild type (WIK strain) zebrafish, but in an Nrf2 mutant zebrafish, their expression was diminished considerably at 24 hpf (*mt2*) and 3 dpf (*gstp*) supporting the theory that *nrf2* mediates the role of oxidative stress in the signalling cascade for *mt2* and *gstp* in fish. Our findings concur with studies in *nrf1* mutant mice, where expression of both metallothionein (Ohtsuji et al., 2008) and GSH were decreased (Itoh et al., 1997) and indicating similarities in the response mechanisms between mice and fish. In the Nrf2 mutant zebrafish exposed to AgNO₃ (at 3 dpf) *gstp* expression was still clearly evident in the neuromasts, suggesting that the response in this tissue could be mediated by a transcriptional oxidative stress pathway different than that of Nrf1 (Biswas and Chan, 2010). Collectively data for the *nrf2* mutant however, shows that the Nrf2 pathway plays an important role in mediating the toxicological response to Ag materials in fish.

Conclusion

Applying *WISH*, we identify target tissues for silver nanomaterials, and they include tissues involved in environmental sensing (olfactory bulbs and neuromasts) and ionocytes involved with ion transport. Furthermore, gene responses associated with detoxification and oxidative stress appear to occur as a consequence of silver ions rather than a physical effect of the materials. We further show that using an Nrf2 mutant zebrafish, that *Nrf2* is an important transcription factor in the toxicological response of *mt2* and *gstp* to AgNPs. Our work illustrates the potential for the use of mutant lines of zebrafish in combination with whole mount *in situ* hybridisation to better delineate pathways of effect for nanomaterials.

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Declaration of Interest

The author's affiliation is as shown on the cover page and the authors have sole responsibility for the writing and content of the paper. This work was funded by the Natural Environmental Research Council NE/L007371/1 and NE/H013172/1) and the European Union 7th Framework programme (Nanomile; Engineered nanoparticles and mechanisms of interactions with living systems and the environment: a universal framework for safe nanotechnology. The literature review conducted, the interpretations made and the conclusions drawn are exclusively those of the authors. None of the authors has any actual or potential competing financial interests.

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Table and Figure Legends

Tables

Table I. Quantification of target gene responses derived from *in situ* hybridisations showing fold change in expression compared with controls. A. *mt2*, *gstp*, *gstm1*, *hmox1* and *fth1* at 24 hpf. B. *gstp* at 48 hpf; C. *mt2*, *gstp* and *gstm1* at 4 dpf. D. *gstm1*, *hmox1* and *fth1* at 5 dpf.

Figures

Figure 1. Expression of *mt2* in zebrafish embryos/larvae at 24 hpf-after exposure to AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation. Arrow indicates focal target areas affected *i.e.* where *mt2* expression is observed. Ai. Control embryo, Aii. 500 µg AgNPCi/l, Aiii. 500 µg AgBCi/l, Aiv. 20 µg AgNO₃/l (all 24 hours post fertilisation). Bi. Control embryo, Bii-BvAgNP, at (Bii) 4 µg AgNP/l, (Biii) 8 µgAgNP/l, (Biv) 16 µgAgNP/l and (Bv) 32 µgAgNP/l (all 24 hours post fertilisation). Ci-ii AgNPCi, Ci. Control larvae, Cii 1 000 µg AgNPCi /l (all exposed at 4 days post fertilisation for 24 h). Percentage values given are proportion of embryos affected in the treatments (numbers of animals was between 15 and 20 for each treatment). Relative changes in gene expression compared with controls are reported in Table I). Scale bars= 500 µm

Figure 2. Expression of *gstp* in zebrafish embryos/larvae at 24 hpf-after exposure to AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation. Arrow indicates focal target areas affected *i.e.* where *gstp* expression is observed. Ai. Control embryo, Aii. 500 µg AgNPCi/l, Aiii. 500 µg AgBCi/l, Aiv. 20 µg AgNO₃/l (all 24 hours post fertilisation). Bi. Control embryo, Bii-Bv AgNP, at (Bii) 5 µg AgNP /l, 50 µg AgNP /l (Biii), 500 µg AgNP /l (Biv) and 5 000 µg AgNP /l (Bv); (Ci) 20 µg AgNO₃/l Cii-Cv AgB, (Cii) at 5 µg AgB /l, (Ciii) 50 µg AgB /l, (Civ) 500 µg AgB /l and (Cv) 5 000µg AgB /l and (all 48 hours post fertilisation). Di-Div Embryos exposed to AgNPs and subjected to *in situ* hybridisation for

gstp and antibody staining with vHATPase (green) and NaKATPase (red) to detect ionocytes. Di. Exposure to 500 µg AgNP/l showing *in-situ* staining at 50 hpf, Dii. Enlarged view of embryo at 50 hpf after application of antibody staining (green -vHATPase, and red -NaKATPase) to reveal ionocytes on yolk sac, Diii. 500 µg AgNP/l dosed embryo showing skin cell staining on extended yolk sac, Div. embryo after application of antibody staining (green -vHATPase, red -NaKATPase) indicating ionocytes on yolk sac. Ei-ii AgNPCi, Ei. Control larvae, Eii 1 000 µg AgNPCi,/l (all exposed at 4 days post fertilisation for 24 h). Percentage values given are proportion of embryos affected in the treatments (numbers of animals was between 15 and 20 for each treatment). Relative changes in gene expression compared with controls are reported in Table I). Scale bars= 500 µm

Figure 3. Expression of *gstm1* in zebrafish embryos/larvae at 24 hpf-after exposure to citrate-coated AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation. Arrow indicates focal target areas affected *i.e.* where *gstm1* expression is observed. Ai. Control embryo, Aii. 500 µg AgNPCi/l, Aiii. 500 µg AgBCi/l, Aiv. 20 µg AgNO₃/l (all 24 hours post fertilisation). Bi. Control embryo, Bii. 1 000 µg AgNPCi/l, Biii. 1 000 µg AgBCi/l, Biv. 20 µg AgNO₃/l (all 4 days post fertilisation). Ci-ii AgNPCi, Ci. Control larvae, Cii 1 000 µg AgNPCi /l (all exposed at 5 days post fertilisation for 6 h). Percentage values given are proportion of embryos affected in the treatments (numbers of animals was between 15 and 20 for each treatment). Relative changes in gene expression compared with controls are reported in Table I). Scale bars= 500 µm

Figure 4. Expression of *hmox1* and *fth1* in zebrafish embryos/larvae at 24 hpf-after exposure to coated AgNPCi, AgBCi and AgNO₃ as determined by whole mount *in situ* hybridisation. Arrow indicates focal target areas affected *i.e.* where *hmox1* and *fth1* expression is observed. *Hmox1*: Ai. Control embryo, Aii. 500 µg AgNPCi/l, Aiii. 500 µg AgBCi/l, Aiv. 20 µg AgNO₃/l (all 24 hours post fertilisation). Bi-ii AgNPCi, Bi. Control larvae, Bii 1 000 µg/l (all exposed at 5 days post fertilisation for 6 h). *Fth1*: Ci. Control embryo, Cii. 500 µg AgNPCi/l, Ciii. 500 µg AgBCi/l, Civ. 20 µg AgNO₃/l (all 24

hours post fertilisation). Di-ii AgNPCi, Di. Control larvae, Dii 1 000 µg AgNPCi /l (all exposed at 5 days post fertilisation for 6 h). Percentage values given are proportion of embryos affected in the treatments (numbers of animals was between 15 and 20 for each treatment). Relative changes in gene expression compared with controls are reported in Table I). Scale bars= 500 µm

Figure 5 Expression of *mt2* and *gstp* in zebrafish embryos (WIK and *nrf2* mutant) -after exposure to AgNPCi and AgNO₃ as determined by whole mount *in situ* hybridisation. Arrow indicates focal target areas affected *i.e.* where *mt2* and *gstp* expression is observed. - *Mt2*: Ai. AgNPCi (WIK) Aii. AgNPCi (*nrf2* mutant), Aiii. AgNO₃ (WIK), Aiv. AgNO₃ (*Nrf2* mutant) –all at 24 hpf. *Gstp*: Bi. AgNPCi (WIK) Bii. AgNPCi (*Nrf2* mutant) Biii. AgNO₃ (WIK) Biv. AgNO₃ (*Nrf2* mutant)- all at 3 dpf. Percentage values given are proportion of embryos affected in the treatments (numbers of animals was between 15 and 20 for each treatment). Scale bars= 500 µm