Transgenic zebrafish larvae as a non-rodent alternative model to assess pro-inflammatory (neutrophil) responses to nanomaterials

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ABSTRACT

Hazard studies for nanomaterials (NMs) commonly assess whether they activate an inflammatory response. Such assessments often rely on rodents, but alternative models are needed to support the implementation of the 3Rs principles. Zebrafish (Danio rerio) offer a viable alternative for screening NM toxicity by investigating inflammatory responses. Here, we used non-protected life stages of transgenic zebrafish (Tg(mpx:GFP)i114) with fluorescently-labeled neutrophils to assess inflammatory responses to silver (Ag) and zinc oxide (ZnO) NMs using two approaches. Zebrafish were exposed to NMs via water following a tail fin injury, or NMs were microinjected into the otic vesicle. Zebrafish were exposed to NMs at 3 days post-fertilization (dpf) and neutrophil accumulation at the injury or injection site was quantified at 0, 4, 6, 8, 24, and 48 h post-exposure. Zebrafish larvae were also exposed to fMLF, LTB₄, CXCL-8, C5a, and LPS to identify a suitable positive control for inflammation induction. Aqueous exposure to Ag and ZnO NMs stimulated an enhanced and sustained neutrophilic inflammatory response in injured zebrafish larvae, with a greater response observed for Ag NMs. Following microinjection, Ag NMs stimulated a time-dependent neutrophil accumulation in the otic vesicle which peaked at 48 h. LTB₄ was identified as a positive control for studies investigating inflammatory responses in injured zebrafish following aqueous exposure, and CXCL-8 for microinjection studies that assess responses in the otic vesicle. Our findings support the use of transgenic zebrafish to rapidly screen the pro-inflammatory effects of NMs, with potential for wider application in assessing chemical safety (e.g. pharmaceuticals).

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Introduction

The exploitation of engineered nanomaterials (NMs) in products such as cosmetics, electronics, textiles, and medicines is increasing rapidly, with the nanotechnology market expected to be worth $125 billion (USD) by 2024 (Research and Markets 2018). The hazards of NMs need to be thoroughly assessed to ensure the safe and responsible exploitation of nanotechnology, but this is challenging given the rapid growth of the nanotechnology industry and the production of a huge diversity of NMs. Silver (Ag) and zinc oxide (ZnO) NMs are some of the most widely used NMs (Vance et al. 2015) and existing in vitro and in vivo studies have shown that they can cause toxicity to different target tissues in mammalian models (e.g. lung, immune system, liver) and environmental species (reviewed by Vandebriel and De Jong 2012; Landsiedel et al. 2014; Akter et al. 2018; Tortella et al. 2020; Ferdous and Nemmar 2020).

Investigation into whether NMs activate a pro-inflammatory response is routinely assessed by in vitro and in vivo nanotoxicology studies as inflammation is hypothesized to be a key mechanism underlying NM toxicity (Stone, Johnston, and Clift 2007; Johnston et al. 2018). There is extensive evidence in the published literature that Ag and ZnO NMs can activate inflammatory responses. For example, rodent studies have shown that Ag and ZnO NMs stimulate an influx of neutrophils into organs including the lung and liver (e.g. Cho et al.
2012; Kermanizadeh et al. 2012; Gaiser et al. 2013; Adamcakova-Dodd et al. 2014; Sha et al. 2014; Smulders et al. 2014; Abbasalipourkabir et al. 2015; Awasthi et al. 2015; Patlolla, Hackett, and Tchounwou 2015; Smulders et al. 2015; Almansour et al. 2017). Furthermore, Ag and ZnO NMs stimulate pro-inflammatory cytokine production (e.g. interleukin (IL)-8 and tumor necrosis factor (TNF)-α) in vivo and in vitro, which is likely due to the activation of redox-sensitive pro-inflammatory signaling pathways (e.g. NF-κB) (e.g. Cho et al. 2012; Kermanizadeh et al. 2013a, 2013b; Johnston et al. 2015).

Traditionally, toxicology studies for protecting human health have relied on the use of rodents (Burden et al. 2017). When assessing inflammatory responses, the ability to quantify and visualize the accumulation of immune cells in specific target sites (e.g. lungs) in rodents provides benefits over in vitro models which lack the complexity to investigate such responses. However, in vivo (rodent) studies are costly and time-consuming, their use raises ethical concerns, and there is also evidence that rodents do not accurately predict adverse outcomes in humans due to species differences (Burden et al. 2017; Johnston et al. 2018). Consequently, there has been a strong drive toward the more widespread implementation of the 3Rs principles of Reduction, Refinement, and Replacement of animals use in nanotoxicology research (Burden et al. 2017). We propose that zebrafish (Danio rerio) can be used as an alternative to rodents to screen NM hazards, and more specifically to assess whether NMs activate an inflammatory response.

Zebrafish are not protected by current EU and UK legislation until they have reached the stage of exogenous feeding at 5 days post-fertilization (dpf) (Home Office 1986; European Commission 2010). Thus, the use of non-protected life stages of zebrafish for scientific research requires no approval from responsible authorities. There are many other advantages to using the early life stages of zebrafish as a testing model including their small size, and their transparency which enables visualization of organ and tissue development. Furthermore, the zebrafish genome is remarkably homologous to the human genome, with 84% of disease-associated genes having a zebrafish counterpart (Howe et al. 2013; Cassar et al. 2020). The zebrafish genome is also highly amenable to genetic manipulation, and the optical transparency of zebrafish larvae coupled with the transgenic expression of fluorescent proteins enables real-time visualization of specific cellular processes, cells, and tissues, which is not possible in rodents (He et al. 2014). Indeed, many transgenic zebrafish strains have been developed for a wide range of targets spanning: immune cells (Renshaw et al. 2006; Ellett et al. 2011), estrogen receptors (Lee et al. 2012; Green et al. 2018), markers of oxidative stress (Kusik, Carvan, and Udvadia 2008; Bar-Ilan et al. 2012; Mourabit et al. 2019), neurons (Park et al. 2000; Winter et al. 2021), blood vessels (Gao et al. 2016), the liver (Zhang, Li, and Gong 2014), and cell organelles (e.g. mitochondria (Kim et al. 2008)) (reviewed by Lee, Green, and Tyler 2015; Bai and Tang 2020; Choe et al. 2021). As a consequence, early life stages of transgenic zebrafish are now increasingly used in biomedical research (e.g. reviewed by Dodd et al. 2000; Renshaw et al. 2007; Lieschke and Currie 2007; Choe et al. 2021). However, transgenic zebrafish have not been widely used for assessing the impact of NMs on human health. Instead, the majority of existing published studies using zebrafish have focused on assessing the ecotoxicity of NMs via investigation of zebrafish development, hatching rate, behavior, mortality, teratogenicity, and oxidative stress (Asharani et al. 2008a, 2008b; Zhu et al. 2008; Bai et al. 2010; Bar-Ilan et al. 2012, 2013; Duan et al. 2013 Osborne et al. 2013; Kovrižnych et al. 2013; Clemente et al. 2014; Hua et al. 2014; reviewed by Bai and Tang 2020; d’Amora et al. 2022).

The zebrafish offers an attractive alternative model for assessing the inflammatory effects of NMs due to their well-characterized innate immune system, with neutrophils and macrophages (that are functionally equivalent to those in mammals) active from 1 dpf (Lieschke and Trede 2009; Novoa and Figueras 2012). There are a number of transgenic zebrafish lines available that express fluorescent proteins in specific immune cell types including neutrophils (Tg(mpx:GFP), macrophages (Tg(mpeg1):GFP) (Renshaw et al. 2006; Ellett et al. 2011) or both (e.g. Tg(mpx:GFP/mpeg1:mCherry)) (Ellett et al. 2011). Accordingly, transgenic lines have been exploited effectively in zebrafish embryo-larval life stages to visualize and quantify
inflammatory responses to various stimuli including lipopolysaccharide (LPS) (e.g. Yang et al. 2014; Zhang et al. 2016), neutrophil chemoattractants [e.g. chemokine (CXC) ligand-8 (CXCL-8), N-Formyl-Met-Leu-Phe (fMLF), Leukotriene B₄ (LTB₄)] (Elks et al. 2011; de Oliveira et al. 2013), tail fin wound injury (e.g. Renshaw et al. 2006; Ellett et al. 2011; Hoodless et al. 2016), cardiac injury (e.g. Kaveh et al. 2020), micro-organisms (e.g. Nguyen-Chi et al. 2014; Benard et al. 2012; Jim et al. 2016) and NMs, such as silica (e.g. SiO₂) (Sharif et al. 2012; Duan et al. 2016; Zhang et al. 2016; Brun et al. 2018). Of major benefit is that inflammatory responses to such stimuli can be monitored in the same zebrafish over time, which is not possible in rodents.

The tail fin injury technique has been a popular method used to induce an inflammatory response in transgenic zebrafish larvae [e.g. Tg (mpx:EGFP)] whereby part of the median tail fin is removed using a scalpel, needle, or laser, and the inflammatory response (e.g. neutrophil accumulation) at the injury site is monitored over time via fluorescent microscopy (Renshaw et al. 2006; Cvejic et al. 2008; Loynes et al. 2010). This model has been widely used to investigate the dynamics of inflammatory responses to injury to better understand tissue repair and wound resolution processes (Renshaw et al. 2006; Ellett et al. 2011; Feng et al. 2010; Cordero-Maldonado et al. 2013; Lucas et al. 2013; Nguyen-Chi et al. 2017). In addition, injured zebrafish larvae have been exposed to compounds, such as pharmaceuticals (e.g. anti-inflammatory drugs) and pathogens (e.g. bacteria) via water to investigate their impact on the inflammatory response at the injury site (Loynes et al. 2010; Gratacap, Rawls, and Wheeler 2013; Hoodless et al. 2016; Miskolci et al. 2019). Inflammatory responses to a range of stimuli (such as LPS, C5a, and CXCL-8), and micro-organisms (e.g. bacteria, fungi) have also been investigated following their microinjection into several body sites in zebrafish larvae including the otic vesicle, swimbladder, hindbrain ventricle, notochord, and caudal vein (Gratacap, Rawls, and Wheeler 2013; Nguyen-Chi et al. 2014; Benard et al. 2012; Jim et al. 2016; Harvie and Huttenlocher 2015; Zhang et al. 2016; de Oliveira et al. 2013). Following injection, inflammatory responses are monitored at the injection site (and sometimes other locations) over time.

The majority of existing studies using transgenic zebrafish to investigate inflammatory responses have done so to assess for effects of pathogens and physical injury. A limited number of published studies have investigated inflammatory responses to NMs using transgenic zebrafish but they are limited with respect to the number of NMs tested, life stages of zebrafish, exposure routes, and time points used. Examples of these studies include the investigation into whether silica NMs activate a neutrophil response in the cardiac region following microinjection into the Duct of Cuvier (equivalent to intravenous injection) in Tg(mpo:GFP) zebrafish up to 24 h post-injection (Duan et al. 2016). Zhang et al. (2016) assessed neutrophil responses to silica NMs 4 h post-microinjection into the swim bladder of Tg(mpo:GFP) zebrafish. Sharif et al. (2012) investigated neutrophil responses to silica NMs in lysC::DsRED2 zebrafish at 2 h and 3 days following microinjection into the flank of zebrafish. Brun et al. (2018) investigated the migration of neutrophils and macrophages to the tail following aqueous exposure and intravenous injection to CuO and polystyrene NMs using Tg(mpx:eGFP) and Tg(mpeg1:eGFP) zebrafish strains for up to 120hpf.

This study aimed to investigate the suitability of using transgenic zebrafish larvae as a testing model for screening the toxicity of NMs via assessment of neutrophil responses. We prioritized the use of Ag and ZnO NMs as it is already well-established that these NMs stimulate a pro-inflammatory response in rodent and in vitro models. More specifically, there is extensive evidence that Ag and ZnO NMs activate inflammatory responses that are characterized by the accumulation of neutrophils in vivo (rodents) and enhanced production of neutrophil chemoattractants in vitro and in vivo (e.g. Kermanizadeh et al. 2013b; Smulders et al. 2015; Almansour et al. 2017; Verdon et al. 2021). Despite evidence that Ag and ZnO NMs stimulate pro-inflammatory responses, no studies have yet used transgenic zebrafish to monitor inflammatory responses to these NMs. Accordingly, Tg(mpx:GFP)i114 zebrafish (with fluorescent neutrophils) were selected to assess neutrophil responses to Ag and ZnO NMs in our study. We investigated the dynamics of the inflammatory response following NM exposure by monitoring neutrophil...
accumulation over time, as existing studies using NMs have often only used a limited number of time points.

We used non-protected life stages of zebrafish (≤5dpf) seeking to further advocate the implementation of the 3Rs principles in nanotoxicology. The use of this life stage however is restrictive in the sense of what exposure and target sites can be used to assess inflammatory response in zebrafish as not all tissues and organs are fully developed in non-protected life stages. The use of non-protected life stages also limits the length of exposure and monitoring of responses to NM exposure. Aqueous exposure is the most convenient (and environmentally relevant) way of exposing zebrafish to NMs (as well as other substances and pathogens). Given that the tail fin injury approach has been used extensively in the published literature to investigate the dynamics of inflammatory responses, we chose this approach to investigate inflammatory responses to NMs following aqueous exposure. It was hypothesized that both Ag and ZnO NMs would enhance and prolong the neutrophil response that was activated at the injury site.

Whilst microinjection requires more specialized equipment and is more technically challenging than aqueous exposure, it allows inflammatory responses to be investigated at the injection site (as well as other locations) over time. Accordingly, we microinjected NMs into the otic vesicle of zebrafish larvae (the primordial ear of the larvae) and quantified the inflammatory response at the injection site. Here, we hypothesized that both Ag and ZnO NMs would stimulate the infiltration of neutrophils into the otic vesicle following microinjection. We selected the otic vesicle as a discrete target site for injection to induce a localized neutrophil response (Levraud et al. 2008; Harvie and Huttenlocher 2015; Buchan et al. 2020). Whilst other injection sites (e.g. swim bladder, caudal vein, hindbrain ventricle) have been used in the literature to investigate inflammatory responses to a range of stimuli, not all these sites can be used in non-protected life stages and the otic vesicle offers a suitable target site for assessing localized inflammatory responses as it is typically devoid of neutrophils and as there is evidence that they are recruited to this site in response to injury or infection (Levraud et al. 2008; Harvie and Huttenlocher 2015). To date, no published studies have investigated inflammatory responses to NMs following the aqueous exposure of injured zebrafish or following their microinjection into the otic vesicle.

The administration routes we have adopted are primarily intended as a convenient way of administering NMs to screen their safety, when monitoring neutrophil responses as an indicator of toxicity, and are not intended to necessarily represent expected routes of human or fish exposure to NMs. Zebrafish hatching occurs between 2 and 3 dpf, and the presence of the chorion can limit the interaction of the zebrafish embryos with NMs. We exposed hatched larvae to NMs at 3dpf, as whilst zebrafish embryos can be exposed before hatching (when in their chorion) or can be dechorionated to allow for exposure at earlier time points, this is time-consuming and can cause damage to the zebrafish. As we worked with non-protected life stages of zebrafish, this allowed for inflammatory responses to be monitored up to 48 h post-exposure. Previous studies have demonstrated that the activation and resolution of inflammatory responses to various stimuli (e.g. injury) can be captured within this timeframe.

Interestingly, existing studies investigating inflammatory responses in zebrafish often neglect to include a positive control. Thus, for both exposure routes, we aimed to identify a suitable positive control as a comparator, and for this, we included LPS, and the neutrophil chemoattractants fMLF, CXCL-8, LTB4, and C5a.

Materials and methods

Ethics statement

Experiments were conducted in accordance with the accepted standards of animal care, under the regulation of the UK Animals (Scientific Procedures) Act (ASPA) 1986, and EU Directive 2010/63/EU (European Commission 2011; Home Office 1986). Zebrafish adults were housed in a UK Home Office-approved facility within the University of Edinburgh. Experiments were conducted with non-protected life stages of zebrafish aged between 0 and 5 dpf.

Characterization of nanomaterials

Ag NMs (NM-300K) and ZnO NMs (NM-110) were obtained from the Joint Research Center (JRC)
nanomaterial repository. Ag NMs were provided as a colloidal aqueous dispersion and the ZnO NMs were provided as a dry powder. All NMs were prepared according to a protocol developed for the FP7-funded project ENPRA (Jacobsen et al. 2009). More specifically, a 1 mg/mL stock suspension of each NM was prepared in 2% heat-inactivated fetal bovine serum (HI-FBS) in sterile water and sonicated at full power in a Q-Series Ultrasonic cleaning bath (Ultrawave Ltd., UK) for 16 min. The NMs used have previously been characterized extensively as they are commonly used as benchmark materials by the nanotoxicology community (Table 1).

The NMs were characterized in media relevant to this study via measurement of the hydrodynamic diameter (i.e. size), zeta potential (surface charge), and polydispersity index (PdI) using dynamic light scattering (DLS), using a Zetasizer Nano Series (Malvern Instruments, UK). More specifically, a 1 mg/mL stock suspension of each NM was prepared in 2% heat-inactivated fetal bovine serum (HI-FBS) in sterile water and sonicated at full power in a Q-Series Ultrasonic cleaning bath (Ultrawave Ltd., UK) for 16 min. The NMs used have previously been characterized extensively as they are commonly used as benchmark materials by the nanotoxicology community (Table 1).

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The physio-chemical characteristics of the NMs under investigation have been extensively characterized previously. A summary of the physico-chemical characteristics of the tested NMs is provided based on information provided in the published literature. References are included to indicate the source of the information.

**Table 1. Physico-chemical properties of Ag and ZnO NMs.**

| NM          | Supplier: | Size         | Shape                                  | Surface area (BET) m²/g | Solubility      | References                                      |
|-------------|-----------|--------------|----------------------------------------|-------------------------|-----------------|------------------------------------------------|
| Ag (NM-300K)| Supplier: | <20 nm       | The majority of particles are spherical, however there are also some triangular or trapezium-like particles | N/A                     | Slow dissolving | Klein et al. 2011; Kermanizadeh et al. 2013a; Donnellan et al. 2016; Peijnenburg et al. 2020 |
|             | TEM:      | 15 nm (99% of particles are <20 nm) | Not agglomerated/aggregated          | *Cannot be measured as supplied as an aqueous suspension |                 |                                                 |
| ZnO (NM-110)| Supplier: | 100 nm       | Heterogeneous shape (e.g. bottle-like, rod-shaped, rectangular) | 13                      | Fast dissolving | Singh et al. 2011; Kermanizadeh et al. 2013a; Tavares et al. 2014; Keller et al. 2020, 2021; Keller Peijnenburg et al. 2020 |
|             | TEM:      | 20-350nm     | Highly agglomerated/aggregated        |                         |                 |                                                 |
|             | XRD:      | 42 nm        |                                        |                         |                 |                                                 |

TEM: transmission electron microscopy; XRD: X-ray diffraction; BET: Brunauer–Emmett–Teller.

Zebrafish adults (Tg(mpx:GFP)i114) were housed at the University of Edinburgh (UK) which maintained a 14:10 h light:dark photoperiod, at an ambient temperature of 28.5 °C (Westerfield 2000). Embryos were collected either by pair mating or by group spawning of adults and placed in 100 mL sterile Petri dishes (Sigma–Aldrich) containing E3 medium supplemented with 1% methylene blue (an antibacterial additive) (ZMS Systems Ltd) and kept at 28.5 °C until they reached 3 days post-fertilization (dpf). Experiments were carried out with larvae (hatched embryos) at 3 dpf. At 5 dpf, all experimental larvae were euthanized using 100% tricaine, in accordance with Schedule 1 methods as described by the Animals (Scientific Procedures) Act 1986 (Home Office 1986).

**Tail fin injury (transection)**

Zebrafish larvae (3 dpf) were anesthetized in 4.2% tricaine (MS-222, Sigma-Aldrich), in E3 medium (adjusted to pH 7) for 15 min. Anesthetized larvae were then placed onto a clean glass microscope slide (Fisher-Scientific, UK) on the stage of a Leica M205 FCA fluorescence stereomicroscope. The larvae were manipulated into a right lateral recumbent position, using a superfine eyelash tool (Agar Scientific, UK). Using a sterile #23 scalpel blade (Swann-Morton, UK), the tail fins of the larvae were transected distally from the tip of the notochord to induce a moderate injury (Figure S1). The larvae were then imaged in a bright field and with an EGFP filter (488 nm), using a Leica DFC7000 T digital color camera connected to the Leica M205 FCA microscope. Larvae were imaged again at 4, 6, 8, 24, and 48 h post-injury.

**Post-injury aqueous exposure to positive controls**

To identify a positive control for the tail fin injury experiments, the neutrophil chemoattractants fMLF, LTB₄, CXCL-8, and C5a and the pro-inflammatory
compound LPS were used. Concentrations of each compound were selected based on information obtained from the published literature; 10, 20, 40, 75, and 100 μg/mL for LPS (Yang et al. 2014), 10 μM of fMLF and C5a (Bernut et al. 2016), 300 nM LTβ4 (Vincent et al. 2017), and 10 nM CXCL-8 (Bernut et al. 2016). All compounds were prepared in an E3 medium.

Following tail fin transection (as previously described), larvae were transferred to a 24-well plate containing the appropriate treatment (2.5 mL/well) and incubated at 28.5°C. Anesthetized larvae were imaged at 0, 4, 6, 8, 24, and 48 h post-injury.

Post-injury aqueous exposure to nanomaterials

Following tail fin transection (as previously described), larvae were immediately transferred to a 24 well plate containing either Ag NMs (0.02, 0.375, 0.75, and 1.5 Ag μg/mL), ZnO NMs (1, 5, and 7.5 Zn μg/mL), AgNO3 (0.02 and 1.5 Ag μg/mL), ZnCl2 (0.5 Zn μg/mL), or E3 medium (negative control) at a volume of 2.5 mL/well and incubated at 28.5°C. Anesthetized larvae were then imaged at 0, 4, 6, 8, 24, and 48 h post-injury. For all experiments, existing knowledge of the LC50 values (concentration required to kill 50% of the animals exposed) for each NM and metal salt informed the selection of doses used (e.g. Massarsky et al. 2013; Muth-Köhne et al. 2013; Osborne et al. 2013; Küçükoğlu, BiNokay, and Pekmezekmek 2013; Choi et al. 2016).

Microinjection of the otic vesicle

Microinjection needles were prepared using glass capillaries (1.0 mm outer diameter × 78 mm inner diameter × 100 mm long) (Harvard Apparatus, UK) that were pulled using a P-97 Flaming/Brown micropipette puller (Sutter Instrument Company, USA). The needle was opened using Dumont #5 fine forceps and then backfilled with ~5 μL of the appropriate treatment using a finely tapered microloader pipette tip (Eppendorf Ltd, UK). The filled needle was secured onto a micromanipulator, which was mounted on a magnetic stand and connected to a PV820 Pneumatic PicoPump microinjector (World Precision Instruments, Europe), which was connected to a compressed air cylinder. The micromanipulator was positioned over the stage of a Leica M205 fluorescence stereo microscope. The bolus size was calibrated to deliver a volume of 0.5 to 1 nL per injection.

Anesthetized larvae were placed into a polydimethylsiloxane (PDMS) microinjection device, manufactured by Dr. Dean Crawford and Dr. Graeme Whyte at Heriot-Watt University, using the master wafer as a mold, which was a kind gift from Dr. Felix Ellett (Ellett and Irimia 2017a, 2017b). The larvae were positioned using a superfine eyelash tool (Agar Scientific) and manipulated into the funnel entrance of the appropriate channel until secured in place in the lateral recumbent orientation, with the left otic vesicle accessible (Figures S2(A,B)). Using the micromanipulator, the tip of the needle was inserted into the otic vesicle and a bolus of the solution was delivered (Figures S2(C,D)). The following treatments were injected into the otic vesicle; LPS (250 μg/mL), fMLF (100 nM), CXCL-8 (100 nM), C5a (100 nM), LTβ4 (1 μM), Ag NMs (1.95, 3.9, and 7.81 Ag μg/mL) or the vehicle control (1% dextran in PBS only). All treatments were prepared in a fluorescently-labeled 1% dextran tracer, in PBS (10 kDa, Alexa Fluor™ 594, 590/617 nm ex/em; Fisher-Scientific). Anesthetized larvae were imaged at 0, 4, 6, 8, 24, and 48 h post-injection.

Image analysis (quantifying the inflammatory response)

All images were analyzed using ImageJ software (Schneider, Rasband, and Eliceiri 2012). For the tail fin injury experiments, a selection area measuring 250 μm in length was applied to the tail fin injury region in ImageJ, so that the right vertical line of the selected area was in line with the transected edge of the tail fin, and all neutrophils within this region were counted manually. For the microinjection images, the otic vesicle was identified by eye and the neutrophils within the vesicle were counted manually using the ImageJ multi-point tool. The neutrophil counts in each image were scored ‘blind’ such that the researcher was not aware of what treatment what being assessed.

Statistical analysis

Unless otherwise stated, each experiment was repeated at least three times, on three separate
occasions. At least 12 larvae were included in each treatment group for each experiment. Data were analyzed using Minitab Statistical Software (Version 17, Pennsylvania, USA). A one-way analysis of variance (ANOVA) was performed, followed by a post-hoc Tukey’s test. A p-value of <0.05 was considered to be significant.

Results

Characterization of nanomaterials

DLS was performed to determine the hydrodynamic diameter (i.e. size), zeta potential (charge), and PdI of the Ag and ZnO NMs in media relevant to this study (Table 2). Ag NMs had a hydrodynamic diameter <62 nm in E3 medium and PBS. ZnO NMs had a hydrodynamic diameter of <883 nm in E3 medium. The zeta potential of Ag and ZnO NMs was <−30 mV, indicating a negative surface charge and a stable dispersion (Bilberg et al. 2012; Brun et al. 2014). Ag NMs were monodispersed, with PdI values of <0.5 at all-time points and in both E3 medium and PBS. The PdI values for ZnO NMs in the E3 medium are >0.5 at 24 and 48 h, suggesting that ZnO NMs are agglomerated.

Post-tail fin injury exposure to chemo-attractants

In control zebrafish (exposed to E3 medium alone), the tail fin injury stimulated a time-dependent increase in neutrophils in the injury site, which peaked at 6 h post-injury and was resolved 48 h post-injury (Figure 1). LPS did not enhance the neutrophil response in the injury site of injured larvae at any time point (data not shown). fMLF caused a significantly higher count of neutrophils at the injury site at 8 and 48 h post-injury when compared to the control (Figure 1(A)). There was also a higher count of neutrophils at the injury site in response to LTB₄ at 24 and 48 h when compared to the control (Figure 1(B)). CXCL-8 caused an increased neutrophil response at the injury site at 6, 8, 24, and 48 h post-injury when compared to the control (Figure 1(C)). There was a higher number of neutrophils at the injury site in response to C5a at 8 h only in comparison to the control (Figure 1(D)). As LTB₄ induced an enhanced and prolonged neutrophil response, it was identified as the most appropriate positive control.

Table 2. Characterization of Ag and ZnO NMs using DLS.

| Dispersant | Time (h) | Hydrodynamic diameter (nm) | Zeta potential (mV) | PdI (units) |
|------------|----------|-----------------------------|---------------------|-------------|
| E3 medium (Ag NMs) | 0        | 60.4 ± 0.6                 | −12.9 ± 0.1         | 0.47 ± 0.02 |
|            | 24       | 55.4 ± 0.6**               | −13.8 ± 0.4         | 0.49 ± 0.05 |
|            | 48       | 52.6 ± 1.4**               | −14.5 ± 0.2**       | 0.48 ± 0.01 |
| E3 medium (ZnO NMs) | 0        | 630.9 ± 15.4               | −15.4 ± 0.2         | 0.34 ± 0.01 |
|            | 24       | 640.5 ± 32.7               | −14.7 ± 0.1**       | 0.51 ± 0.03*|
|            | 48       | 883.5 ± 133.5              | −12.6 ± 0.05***     | 0.66 ± 0.08*|
| PBS (Ag NMs) | 0        | 50.8 ± 1.0                 | −6.8 ± 0.5          | 0.46 ± 0.01|
|            | 24       | 62.8 ± 0.6***              | −10.5 ± 0.4**       | 0.45 ± 0.001|
|            | 48       | 60.3 ± 0.2**               | −10.2 ± 0.5*        | 0.28 ± 0.002***|

NB: ZnO NMs were not characterized in PBS as no microinjection experiments were performed with this material.

Ag and ZnO NMs were dispersed in media relevant to aqueous exposure (E3 medium) or microinjection (PBS) of zebrafish at a concentration of 125 µg/ml and then DLS was performed immediately (0 h), and at 24 and 48 h following incubation at 28.5 °C to assess hydrodynamic diameter, zeta potential, and PdI. Data are shown as mean ± SEM (n = 3). Significance (compared to 0 h) is indicated by *p < 0.05, **p < 0.01, ***p < 0.001.
neutrophil accumulation over time at the injury site following exposure to Ag NMs are presented in Figure S3.

At 4 h post-injury, exposure to ZnO NMs at a concentration of 5 Zn μg/mL resulted in a greater number of neutrophils at the injury site when compared with the untreated injured control (Figure 3). No other changes in the neutrophil response were seen at this time point, or at 6 h post-injury. At 8 h post-injury, exposure to all three concentrations of ZnO NMs (1, 5, and 7.5 Zn μg/mL), and ZnCl₂ (0.5 Zn μg/mL) resulted in a higher number of neutrophils at the injury site in comparison to the untreated injured control (Figures 3(A,B)). At 24 h post-injury, ZnO NMs resulted in a higher number of neutrophils at the injury site at exposure concentrations of 1, 5, and 7.5 Zn μg/mL, in comparison to the untreated injured control (Figures 3(A,B)). At 48 h post-injury, all three concentrations of ZnO NMs (1, 5, and 7.5 Zn μg/mL), and ZnCl₂ (0.5 Zn μg/mL) resulted in higher numbers of neutrophils at the injury site when compared to the untreated injured control (Figure 3(A,B)). Overall, the neutrophil responses observed to ZnO NMs and ZnCl₂ were comparable to those observed in response to the positive control (LTB₄) except for ZnO NMs at a concentration of 7.5 μg/mL which stimulated a significantly higher number of neutrophils at the injury site in comparison to LTB₄ 48 h post-injury. Representative images of neutrophil accumulation over time at the injury site following exposure to ZnO NMs are presented in Figure S4.

Systemic inflammatory responses activated by nanomaterials in injured larvae

Whilst our studies aimed to assess local inflammatory responses at the injury site, we also observed
(via qualitative assessment) that systemic inflammatory responses were activated by the NMs (Figure 4(A–J)). In comparison to the control the abundance of neutrophils present throughout the larval body at 48 h post-injury was higher in response to exposure to Ag (Figure 4(C–F)) and ZnO (Figure 4(G–J)) NMs. The greatest effect was observed for Ag NMs.

**Microinjection of positive controls**

Microinjection of fMLF, LPS, C5a, and LTB₄ did not result in higher numbers of neutrophils in the otic vesicle in comparison to the vehicle control at any of the time points investigated (data not shown). Only CXCL-8 exposure induced higher numbers of neutrophils in the otic vesicle, and at 48 h post-injection only (Figure 5). Therefore, CXCL-8 was selected as the positive control for microinjection studies.

**Microinjection of nanomaterials**

Following microinjection, Ag NMs caused a time-dependent inflammatory response in the otic vesicle that was greatest at 48 h post-injection (Figure 6(A,B)). The neutrophil response stimulated by Ag NMs in the otic vesicle was not different from the vehicle control (0 Ag µg/mL) between 4 and 24 h post-injection at all concentrations tested. In response to microinjection of Ag NMs at a concentration of 1.95 and 3.9 Ag µg/mL, there was a higher neutrophil accumulation in the otic vesicle at 48 h post-exposure in comparison to the vehicle control (Figure 6(A,B)). AgNO₃ at a concentration of 1.95 Ag µg/mL, resulted in greater numbers of...
neutrophils in the otic vesicle at 48 h only, in comparison to the vehicle control (Figure 6(A,B)). Representative images of neutrophil accumulation over time at the injection site (otic vesicle) following exposure to Ag NMs are presented in Figure S5.

Discussion

Our findings demonstrate that inflammatory (neutrophil) temporal responses to NMs can be effectively assessed in non-protected life stages of transgenic zebrafish. Both Ag and ZnO NMs activated a pro-inflammatory response that we evidenced by an enhanced and sustained accumulation of neutrophils at the injury site of injured zebrafish larvae following aqueous exposure. It was shown that Ag NMs stimulated a greater inflammatory response than ZnO NMs, and thus Ag NMs were prioritized for experiments assessing inflammatory responses following microinjection. Ag NMs also stimulated a small infiltration of neutrophils into the otic vesicle following microinjection, further illustrating the capacity for Ag NMs to stimulate a pro-inflammatory response. These findings thus provide the nanotoxicology community with an alternative approach for investigating NMs for inflammatory responses. More specifically, we suggest that non-protected life stages of transgenic zebrafish larvae are used to reduce the current reliance placed on rodent testing.

Characterization of nanomaterials

Ag and ZnO NMs were obtained from the JRC nanomaterial repository which supplies reference (benchmark) materials for use in nanotoxicology studies. As a consequence, the physico-chemical properties of these NMs have been extensively characterized previously (Table 1). We, therefore, performed limited characterization of the NMs in biological media relevant to our study. Previous studies using the same Ag NMs tested in this study have shown a primary particle size of ~15 to 20 nm (Klein et al. 2011). In media relevant to this study the hydrodynamic diameter of Ag NMs was...
~50–60 nm, which suggests that there is limited agglomeration, which aligns with other findings in the published literature (e.g. Bilberg et al. 2012; Johnston et al. 2015; Budama-Kilinc et al. 2018; Lebedová et al. 2018). The diameter of the ZnO NMs used in this study was more heterogeneous and the primary particle size reported previously varies depending on the method used to quantify particle size (e.g. XRD vs TEM, Table 1) (Singh et al. 2011; Keller et al. 2021). We observed that the NM suspensions had a hydrodynamic diameter >600 nm, which suggests the ZnO NMs agglomerated in the exposure medium, and this is consistent with previous reports in the published literature (e.g. Donnellan et al. 2016; El Yamani et al. 2017; Da Silva et al. 2019). The lower tendency of Ag NMs to agglomerate could help explain, at least in part, why they were more toxic than ZnO NMs in our study. Future studies could therefore test a wider panel of NMs with more varied physico-chemical characteristics to identify which properties confer toxicity.

Assessment of dissolution of metallic NMs is common in nanotoxicology studies to identify whether any toxic effects are driven by ions, particles, or both. The dissolution of the NMs we tested has been reported previously (e.g. Bar-llan et al. 2009; Massarsky et al. 2013; Osborne et al. 2013; van Aerle et al. 2013; Brun et al. 2014; Odzak et al. 2014; Wehmas et al. 2015; Gao et al. 2016; Keller et al. 2020, 2021; Peijnenburg et al. 2020) and it has been consistently demonstrated that ZnO NMs dissolve quickly, whereas Ag NMs are more slowly dissolving (Table 1). We included ionic controls (e.g. AgNO₃ and ZnCl₂) in all of our experiments to assess the contribution of metal ions to the observed responses (see below).

Post-tail fin injury exposure to negative and positive controls

The findings from the tail injured larvae without NM treatment show that the neutrophil response to the injury has been initiated by 4 h post-injury and
is resolved by 48 h post-injury, with the peak of the inflammatory response to an injury occurring at 6 h. These findings are consistent with those of previous studies (Renshaw et al. 2006; Loynes et al. 2010; Elks et al. 2011; Gray et al. 2011; Ellett et al. 2015; Ren et al. 2015a, 2015b).

There has been a lack of use of positive controls in published studies investigating inflammatory responses in transgenic zebrafish. The inclusion of such positive controls is important to allow the sensitivity and reproducibility of studies to be assessed, as well as to allow benchmarking of substances of better-understood inflammogenicity. We found enhanced and prolonged neutrophil responses to fMLF, CXCL-8, and LTB₄ which corresponded with existing findings (Elks et al. 2011; LTB₄. Yoo et al. 2011; Deng et al. 2013; Vincent et al. 2017). Interestingly, neutrophil recruitment in zebrafish larvae is dependent on zebrafish CXCL-8 homologs (de Oliveira et al. 2013). C5a is known to be a potent chemoattractant for neutrophils in humans (Metzemaekers, Gouwy, and Proost 2020), but in this study exposure of zebrafish larvae to C5a only resulted in higher levels of neutrophil accumulation at 8 h post-injury, after which the neutrophil response to C5a rapidly resolved. We did not observe an increase in neutrophil accumulation at the injury site following exposure to LPS which is in contrast to previous studies where LPS enhanced the neutrophil response to tail fin injury in zebrafish larvae or stimulated a neutrophil response in zebrafish in immersion studies without a tail fin injury (Cordero-Maldonado et al. 2013; Philip et al. 2017). LPS toxicity has been shown to vary between bacterial sources and extraction methods, as well as between batches of the same LPS (Bates et al. 2007; Mottaz et al. 2017). The LPS obtained in our study was sourced from *Escherichia coli*, however other studies have used LPS sourced from *Salmonella typhosa*, *Aeromonas hydrophila*, and *Pseudomonas aeruginosa*. Therefore, future studies might seek to compare the inflammatory effects of LPS obtained from different sources on inflammatory responses in zebrafish. In addition, zebrafish are not maintained in a sterile environment, which might also explain why LPS was not effective at eliciting an inflammatory response.

LTB₄ and CXCL-8 were thus identified as the best candidates for use as a positive control in zebrafish larvae as they enhanced and prolonged the neutrophil response at the injury site, with LTB₄ producing more reliable and reproducible results. Interestingly, however, there is evidence that exposure to a mixture of chemoattractants, e.g. CXCL-8 and LTB₄, may be most effective in inducing an enhanced neutrophil response (Subramanian, Moissoglu, and Parent 2018), and therefore a co-exposure to chemoattractants might be usefully investigated in the tail fin injury assay in future.

**Post-tail fin injury exposure to nanomaterials**

We demonstrated that Ag and ZnO NMs enhanced and prolonged the neutrophil response at the injury site in injured zebrafish following aqueous exposure to these materials. Existing *in vitro* and *in vivo* (rodent) studies have also demonstrated that Ag NMs activate pro-inflammatory responses (Carlson et al. 2008; Foldbjerg, Dang, and Autrup 2011; Kermanizadeh et al. 2012; Gaiser et al. 2013; Smulders et al. 2014; Awasthi et al. 2015; Johnston et al. 2015; Patlolla, Hackett, and Tchounwou 2015; Smulders et al. 2015). Similarly, *in vitro* and *in vivo* (rodent) studies have shown that ZnO NMs stimulate neutrophil-driven inflammatory responses (Cho et al. 2012; Kermanizadeh et al. 2013b; Adamcakova-Dodd et al. 2014; Sha et al. 2014; Abbasalipourkabir et al. 2015; Johnston et al. 2015; Almansour et al. 2017). Whilst ZnO NMs enhanced the neutrophil response at the injury site, the effect was not as marked as that of the Ag NMs, with fewer neutrophils observed in response to ZnO NM treatment. This is supported by existing studies that have shown that Ag NMs are typically more toxic and inflammogenic than ZnO NMs (e.g. Bondarenko et al. 2013; Johnston et al. 2015; Verdon et al. 2021). Following exposure of injured zebrafish to Ag and ZnO NMs, the inflammatory response was not resolved within 48 h. As our experiments were focused on using non-protected life stages of zebrafish, we did not investigate what impact Ag and ZnO NMs had on the inflammatory response after 48 h, but this could be investigated in future studies to more fully assess the dynamics of the inflammatory response and how it resolves over time.

A common consideration in existing *in vitro* and *in vivo* nanotoxicology studies is whether ions released from NMs contribute to their toxicity.
Interestingly, whilst Ag NMs stimulated a neutrophil response, there were no significant increases in neutrophils at the injury site for AgNO₃. Previous studies have demonstrated that ionic forms of silver are more toxic than Ag NMs. For example, reported LC₅₀ values for zebrafish (concentration required to kill 50% of the animals exposed) of between 0.07 and 0.1 Ag µg/mL for ionic silver (in the form of AgNO₃), whereas for Ag NMs the LC₅₀ values in these studies were reported to be much higher measuring between 0.5 and 1.79 µg/mL (Massarsky et al. 2013; Muth-Köhne et al. 2013; Osborne et al. 2013). Whilst Ag NMs stimulated an inflammatory response at concentrations >0.375 Ag µg/mL, the high toxicity (mortality) of AgNO₃ (data not shown) meant that the maximal concentration of AgNO₃ that could be tested was 0.02 Ag µg/mL. Several studies have reported that Ag⁺ dissolution from the (NM300) Ag NMs suspended in zebrafish medium ranges from 0.1 to 2% over a 48 to 72 h period (Bar-Ilan et al. 2009; Massarsky et al. 2013; Osborne et al. 2013; van Aerle et al. 2013; Gao et al. 2016). As the Ag NMs are of low solubility, it may be that the inflammatory response activated by Ag NMs is driven by particles and/or that the particles more effectively deliver ions to the target tissue.

Zinc ions are highly toxic to zebrafish, with LC₅₀ values of between 1.3 and 1.7 µg/mL (Küçükoğlu, BiNokay, and Pekmezekmek 2013; Choi et al. 2016). The ZnO NMs tested in this study have been shown to rapidly dissolve, with several studies reporting up to ~80% dissolution of these NMs over a period of 3–120 h (Brun et al. 2014; Odzak et al. 2014; Wehmas et al. 2015). Thus, it is likely that ion release from ZnO NMs contributes to the observed response.

**Systemic inflammatory responses activated by NMs in injured zebrafish**

Whilst we focused on quantifying responses at the injury site, we observed systemic inflammatory responses activated by exposure to both Ag and ZnO NMs. This may be due to an increase in hematopoiesis. For example, Hall et al. (2012) and Hou et al. (2016) demonstrated that bacterial infections can cause an expansion in neutrophil numbers (granulopoiesis) in zebrafish due to stimulation of hematopoietic stem cells and increased progenitor cell differentiation. Alternatively, NMs may delay neutrophil apoptosis to prolong the inflammatory response. For example, Goncalves and Girard (2014) demonstrated that ZnO NMs can delay human neutrophil apoptosis in vitro. Further studies would be needed to test these hypotheses. However, it is advised that future studies assess not only local responses in the injury site but explore and quantify the systemic inflammatory response that is activated in injured zebrafish larvae.

**Microinjection of chemoattractants**

CXCL-8 stimulated an inflammatory response following microinjection into the otic vesicle in our study, as has been shown previously (Yang et al. 2012; de Oliveira et al. 2013; Deng et al. 2013; Powell et al. 2017; Torraca et al. 2017) and was therefore identified as an effective positive control for studies investigating inflammatory responses in the otic vesicle following microinjection.

Following the microinjection of LPS, LTB₄, C5a, and fMLF, there were no increases in neutrophils in the otic vesicle of zebrafish. This finding contrasts with previous studies that have shown microinjection of LPS (into various target sites, such as the swim bladder and the yolk sac), LTB₄ (into the otic vesicle), and fMLF (into the hindbrain ventricle and the otic vesicle) elicited inflammatory responses in zebrafish larvae (Deng, Harvie, and Huttenlocher 2012; Yang et al. 2012; de Oliveira et al. 2013; Deng et al. 2013; Yang et al. 2014; Torraca et al. 2015; Zhang et al. 2016; Yang et al. 2017; Kim et al. 2019). Only a limited number of concentrations of each chemoattractant were tested in the microinjection studies, however, as they were more technically challenging and time-consuming to perform, which limits the number of treatment groups included in each experiment. Future studies should therefore assess a wider range of concentrations of these chemoattractants to identify whether they can be used as a positive control for microinjection studies.

**Microinjection of nanomaterials**

Ag NMs were prioritized for the microinjection experiments as we had shown that they stimulated a greater inflammatory response following aqueous exposure, together with extensive evidence in the
published literature indicating that Ag NMs are more inflammogenic than ZnO NMs. We showed the capacity for Ag NMs to stimulate a neutrophil response following microinjection into the otic vesicle. Microinjection of NMs into the otic vesicle may therefore be a suitable exposure route to assess inflammatory responses in zebrafish. Other microinjection sites have been used in the published literature to investigate inflammatory responses to other stimuli (e.g., bacterial or fungal infection), such as the duct of Cuvier, caudal artery, yolk sac, swim bladder, hindbrain ventricle, and the tail muscle (Benard et al. 2012; Li and Hu 2012; Yang et al. 2014; Liang et al. 2016; Zhang et al. 2016; Díaz-Pascual et al. 2017; Varas et al. 2017). Whether these injection sites offer a more effective alternative to the otic vesicle would be worthwhile to investigate in the future to assess the advantages and limitations of different exposure sites and to study the response of different locations. However, not all of these sites will be feasible in the non-protected early life stages of zebrafish as they are not all fully developed. For example, the swim bladder is not fully developed until 4–5 dpf and thus investigation of NM-mediated inflammatory responses in the swim bladder would require the use of protected life stages of zebrafish with more ethical implications associated with this research.

**Conclusion**

We demonstrated that aqueous exposure of injured zebrafish to neutrophil chemoattractants (fMLF, LTB₄, and CXCL-8), Ag NMs, and ZnO NMs enhanced and prolonged the neutrophil response at the injury site. Furthermore, Ag NMs and the neutrophil chemokine CXCL-8 stimulated a small neutrophil response following microinjection into the otic vesicle. LTB₄ was identified as the most suitable positive control for studies investigating inflammatory responses in injured zebrafish following aqueous exposure, and CXCL-8 for microinjection studies that assess responses in the otic vesicle. Our findings align with the existing in vitro and in vivo (rodent) literature which shows that Ag and ZnO NMs are highly effective in eliciting pro-inflammatory responses. Thus, non-protected life stages of transgenic zebrafish can be effectively harnessed to investigate the inflammatory effects of NMs more widely. It may be prudent to prioritize the use of the injury model as it is less technically challenging and requires less technical skill or specialized equipment compared with microinjection of the otic vesicle, and as the levels of neutrophil accumulation are greater in the injury model. The methods applied here might also be usefully adopted with other zebrafish reporter lines for assessing immunological responses to NM exposure. As an example, Tg (mpeg1:mCherry/mpx:EGFP) zebrafish (with mCherry-labeled macrophages and GFP-labeled neutrophils) would allow for simultaneous investigation into both neutrophil and macrophage responses (Davis et al. 2016; Barros-Becker et al. 2017; Ellett et al. 2018; Bos et al. 2019). The use of transgenic zebrafish to monitor inflammatory responses to NMs is still in its infancy and the use of a wider range of transgenic strains and consideration of other biomarkers of inflammatory and oxidative stress responses (e.g., cytokine expression) in the future could further probe for the mechanism(s) of NM toxicity. In addition, future studies could consider investigating responses at other target sites in zebrafish (e.g., gills, intestine, swim bladder, hindbrain ventricle), following administration via different routes. Furthermore, combining these transgenic models with assessments of the uptake and translocation of the NMs (via techniques, such as electron microscopy or Coherent anti-Stokes Raman scattering (CARS) microscopy (e.g. Osborne et al. 2013)) would allow for a better understanding of the fate of the NMs in the exposed zebrafish larvae and the wider, systemic, immunological (e.g., neutrophil) responses seen in our studies.

The number of neutrophils that accumulated at the injury or injection site was often low, particularly following microinjection into the otic vesicle, which might be regarded as a limitation of using this model. Our study used non-protected life stages of zebrafish which restricted the length of exposures that were used. In our analyses, we observed that inflammatory responses to NMs following aqueous exposure of injured zebrafish, and following microinjection into the otic vesicle often peaked at 48h post-exposure. Future studies might usefully consider monitoring the inflammatory response to NMs over a longer time period to capture the activation and resolution (or lack thereof)
of inflammatory responses (albeit this would require the use of protecting fish life stages).

To conclude, the use of selected lines of transgenic zebrafish larvae can help to better understand pro-inflammatory responses to NMs and provide us with considerable potential for reducing our reliance on in vivo rodent studies, and in turn better align nanotoxicology with the 3Rs principles, to support the sustainable and responsible exploitation of nanotechnology. We also advocate the more widespread use of transgenic zebrafish to screen the toxicity of other compounds (e.g. pharmaceuticals) and pathogens as an alternative to rodents.

Notes

1. Pigmentation begins to develop from 1 dpf, however it is not fully developed until beyond 14 dpf (Rawls, Mellgren, and Johnson 2001).

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