Research Article

Toxicity of polyelectrolyte-functionalized titania nanoparticles in zebrafish (*Danio rerio*) embryos

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Abstract

We investigated the effects of short-term exposure of bare TiO_2NPs and polyelectrolyte-coated TiO_2NPs in the 5–25 nm size range, at relatively high concentrations (of 500 and 1000 mg/L) under light or dark conditions, in *D. rerio* embryos. The biological endpoints investigated included embryo viability and mRNA transcript levels of antioxidant and membrane transport genes relative to control embryos. The presence of nanoparticles on the surface of embryos was assessed using TEM. The results confirm an accumulation of TiO_2NPs on the outer surface (chorion) of the embryo, but not within the embryo. No significant difference in embryo viability was detected following each exposure regime. The expression of antioxidant biomarker, *SOD2,* was significantly impacted by the type of TiO_2NP, with TiO_2NPs/PSS/PAH coating exposure showing down regulation; the concentration of the nanoparticles, with down regulation at 500 mg/L; and dark/light condition with down regulation in the light. The expression levels of the hypoxia and membrane markers, *HIF1* and *Pxmp2,* were not significantly impacted by any factor. The study indicates that *SOD2* mRNA expression levels may be useful in the detection of apparent oxidative stress induced by the titania nanoparticle build up on the embryo chorion surface.

Keywords Titania nanoparticles · Zebrafish · Embryos · Oxidative stress

1 Introduction

One of the most widely used engineered nanoparticles (NPs) worldwide is nano-sized titanium dioxide NPs (TiO_2NPs) that is used for its photocatalytic properties [1]. TiO_2NPs are used in sunscreens and cosmetic creams [2] for their ability to block UV light [3]. Titania is also used as a pigment in toothpaste [4], in skin treatments [5], and paints, as well as food industry applications [6, 7]. They have further wide-ranging uses from photosensitizing agents for photodynamic therapy of endobronchial and esophageal cancers [8], as disinfection agents in wastewater treatment [9], as well as in the environmental decontamination of soil, air, and water [10, 11].

TiO_2NPs may be toxic when released into the aquatic environment as they form superoxide and hydroxyl radicals on exposure to sunlight (UV) and oxygen, which could then lead to damage of the cell contents if taken up by organisms [12]. TiO_2NPs have been categorised into different forms: rutile, anatase, and amorphous [13] whereby the anatase forms are generally found to be more photoactive than rutile TiO_2NPs. Al-Awady et al. [14] reported a

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significant nanotoxicity of TiO_2NPs against algae and yeast, which depends on the particle surface charge. Nanoparticles with cationic surfaces are generally found to be more toxic than those with anionic surfaces [15]. Several sunscreen products contain anatase TiO_2NPs, which has been found to generate reactive oxygen species (ROS) upon illumination with UV light [16]; this potentially leads to biological damage [17]. Previous studies have shown that TiO_2NPs (at an exposure level of 5 mg/L) induce oxidative stress in the liver of zebrafish [18] and in the brain tissue of rainbow trout (*Oncorhynchus mykiss*) [19]. In contrast, the potential of TiO_2NPs to produce oxidative damage to DNA without photoactivation is still unclear [20].

TiO_2NP toxicity has been shown to be dependent on both particle size and the degree of particle aggregation. Smaller nanoparticles have been found to have higher mobility between biological compartments. For example, the 4 nm TiO_2NPs have been observed penetrating into the deeper layer of the epidermis (to the basal cell layer) in hairless mice [21]. In contrast though, TiO_2NP aggregation, increasing the size factor, has been shown to have a larger effect on cell viability and gene expression of biomarkers focused on stress, inflammation, and cytotoxicity in human acute monocytic leukemia and bronchial epithelial cell lines when compared with smaller aggregates of 166 nm [22]. Encapsulating the TiO_2NPs with coating agents (such as inert oxides of silica, alumina, or zirconium) also mediates the ROS associated impacts [23] by reducing or eliminating them. Coatings based on silicon dioxide have also been found to reduce the oxidative activity of TiO_2NPs on pig skin [24].

Overall, the increased use of TiO_2NPs, and their ultimate release into the environment, suggests an increasing need to evaluate their potential toxicity, including the effect of the nanoparticle coating and size range. In this study, *D. rerio* embryos were used to study the effect of TiO_2NPs coated with different number of layers of anionic and cationic polyelectrolytes on embryo viability. Selected oxidative stress markers, which are conserved in all vertebrate species, were also measured including *superoxide dismutase 2* (*SOD2*), *hypoxia inducible factor 1* (*HIF1*) and peroxisomal membrane transporter protein (*Pxmp2)* gene expressions.

2 Materials and methods

2.1 Preparation and characterisation of TiO_2NPs

Three different batches of TiO_2NPs were prepared in Milli-Q water: bare titania NPs (TiO_2NPs), anionic NPs prepared with poly (sodium 4-styrene sulfonate) sodium salt (PSS) as TiO_2NPs/PSS, and the cationic NPs prepared with poly (allylamine hydrochloride) (PAH) as TiO_2NPs/PSS/PAH. The synthesis of TiO_2NPs was conducted using the procedure described by Al-Awady et al. [14]—see Fig. 1a. Briefly, 1 M

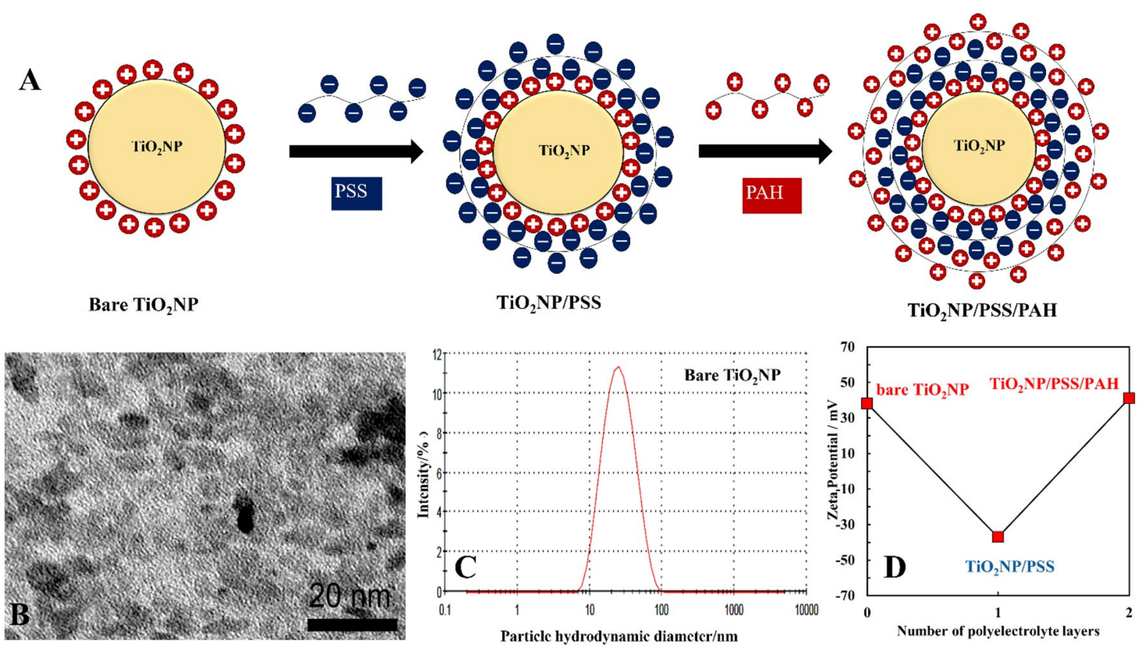

Fig. 1 **a** Schematic diagram of the coating of the bare TiO_2NP with polyelectrolytes (PSS and PAH), **b** TEM image of the titania produced by annealing at 70 °C, **c** Size distribution of dispersed titanium dioxide NPs synthesised by hydrolysis and condensation of titanium isopropoxide at acidic medium for 20 h at 70 °C. **d** Zeta-potential of the bare TiO_2NP, TiO_2NP/PSS and TiO_2NP/PSS/PAH at pH 5.5

HNO_3 was added drop-wise to 250 mL of Milli-Q water to adjust the pH to 2 followed by dropwise addition of a mixture consisting of 15 mL aliquot of isopropanol and 5.0 mL of titanium isopropoxide (TTIP) to the former solution with vigorous stirring, leading to the formation of a white turbid suspension as a result of the hydrolysis of TTIP. The suspension of Ti $(OH)_4$ was heated to 70 °C for 20 h to form a yellow-white precipitate of titania that was filtered, washed with ethanol and further dried under vacuum (Gallenkamp vacuum oven) at 100 °C for 2 h. Aqueous dispersions of TiO_2NPs was prepared by dispersing 4 mg of the titania sample in 10 mL aliquots of 20 mM aqueous solution of NaCl at pH 4 using a digital sonicator (Branson 450, 5 mm tip, 400 W maximal power) at 40% of the maximum power for 10 min at 1 s ON/1 s OFF pulse time and followed by filtration through a syringe filter of pore size 0.22 µm. The TiO_2NPs were characterised in terms of size distribution and zeta potential in aqueous solutions using a Zetasizer Nano ZL (Malvern, U.K.). Transmission electron microscopy (TEM) images of the particle samples were obtained using JEM 2011 (JEOL, Japan) running at 200 kV.

2.2 Layer-by-layer polyelectrolyte-coated TiO_2NPs

Polyelectrolyte-coated TiO_2NPs were prepared using titania synthesised and annealed at 100 °C (anatase). 10 mL of 1500 µg mL^{-1} TiO_2NPs dispersion in Milli-Q water was added drop-wise to an equal amount of 10 mg mL^{-1} of solution of PSS (M.W. ~ 70 kDa) dissolved in 1 mM NaCl solution. After shaking for 20 min, the particles were washed three times by centrifugation for 1 h at 8000 rpm to remove the excess of PSS and were finally re-dispersed in 10 mL of Milli-Q water. The PSS-coated TiO_2NPs were then mixed drop-wise with 10 mL of 10 mg mL^{-1} PAH (M.W. 15 kDa) dissolved in 1 mM NaCl solution, shaken for 20 min and centrifuged again three times at 8000 rpm for 1 h to yield TiO_2NPs/PSS/PAH. For further coating with PSS, the latter was mixed drop-wise with 10 mL of 10 mg mL^{-1} PSS whilst being sonicated. The mixture was shaken for 20 min, centrifuged and dispersed in Milli-Q water to produce TiO_2NP/PSS/PAH/PSS. Furthermore, PSS and PAH of various molar masses (10 kDa and 70 kDa for PSS and 15 kDa and 56 kDa for PAH) were used to examine their effect on the size of the coated TiO_2NPs. After each polyelectrolyte coating, the TiO_2NPs were characterized by the Zetasizer Nano ZL to check their zeta potential and the particle aggregation.

2.3 Embryo exposure to TiO_2NPs

Healthy *D. rerio* embryos (n = 10) at 0–72 hpf, with the chorion intact, were selected and exposed to a treatment dose of test media (bare TiO_2NPs, TiO_2NPs/PSS, or TiO_2NPs/PSS/PAH) and incubated for 3 h in either dark conditions or illuminated with visible light, at particle concentrations of 0, 500 or 1000 mg/L based on published LC_{50} values to increase the likelihood of observable effects [25, 26] rather than environmentally-relevant levels. Healthy *D. rerio* embryos (n = 10) were used as a control group in parallel.

2.4 Embryo viability following exposure to TiO_2NPs

Embryos (at 48–72 hpf, n = 5) from each exposure regime (control, bare TiO_2NPs, TiO_2NPs/PSS, and TiO_2NPs/PSS/PAH) were isolated after the exposure and washed with commercially supplied (nuclease free) molecular-biology grade water (Fisher Scientific, U.K.) three times, and re-dispersed with 1 mL molecular grade water and incubated with a drop of 98% fluorescein diacetate (FDA) (Honeywell Fluka, U.K.) in acetone (0.5 mg/L) for 15 min. This assay is based on accumulation of the fluorescent by-product (fluorescein) inside the viable embryos as a result of the hydrolysis of the diffused FDA by intracellular enzymes (esterases). The embryos were then washed again with deionised water and the cell viability examined using an Olympus BX51 fluorescence microscope attached to a DP70 digital camera and FITC fluorescence filter set. Living cells were identified as having taken up FDA and fluorescent green [27].

2.5 TEM imaging of embryos after exposure to TiO_2NPs

The morphology of *D. rerio* embryos (n = 5) after a 3 h incubation with 0, 500 or 1000 mg/L of bare TiO_2NPs TiO_2NPs/PSS, or TiO_2NPs/PSS/PAH was examined with TEM using the following protocol. The embryos were washed with deionised water and fixed in 2.5% glutaraldehyde (0.5 ml 25% glutaraldehyde stock solution, 4.5 ml 0.1 M cacodylate buffer and glucose (20 mL 0.2 M cacodylate stock, 10 mL Milli-Q water, 0.216 g glucose, pH 7.3, and final volume made up to 40 mL) for 1 h at room temperature. Next, cacodylate buffer was removed and embryos were fixed by 1% osmium tetra-oxide in cacodylate buffer (2.5 mL 2% Osmium tetroxide, 2.5 mL 0.1 M cacodylate buffer and glucose 0.03 M) at 4 °C overnight. After the cacodylate buffer was removed, embryos were stained for 30 min with 1% uranyl acetate (2 ml 2.5% uranyl acetate stock, final volume 3 mL) and washed with solutions of ethanol of increasing concentration (30%, 50%, and 70% overnight). The embryos were washed again the next day with ethanol solutions of 90% and 100%. After standard dehydration, the embryos were embedded in fresh epoxy/araldite at 60 °C for 48 h. The embedded embryo samples were removed from the oven and allowed to stand at room temperature for 48 h, then sectioned using an ultramicrotome.

The Oxford Instruments INCA Energy Dispersive Spectroscopy (EDS) was attached to the TEM and run at 120 kV to identify and semi-quantitatively characterize the TiO_2NPs on the surface, or within, of the *D. rerio* embryo samples. The sectioned samples were imaged using a JEOL 2010 TEM (Japan) operating at 80 kV and images were captured (from one randomly selected embryo per treatment group) with a Gatan Ultrascan 4000 digital camera (Gatan, Pleasanton, U.S.A.) and the corresponding software for imaging was the Digital Micrograph.

2.6 Target gene isolation and characterization

Total RNA was extracted from pooled samples of embryos (0–72 hpf with chorion intact, n = 10) from each treatment group, using the manufacturer's protocol (Roche Diagnostics Ltd., Burgess Hill, U.K.). The embryo pooled sample exposures consisted of bare TiO_2NPs, TiO_2NPs/PSS, or TiO_2NPs/PSS/PAH at 500 or 1000 mg/L particle concentration for 3 h exposure duration, in dark or illuminated with visible light as well as the corresponding control treatment group (n = 10). To assess the integrity of total RNA, samples were analysed on a denaturing agarose gel stained with ethidium bromide (Life Technologies, Paisley, U.K.). 100 ng of pooled RNA was used to generate cDNA using SuperScript VILO cDNA Synthesis reagents and protocol (Life Technologies, Paisley, U.K.) with 14 µL (~ 100 ng) of total RNA. In a 0.2 mL tube, the following reagents were added: 4 µL of 5x VILO Reaction Mix (includes random primers, $MgCl_2$, and dNTPs in a buffer formulation) 2 µL of 10x Superscript enzyme mix. Each reaction was incubated at 25 °C for 10 min, and then 60 min at 42 °C followed by 5 min at 85 °C and a holding step at 4 °C. To degrade any remaining RNA, the following reagents were added: 0.5 µL (5 units) of RNase H (supplied in 100 mM KCl, 20 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 0.1 mM EDTA, 0.1 mM dithiothreitol and 50% glycerol) and 2 µL of 10x RNase H Reaction Buffer (includes 75 mM KCl, 50 mM Tris–HCl, 3 mM $MgCl_2$, 10 mM $MgCl_2$ in pH 8.3 at 25 °C). All reagents were mixed, incubated at 37 °C for 45 min and then stored at − 20 °C.

For the generation of *SOD2*, *HIF1*, and *Pxmp2* PCR products, 1 µL of cDNA was combined with 0.5 µl of 10 mM dNTPs, 5 µL amplification buffer, 0.5 µL of 0.5–4.5 mM $MgCl_2$, 0.5 µL of 1.5 µM for each sense and antisense primers (Table S-1) and 0.25 µL (1.25 units) of Herculase II fusion DNA polymerase (Agilent Technologies, Wokingham, U.K.) for a total reaction volume of 25 µL. *Elongation factor 1* (*EF*), *18S rRNA* (*18S*) and *β tubulin* were evaluated as potential reference genes. Amplifications were carried out using the TC-4000 Thermal Cycler (Techne, Staffordshire, U.K.) equipped with a heated lid. All reactions were initially denatured at 94 °C for 30 s then cycled 35 times with 30 s at 94 °C denaturation, 30 s at 50/55/60 °C annealing and 30 s at 72 °C for the elongation step. A final extension step of 2 min at 72 °C was conducted. The PCR fragments were sequenced commercially by Macrogen (Amsterdam, Netherlands). Identities of PCR fragments were verified using a blastn search on the NCBI database (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and aligned using a multiple sequence alignment program, Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/) to determine the correct isoform.

2.7 Quantitative qPCR analysis of mRNA expression

The qPCRs analyses for each pool of embryo cDNAs from each treatment group (n = 10) were carried out using 20 µL reaction volumes consisting of 10 µL of SYBR Green Master Mix (Roche, U.K.), 7 µL of sterilised water, 1 µL of the cDNA template, and 2 µL of optimised primer concentration (*EF*, *HIF1*: 200 nM; *18S*, *Pxmp2*: 300 nM, *SOD2*: 400 nM). Two reference genes (*EF* and *18S*) were determined as the most stable across treatment groups using geNorm software. Amplifications were carried out using a CFX96 Real-time PCR system, C1000 Thermal Cycler (Bio-Rad, Hemel Hempstead, U.K.), in triplicate and with negative controls. Reactions were started with denaturation at 50 °C for 2 min, 95 °C for 10 min, followed by a three-step protocol of 40 cycles of denaturation at 95 °C for 10 s, annealing at 60 °C for 1 min, then 72 °C for 1 min. At the end, a melting/dissociation curve was conducted. A relative quantification method was used to determine changes in mRNA transcript levels of the targeted genes in the treatment group compared to untreated control samples using the geometric mean of the reference genes for normalization and the ΔΔCt method [28].

2.8 Statistical analysis

Each target gene was tested individually for significant differences among the controls and each treatment group. All data were tested for homogeneity of variances using Levene's test in SPSS. A non-parametric test (Scheirer-Ray-Hare) was used to assess the effect of anatase TiO_2NPs coating type (factor 1), TiO_2NP concentration (factor 2) and the exposure condition (factor 3) and to determine the interactions among them. Significance for relative gene expression, between TiO_2NP of different coatings, concentrations, or conditions was also tested individually using the Kruskal–Wallis non-parametric test. Differences were considered significant at $P < 0.05$.

3 Results

3.1 Characterization of the TiO_2NPs size distribution and zeta potential

Aqueous dispersions of the titania samples were synthesized at different annealing temperatures and prepared by sonication as described by Al-Awady et al. [14]. Figure 1a illustrates schematically the process of coating of the bare TiO_2NPs with consecutive layers of PSS and PAH respectively. Figure 1b shows a typical TEM image of the bare TiO_2NPs used in these experiments. The titania produced was characterized as clusters of smaller crystallites of 5 nm domain size in solid state (Fig. 1b). Upon dispersing in Milli-Q water at pH 5.5, TiO_2NPs of an average diameter 25 nm (Fig. 1c) were produced. The zeta potential of the anatase TiO_2NPs in an aqueous solution decreased gradually from positive at low pH to negative at high pH with an isoelectric point at approximately 6.8 (see Fig. 1d).

3.2 TiO_2NP uptake and impact on *D. rerio* embryo viability

Embryos displayed no significant impact on viability (Fig. 2) when exposed to all the TiO_2NPs types (bare TiO_2NPs, TiO_2NPs/PSS, or TiO_2NPs/PSS/PAH) at each exposure level (0, 500 and 1000 mg/L), in both dark and visible light conditions. *D. rerio* embryos from each of the treatment group were examined using EDS attached to TEM (Fig. 3, Table 1). EDS spectra confirmed the presence of TiO_2NPs on the outer surface (chorion) of *D. rerio* embryo incubated with 500 mg/L of TiO_2NPs/PSS under both dark and visible light conditions (Fig. 3b, c). In the samples examined using TEM, no TiO_2NPs were detected inside of the embryos for any treatment or on the embryo outer surface (chorion) in the control group (Fig. 3a) or after incubation with 500 mg/L of TiO_2NPs/PSS/PAH under both dark and visible light conditions (Fig. 3d, e). TiO_2NPs were however detected on the outer surface (chorion) of embryos incubated with 1000 mg/L TiO_2NPs/PSS/PAH incubated under dark conditions (Fig. 3f). No data was available (due to human error) for embryos incubated with 1000 mg/L TiO_2NPs/PSS/PAH incubated under light conditions.

3.3 qPCR analysis of target gene expression in *D. rerio* embryos following exposure to TiO_2NPs

The expression levels of *SOD2*, *HIF1*, and *Pxmp2* mRNA were analysed in control embryos and embryos pooled from each treatment group (n = 10) of TiO_2NPs with concentrations of 0, 500 and 1000 mg/L in dark and visible light conditions using the optimised qPCR method. Firstly, an overall statistical analysis using the Scheirer-Ray-Hare test (Table 2) showed that for the nanoparticles the TiO_2NP type, concentration, condition, the

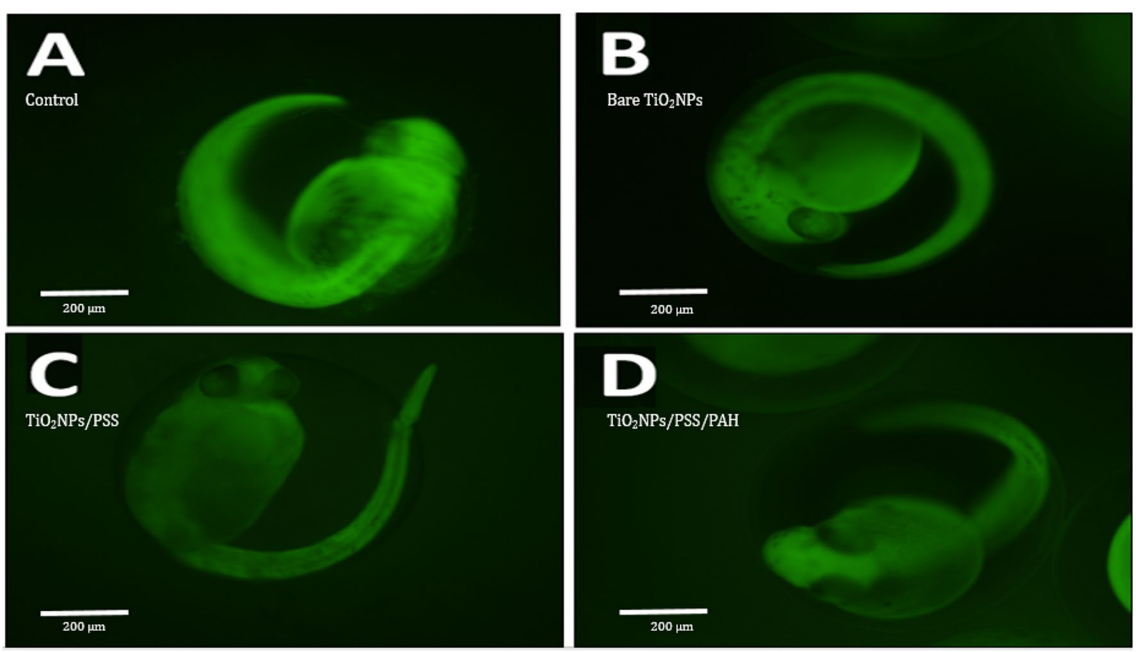

Fig. 2 FDA live/dead assay applied to *D. rerio* embryos (at 48–72 hpf, n = 5) exposed to different types of TiO_2NPs samples at total concentrations of 1000 mg/L for each media and under visible light conditions: **a** Control, **b** bare TiO_2NPs, **c** TiO_2NPs/PSS, **d** TiO_2NPs/PSS/PAH. Scale bars are 200 µm (**a**–**d**). The fluorescence signal indicates that the embryos are still viable after exposure

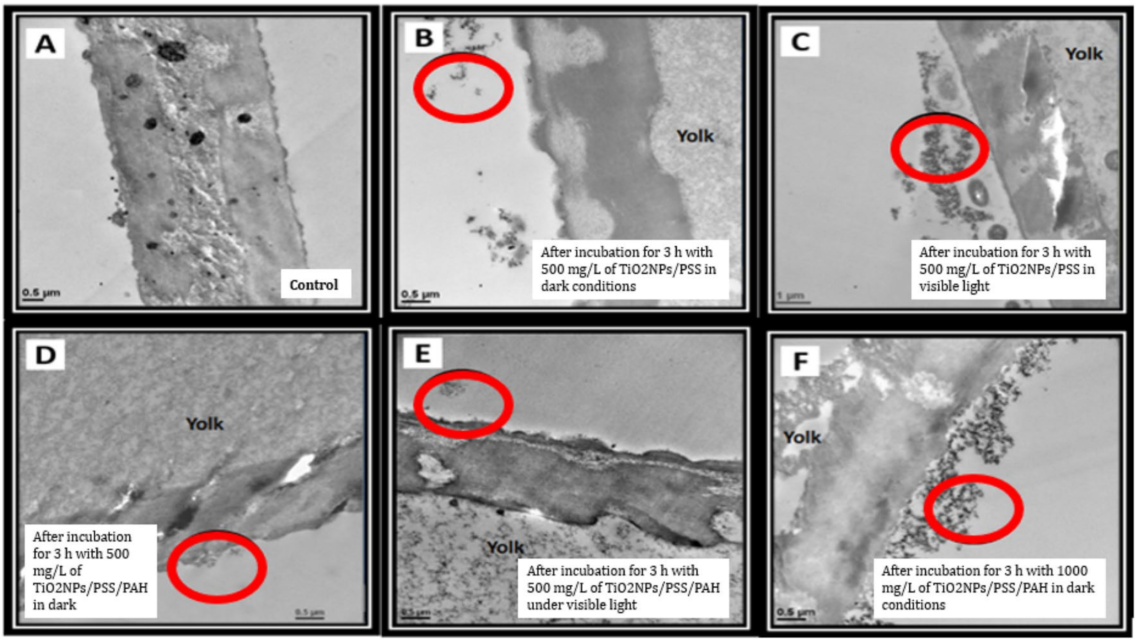

Fig. 3 TEM images of *D. rerio* embryo outer surface (chorion) in **a** control treatment, **b** after incubation for 3 h with 500 mg/L of TiO_2NPs/PSS in dark conditions, **c** after incubation for 3 h with 500 mg/L of TiO_2NPs/PSS in visible light, **d** after incubation for 3 h with 500 mg/L of TiO_2NPs/PSS/PAH in dark, **e** after incubation for 3 h with 500 mg/L of TiO_2NPs/PSS/PAH under visible light, **f** after incubation for 3 h with 1000 mg/L of TiO_2NPs/PSS/PAH in dark conditions. The circles represent the areas used for the EDS spectrum analysis

Table 1 Trace amounts of titania detected on the surface (chorion) of embryo samples (n = 5) exposed to different coated titania treatments using EDS-TEM analysis

Embryo treatment group	Condition	Titania (% of elemental weight)
Control	Light	0
500 mg/L anionic TiO_2NPs/PSS	Dark	2.99
500 mg/L anionic TiO_2NPs/PSS	Light	15.2
500 mg/L cationic TiO_2NPs/PSS/PAH	Dark	0
500 mg/L cationic TiO_2NPs/PSS/PAH	Light	0
1000 mg/L cationic TiO_2NPs/PSS/PAH	Dark	24.85

interaction between the types and concentration; and the interaction between the concentration and condition all significantly affected the relative gene expression levels of *SOD2* mRNA (Table 2). The expression level of *HIF1* was only affected by the condition (light or dark) used in the experiment (Table 2). *Pxmp2* mRNA expression level was not significantly impacted by any of the types of TiO_2NPs, concentration, nor the exposure regime (Table 2).

A Kruskal–Wallis test highlighted further significance within the dataset as follows. Significant difference in *SOD2* expression level was detected as a result of TiO_2NP type,

Table 2 Summary of the statistical analyses on the effect of TiO_2NPs, coated with different anionic (PSS) and cationic (PAH) polyelectrolytes, on the mRNA expression level of *SOD2*, *HIF1* and *Pxmp2* in *D. rerio* embryos. Exposures were conducted at three particle concentrations (0, 500 and 1000 mg/L), for 3 h exposure time, in either dark or visible light conditions

Gene	Scheirer-Ray-Hare test							Kruskal–Wallis test		
	Types	Conc	Cond	Int[1]	Int[2]	Int[3]	Int[4]	Types	Conc	Cond
SOD2	$P < 0.05$	$P < 0.05$	$P < 0.05$	$P < 0.05$	ns	$P < 0.05$	ns	$P < 0.05$	*ns*	$P < 0.05$
HIF1	ns	ns	$P < 0.05$	ns	ns	ns	ns	ns	ns	ns
Pxmp2	ns	ns	ns	ns	ns	ns	ns	ns	ns	$P < 0.05$

Effect of concentration (Conc), Exposure condition (Cond), [1]Interaction between types of TiO_2NPs and concentration (Int[1]), [2]Interaction between types of TiO_2NPs and exposure condition (Int[2]), [3]Interaction between concentration and exposure condition (Int[3]), [4]Interaction between types of TiO_2NPs, concentration, and exposure condition (Int[4]), *ns* not significant

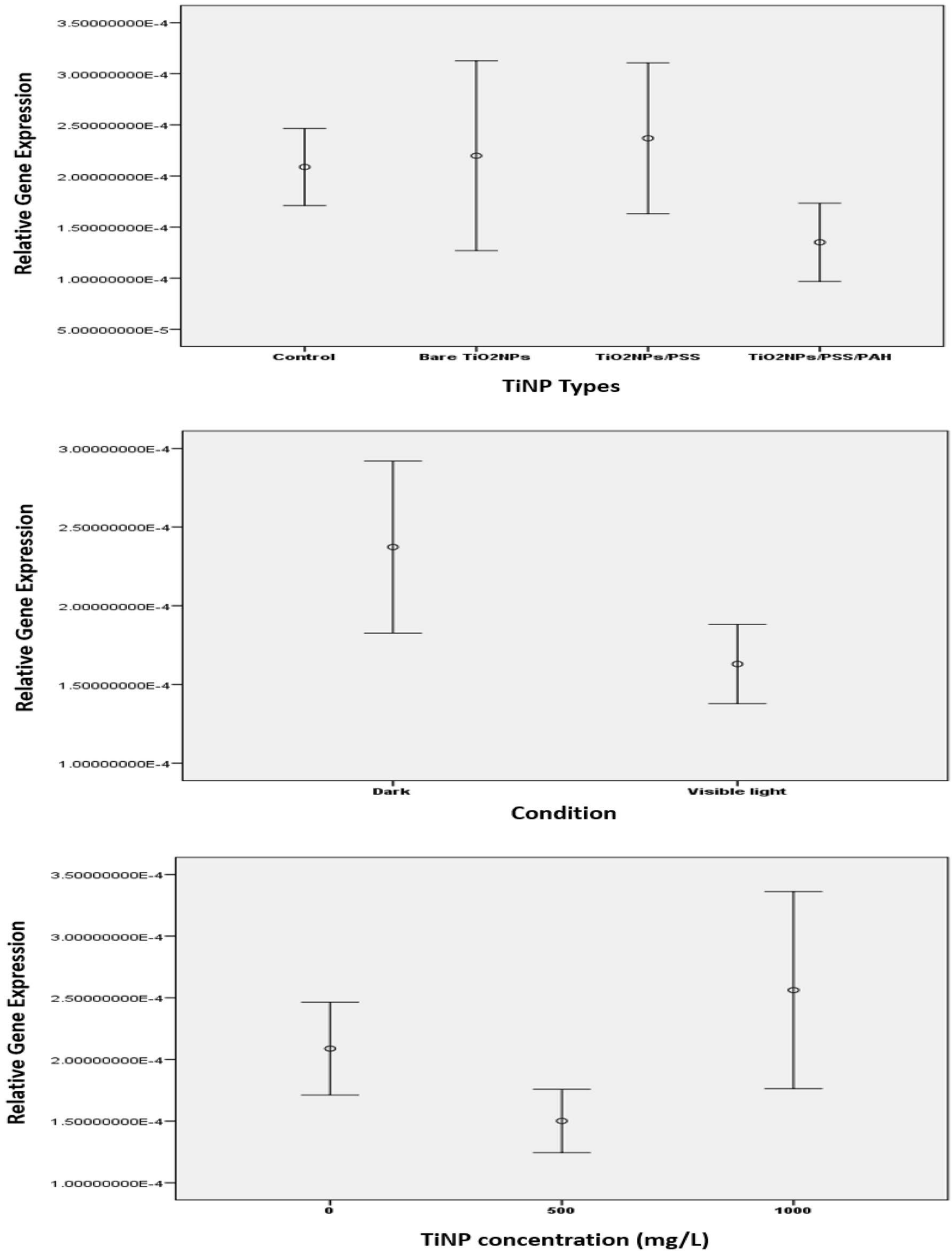

Fig. 4 *SOD2* mRNA shows significant expression levels differences based on titania type. Relative *SOD2* mRNA expression level in pooled embryo samples (0–72 hpf, n = 10) are shown

concentration, and condition (Fig. 4). *HIF1* expression was significantly affected by condition (Fig. 5). *Pxmp2* expression was not affected by any condition (Fig. 6). Separate Kruskal–Wallis tests (Table 2) for one-way analysis of the individual data between TiO_2NPs types, concentration, and condition, revealed that *SOD2* mRNA expression was

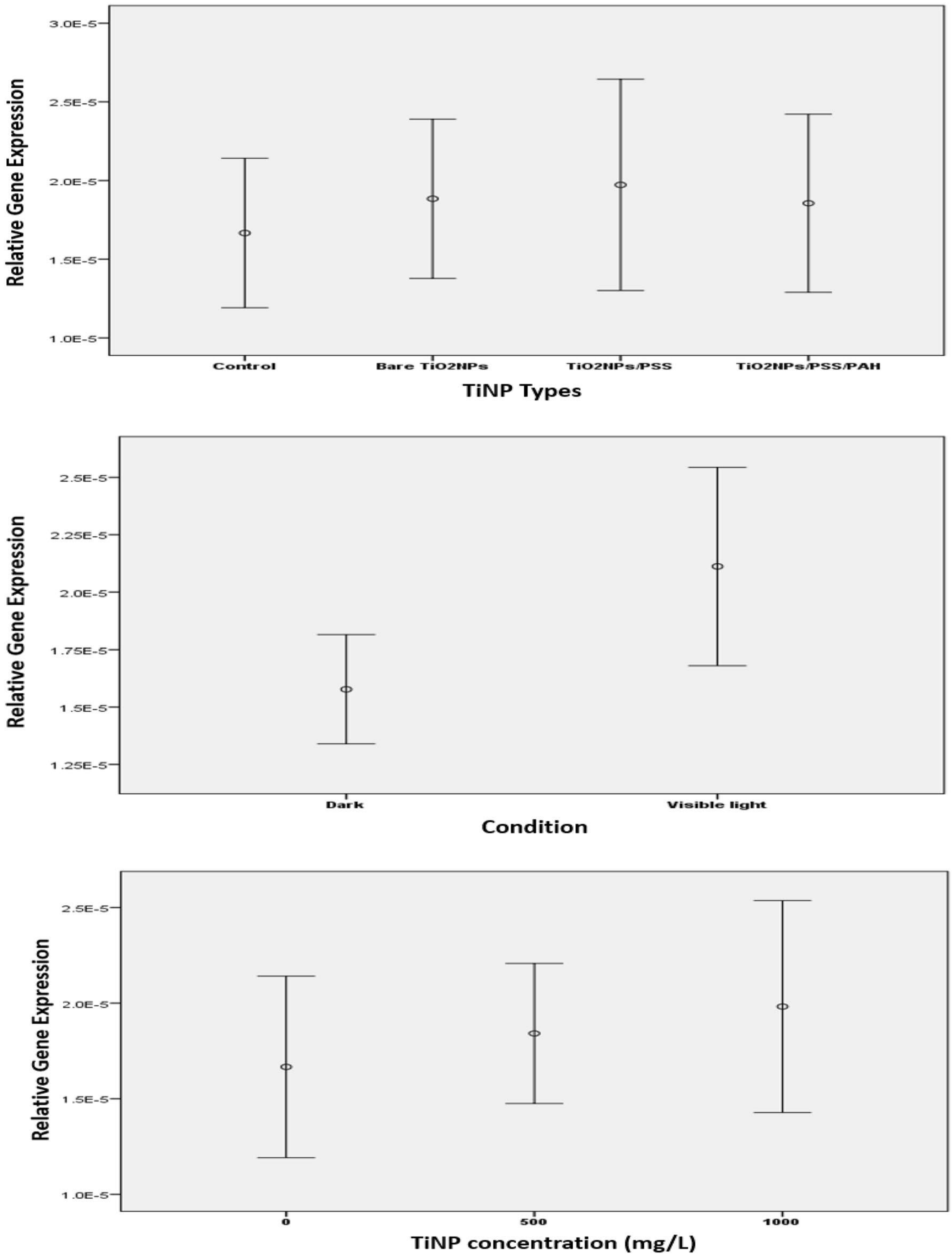

Fig. 5 *HIF1* mRNA shows no significant expression levels differences based on titania type, concentration or condition. Relative *HIF1* mRNA expression level in pooled embryo samples (0–72 hpf, n = 10) following exposure to TiO_2NPs with various coatings, concentrations and dark/light conditions

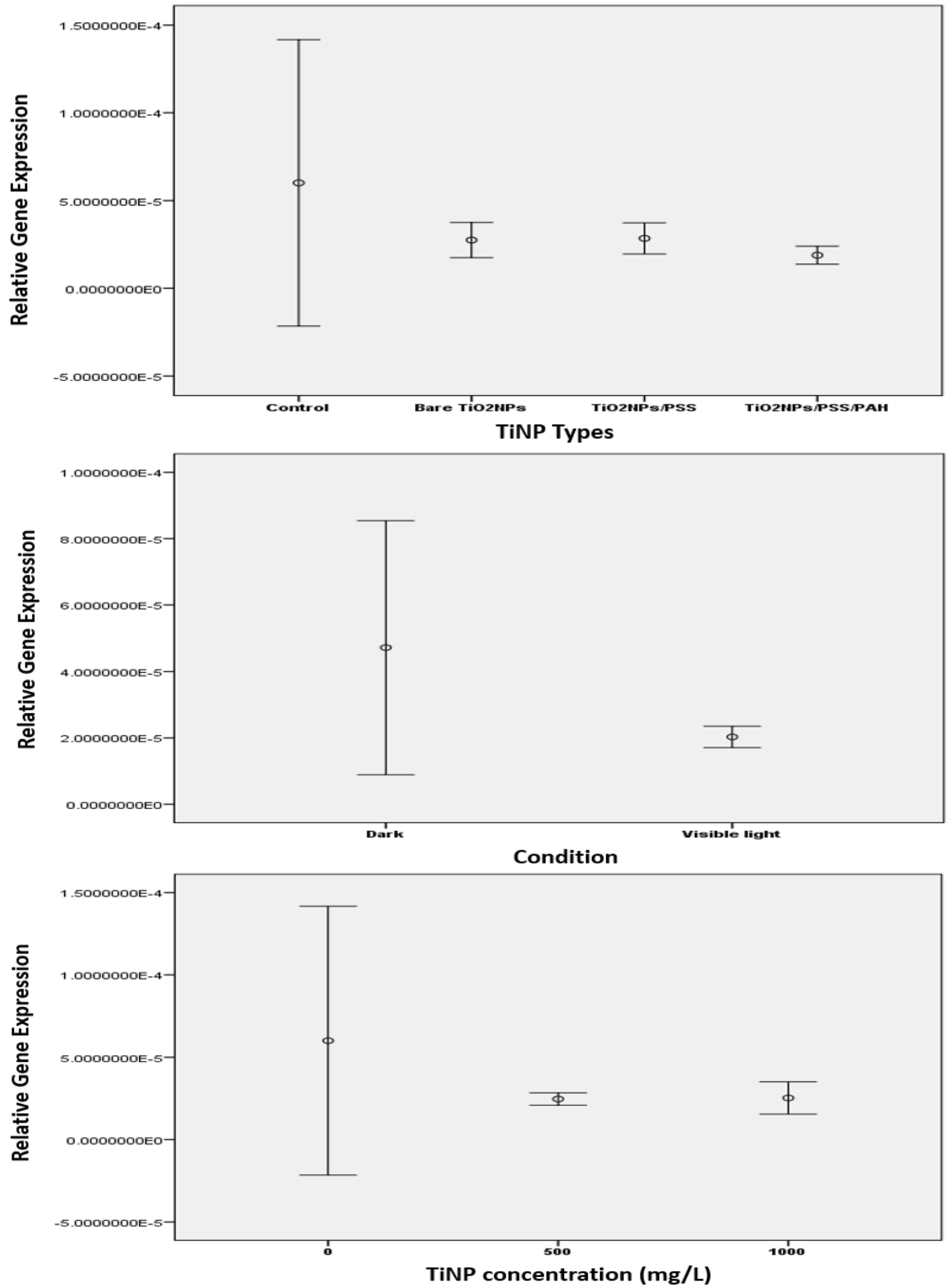

Fig. 6 *Pxmp2* mRNA shows no significant expression levels differences based on titania type, concentration or condition. Relative *Pxmp2* mRNA expression level in pooled embryo samples (0–72 hpf, n = 10) following exposure to TiO_2NPs with various coatings, concentrations and dark/light conditions

significantly affected by both types and condition. *HIF1* mRNA didn't show any significant differences in expression by any factors, and *Pxmp2* mRNA expression shows significant difference only by condition as a result of light/dark effect.

4 Discussion

From this study, using two relatively high exposure levels of uncoated and coated titania NPs (500 and 1000 mg/L), it is evident that overall embryo viability is not affected (Fig. 2), even though titania can be detected on the surface (chorion) of embryos (Fig. 3b, c, f), and significant changes in oxidative stress gene expression, particularly *SOD2* was detected in selected exposure treatment groups (Fig. 4). On the other hand, the markers adopted for hypoxia (*HIF1*, Fig. 5) and membrane function (*Pxmp2*, Fig. 6), suggest that such high exposure levels of these NPs are not having a significant impact on these specific endpoints under this exposure regime.

In terms of NPs availability and uptake, the anatase NPs employed in this study had an average diameter of 25 nm with a range of different coatings. Their impact on viability and their apparent absence within the embryos analysed, would suggest that they are not crossing the embryo and/or causing mortality under the exposure conditions used. This is consistent with studies using eggs and adult zebrafish that reported an LC_{50} value of > 1600 mg/L for uncoated (< 100 nm sized) TiO_2NPs after a 48 h exposure period [29]. Studies using significantly longer exposure times (of 23 days) have shown decreased survival of *D. rerio* embryos exposed to TiO_2NPs at lower concentrations from 10 µg/L to 10 mg/L [30]. Adding UV light has also been shown to increase mortality, in larvae rather than embryos, at concentrations of 1-100 mg/L [31, 32]. The *D. rerio* embryo is ~ 1.5 mm thick [33], is surrounded by a protective chorion, which, at 72 hpf, is considered open to the passage of materials through pores or via passive transport [34], yet these results suggest that no nanoparticle transfer across the chorion has occurred.

Two coatings were compared with each other in terms of relative uptake and toxicity. The TiO_2NPs/PSS/PAH used are cationic and UV-photoactive, similarly to the uncoated TiO_2NPs, i.e. their positive charge is anticipated to promote adhesion on the embryo surface. However these were only identified on the surface of the embryos exposed to the higher 1000 mg/L level (Table 1), with possible disruption of the negatively charged cell membrane [14]. Previous work using microalgae, *Chlamydomonas reinhardtii*, and yeast, *Saccharomyces cerevisiae*, has also confirmed the formation of a significant build-up of NPs on the cell surface for bare and cationic polyelectrolyte-coated TiO_2NPs at pH 5.5 [14]. In contrast, titania was also detected on the outer surface of microtome-sectioned *D. rerio* embryos exposed to 500 mg/L anionic-coated TiO_2NPs/PSS in both dark and visible light conditions (Table 1). Regarding possible mechanisms to understand how such particle build up may occur, the negative charge of the cell membrane has been suggested to facilitate internalization, and affect the toxicity of positively charged coated NPs in other studies, such as gold NPs (AuNPs), which are more toxic than negatively and/or neutrally charged AuNPs [35, 36]. In terms of additional coatings and nanohybrids, the toxicity of TiO_2NP and TiO_2-MWCNT nanohybrid has also been assessed with and without UV light exposure using zebrafish embryos, and neither presented acute toxicity [37]. The acute effects of TiO_2NPs in zebrafish embryos thus depend on both the type of formulation and the illumination condition.

Three biological effects markers of sub-lethal impacts were examined. The expression of *SOD2* mRNA in pooled zebrafish embryos (n = 10) was affected by the type of TiO_2NPs, concentration, and condition (Table 2, Fig. 5) specifically indicating an oxidative stress response. This finding is consistent with those reported by Bar-Ilan et al. [30] whereby exposure to 10 µg/L–10 mg/L TiO_2NPs, illuminated with a lamp, produced toxicity through cumulative reactive oxygen species. The expression levels of the hypoxia and membrane markers, *HIF1* and *Pxmp2,* were not significantly impacted by any factor using the same exposure conditions. The SOD enzyme catalyses the conversion of the reactive superoxide ion (O_2^-) to yield hydrogen peroxide (H_2O_2) and oxygen molecule during oxidative oxygen processes [38]. Other markers of oxidative stress, increased *catalase* and *glutathione S-transferase* expression levels have also been reported in zebrafish embryos, exposed to TiO_2NPs for 96 h, under either visible light or a combination of visible and ultraviolet (UV) light [32]. Felix et al. [39] examined sub-lethal biological effects impacts of 0.1, 1 or 10 mg/L of uncoated TiO_2NPs, poly(acrylic acid)-coated TiO_2NPs, and the polymer coating alone, in the presence or absence of UV light, reporting that uncoated TiO_2NPs produced hydroxyl radicals, delayed hatching, induced lipid peroxidation, increased catalase activity and total glutathione levels, and up-regulated *glutathione peroxidase 1a* gene expression in the presence of UV light, while polymer-coated TiO_2NP increased thiobarbituric acid reactive substances production and total glutathione levels under simulated sunlight illumination. Further experiments are needed, with an increased number of embryos in each treatment group, and a shorter defined embryo stage (from 0 to 72 hpf range), in order to reduce the variation of gene expression within treatment groups observed.

5 Conclusions

In summary, TiO_2NP size, surface charge, concentration and the presence/absence of light have been shown to determine their potential toxicity measured in this study as specific gene expressions. Polyelectrolytes coatings were used in formulations to enhance dispersion stability [40]. The polyelectrolyte multilayer films (PAH/PSS) provide a stable nanocomposite thin film that interacts with the NPs [41]. The nanotoxicty of polyelectrolyte-coated TiO_2NPs have been previously been studied in yeast and microalgae and the results showed that the toxicity of the coated TiO_2NPs changes with their surface charge where cationic polyelectrolyte coating were more toxic than the anionic polyelectrolyte coating [14]. Here, we compare the toxicity of different TiO_2NPs coatings on *D. rerio* embryos and find that *D. rerio* embryos remain viable after exposure to 500 and 1000 mg/L of TiO_2NPs coated with anionic and cationic polyelectrolytes for 3 h. Also, embryos exposed to TiO_2NPs coated with cationic polyelectrolytes showed no Ti on the embryo using EDS while the higher dose of 1000 mg/L of the same coating start to show NPs residues. Importantly, the biological sub-lethal effects marker, *SOD2* expression, showed significant changes related to all factors, indicative of oxidative stress. Similarly, *HIF1* expression showed a significant difference in response to condition. This study focused on a relatively short-term exposure with concentrations that are not environmentally relevant. It would be interesting to understand how NPs at environmentally realistic exposure levels affect *D. rerio* over longer exposure periods, at defined embryo stages, and also several generations.

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Compliance with ethical standards

Conflict of interest The authors report that there are no conflict of interest.

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