Phototoxicity effects of NIR-irradiated cesium tungsten oxide (Cs$_{0.33}$WO$_3$) nanoparticles on zebrafish embryos: A direct immersion study

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**ABSTRACT**

Nanoparticles (NPs) with feature sizes ranging between 1 nm and 100 nm have increasingly gained momentum for their versatile functionality as the pharmaceutical agents in many branches of biomedical research and clinical experiments. However, NPs’ inherent material toxicity and the concomitant adverse effects of their function, such as photo-physical properties, often remain a major concern over the issues of environmental safety and human health, and require a thorough assessment before a wide-spread usage can be compiled. This research herein investigates the intrinsic and photothermal toxicity of Cs$_{0.33}$WO$_3$ NPs solution in zebrafish larvae through a direct immersion method. Experimentally, the survival, hatching and malformation rates of zebrafish embryo/larvae as functions of the NP feature sizes, concentration and duration of photothermal dose were examined and analyzed. This study verified that the Cs$_{0.33}$WO$_3$ NPs has an intrinsic toxicity on a scale of a fraction of 1 mg/ml, and the phototoxicity effect of the NIR-irradiated NPs, when irradiated for 30 min, can affect the embryogenesis of zebrafish larvae and causes 60% and 50% in the survival and delayed hatching rates, respectively, as well as a severe malformation.

1. Introduction

Reducing the feature size of bulk materials down to the nanoscale from 100 nm to 1 nm allows an unprecedented enhancement in many aspects of material properties resulting in many unique chemical, physical, and bio-physicochemical functions for a wide range of scientific disciplines [1]. Medicine compounds containing nanoparticles (NPs), Gold (Au), Iron oxide (Fe$_3$O$_4$), silver (Ag), for instances, have drawn a great interest among the medical research and clinical communities attributing to a broad spectrum of medical usefulness such as, just to name a few, the anti-fungal, anti-bacterial, anti-cancer, anti-amyloidal properties and etc [2–4]. Atop the proven therapeutic effects, the NPs allow an addition of some new visual dimensions to the delicate biological features by augmenting the imaging contrast, sensing the peculiar biomolecules in a biophysical process, and tracking the cellular signal transduction pathways [5]. Inevitably, however, the feature sizes and enhanced biochemical reactivity of NPs can easily disappear into natural environment or sneak into the previously unattainable locations in the human organs with microgranules through the blood streams, raising a grave fear and leaving an immense uncertainty upon the environmental hazard, risks of adverse side-effects and safety for clinical use [6–9]. As a matter of fact, a handful of adverse effects of the direct interaction of NPs with human cell lines with or without photo-irradiation had been confirmed in vitro [10–14]. These studies corroborated a promotion of up-regulation of the cytotoxic reactive oxygenated species (ROS) that can disrupt the cytoskeletal morphology and network, thus rendering some enormous disturbances on the intricate pathways of cellular signaling.

On the organism-level, the larval zebrafish has become a popular and valuable tool for the toxicity assessment of nanomterials due its smallness in size, optical transparency, prolific offspring, and over 80% of genome resemblance to the human kind [15]. A plethora of toxicity studies of inorganic nanoparticles like gold (Au), graphene oxide, SiO$_2$, Cadmium Telluride (CdTe), Fe$_3$O$_4$, TiO$_2$ had been evaluated in zebrafish embryos [16–20]. These investigations revealed a various degree of negative impact on the hatching, heartbeat, mortality and abnormality rates, highly dependent on the NP’s physicochemical properties including, but not limited to, the feature size, shape, concentration, surface defects, aspect ratio, surface coatings, and material composition. Additionally, some reports convincingly attributed the surface charge of

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NPs, dissolved NP ions as well as NP’s photo-oxidation as the main toxicity mechanisms that cause the cellular misdemeanors like detachment, floating and shrinkage, and their consequential disturbance in the cellular metabolism [21–23].

Up-to-date, according to our knowledge, only a few investigations reported the toxic effects of optically irradiated NPs upon zebrafish embryos and larvae. Among them, the pernicious effects of the ultraviolet (UV) or sunlight-illuminated TiO₂ NPs reckon a majority, and can cause the mortality, dwindled growth, reduced metamorphosis, malformation and damages to organs and DNA [24–26]. Likewise, Srivastava et. al. reported a direct exposure of zebrafish embryos to the blue light-illuminated silicon quantum dots (SiQDNP), which induced a pronounced malformation on the larval head, yolk sac and tails, recorded a significant mortality rate when the concentration is beyond 60 µg/ml [27]. Collectively, these studies concluded the reactive oxygen species (ROS) produced through the reactive interactions among electron-hole pairs, oxygen and water as the main inoculation mechanism of photo-toxicity. Atof the ROS production, hyperthermia is an alternative byproduct of the photo-irradiated NPs that could cause the harmful damages to biological organisms since it has been proven to produce an accumulation of the cellular ROS in animal cells, causing the oxidative stress, and the subsequent apoptosis and necrosis [28–30].

Thus far, most of the photothermal toxicity studies of the near-infrared (NIR) irradiated NPs focused on the direct interaction between the material systems, like Au, iron oxide (Fe₃O₄), graphene oxide, CuS, iron platinum (FePt) and NaYF₄, Porous silicon and Cs₃WO₃ with cancer cell lines [27,31–36]. These studies mainly determined the lethal rate of cancer cells without commenting the NPs’ intrinsic toxicity on the healthy, and an evaluation of their potentially concomitant phototoxicity upon larval zebrafish has never been reported previously. Cs₃WO₃ NPs’ high absorbance to a range of the NIR wavelength from 800 nm to 2400 nm upon photothermal conversion, in particular, is appealing to cancer therapeutics for its lowest cytotoxicity at a concentration of 1 mg/ml as well as an effective optical power lower than 50 mW [37]. Moreover, the crystalline structure, chemical composition and size of the NPs’ tungsten bronze structure are vitally important to its strong NIR absorption, and thus, the subsequent heat-releasing [38,39]. Such photothermally converting capability is deduced from the doping of positive Cs ions’ ternary structures into WO₄, the primitive octahedral structure, reducing the oxygen content [40,41], and may induce the harmful ROS and consequential oxidative stresses to biological organisms due the NPs’ innate ionic strength and optically enhanced surface plasmon.

Hence, the research presented herein is poised to interrogate, comprehensively, the intrinsic and photothermal toxicity of the homemade 90 nm-, 140 nm-, and commercial 60 nm-Cs₃WO₃ NPs upon the embryogenesis of larval zebrafish via a direct immersion. The questions to what extend do the physio-chemical and photothermal properties of the NPs, including feature size, concentration and duration of irradiation, influence the early larval development and morphogenesis were to be addressed. Experimentally, the NPs were synthesized, characterized with its structural and photo-physical properties, and applied to the toxicity assays upon zebrafish embryos/larvae to garner the survival, hatching, and malformation rates for statistical analysis, utilizing an optical stereoscope.

2. Material and methods

2.1. Synthesis of Cs₃WO₃ micropowder

Proper mass proportions of the powders of ammonium tungstate [(NH₄)₂WO₄] (99.9% purity, Alfa Aesar) and cesium chloride CsCl (99.9% purity, Alfa Aesar) in a respective ratio of 1:0.33 were dissolved separately in 150 ml of deionized (DI) water at ambient temperature. These two solutions were mixed together in a beaker, stirred at 250 rpm for 1 h (hr) by a magnetic spinner at ambient temperature. The resultant mixture solution was placed on a hotplate inside a fume hood, heated at 180 °C until the water evaporation was completed, and resulted in the form of the dried white powder as a precursor material. The dried precursor powder was put in a quartz boat, placed in the middle section of a high-temperature furnace tube, heated at 500 °C, and blown with a flow of gas mixture of H₂ and N₂ in a ratio of flow rates, 90 and 10 Standard Cubic Centimeter per Minute (SCCM), respectively, for facilitating redox reaction. Afterward, the precursor powder was annealed at 800 °C for 1 hr alongside a gas flow of N₂ at 100 SCCM. Finally, the oven heater was switched off for cooling, and the resultant dark blue micro(µ)-powder of Cs₀.33WO₃ was obtained.

2.2. Grinding process of Cs₀.33WO₃ nanomaterial

The Cs₀.33WO₃ µ-powder obtained from the furnace tube was used to produce the NPs by a two-stage wet nano-grinding process. In details, 15 g of the Cs₀.33WO₃ micropowder was mixed with 3.8 g of the dispersant (Just Nanotech Co., Ltd, Taiwan) and 10 µl of the anti-foaming agent (Just Nanotech Co., Ltd, Taiwan), and afterwards, a proper amount of D.I. water was poured into the mixture until the total mass reached 150 g. For the first stage of the grinding process, also referred as the rough grinding, the mixture solution was incorporated with 600 g of the 0.1 mm-diameter zirconia beads (Orienta Cera TEC, Taiwan) and grinded at a rotational speed of 2400 rpm for 4 h (hrs) while keeping the temperature of the sample chamber at 15 °C. At the end of the 4th hr, the nano-grinder was switched off, and a small amount of the grinded NP solution was obtained from the sample container for analysis of the NP size. Subsequently, a certain volume of D.I. water was added to the grinded NP solution from the first grinding stage until the total content in the container reached 150 g, and grinded with 600 g of 0.05 mm-diameter zirconia beads (Orienta Cera TEC, Taiwan) for 4 hrs. From the above synthetic procedure, the Cs₀.33WO₃ NP solution of two distinct feature sizes, 90 nm and 140 nm, were manufactured by the 1st and 2nd grinding stages, respectively; the commercial 60 nm NPs solution was purchased from Justnano Corporation (Tainan City, Taiwan).

2.3. Characterisation of CsWO₃ nanomaterial

The crystalline formation of Cs₀.33WO₃ µ/nano-materials was characterized by XRD (D2 Phaser, Bruker AXS GmbH, Germany) in the range of detection angle 2θ from 3° to 160°, utilizing an X-ray of 0.15405 nm wavelength. Additionally, the Cs₀.33WO₃ upowerd was characterized by an Energy Dispersive X-ray (EDS), built-in to a field-erosion scanning electron microscope (FE-SEM) (JSM-7000 F, JEOL, Japan) for verification of the NPs’ atomic composition and surface profiles, correspondingly. Moreover, while the contour shape of the Cs₀.33WO₃ NPs was revealed by a Transmission Electron Microscope (FE-TEM) (JEM-2100 F, JEOL, Japan), the statistical distribution of the NPs’ feature size was measured using a Dynamic Light Scattering (DLS) instrument (Delsa Nano C, Beckman Coulter, United States). To measure the photo-absorbance of the Cs₀.33WO₃ NPs solution, which was filled in a quartz cuvette located in the analytic chamber, a visible-near-infrared (VIS–NIR) Spectrometer (V-750, Jasco, Japan) was utilized to register the spectral profiles of an array of solution concentrations in a wavelength range from 400 nm to 1300 nm. The photothermal property of the NIR-irradiated NPs solution was examined by measuring the temporally dependent temperature profile, using a home-built system composed of a temperature sensor for liquid-based material (LP-11, Yotec, Taiwan), a 96-well plate and NIR light lamp (MHAB-100 W-B, Moritech, Japan). To acquire the time-course profile, three wells were filled with 250 µl of the NP solution of each NP size, irradiated with the power-adjustable NIR light set at a distance of 1 cm directly above the well, and monitored by the temperature probe for an hour.
2.4. Zebrafish Husbandry

This research was conducted under the guideline and approval from the institutional animal care and use committee of National Yang Ming Chiao Tung University. 5 pairs of adult wild-type zebrafish were obtained from the zebrafish core facility of Academia Sinica, Taiwan, and raised in a circulating aquarium system conditioned with a light/dark cycle of 14/10 h (hr) and at the temperature of 28.5 °C. The larval and adult zebrafish were raised separately. While the larvae were fed with the live brine shrimp, the adult zebrafish were fed with a combination of the live brine shrimp and supplements of dry shrimp flakes, twice daily. On an evening before the spawning day, the adult male and female fish in a ratio 2:1 were placed in a hatching box with a spacer plate that separates the male from female. In the next morning, the spawning action was initiated once the light was lit. Afterward, every embryo was examined utilizing a stereomicroscope (Nikon H550S, Tokyo, Japan) and the healthfully fertilized, normally developed ones were collected prior to the immersion experiment commencing at 1.5 hrs post-fertilization (hpf), which complies the OECD TG No. 236 outlining the guideline for the acute testing of chemicals with zebrafish embryos [42].

2.5. Direct immersion toxicity assay

The normally developed zebrafish embryos were allocated, individually, in the wells of a 96-well plate filled with 250 μL of the Cs0.33WO3 NPs solution at various concentrations, 0 (untreated), 0.1, 0.25, 0.5, 1, 2, 5 mg/ml. Throughout this assay, a total of 24 embryos (N = 24) were used for every testing concentration and observed, alongside a renewal of the NP solution, at specified time-points, 0-, 24-, 48-, 72-, and 96-hpf, utilizing the stereomicroscope. To analyze the NP’s intrinsic toxicity, the survival rate of the embryo/larvae is expressed as

\[
\text{Survival} = \frac{\sum (#\text{Alive})}{\sum (#\text{Total})} \times 100\%
\]

where an occurrence of any one of the following conditions (see Fig. S1 for illustrations) to the embryos, (1) coagulated embryos, (2) non-detachment of the tail, (3) a lack of somite formation, (4) a lack of heartbeat, at any of the observation points, indicates the embryonic death. Likewise, the hatching rate is calculated as

\[
\text{Hatching} = \frac{\sum (#\text{Hatched})}{\sum (#\text{Total})} \times 100\%
\]

where a larva is considered hatched when all the somatic body (from tail to head) is out of the chorion [43]. In addition, the malformation rate was evaluated at 96-hpf [44] and is defined as

\[
\text{Malformation} = \frac{\sum (#\text{Abnormal})}{\sum (#\text{Alive})} \times 100\%
\]

2.6. Photothermal toxicity assay

To carry out the assay, the experimental parameters including the safety concentration determined from the intrinsic toxicity assay, the power densities of NIR irradiation, 0.04 W/cm², 0.09 W/cm², 0.12 W/cm², and the irradiation duration, 10 min, 30 min, 60 min were implemented. As shown in Fig. 1(a) NIR light source was set vertically 1 cm from the top surface of a well of a 96-well plate to assess the toxicity caused by the NIR-irradiated NPs’ photothermal effect. For each experimental trial, 12 2-hpf embryos (N = 12) were packed in a well of the 96-well plate filled with 250 μL of the NP solution for the NIR irradiation. When the irradiation was finish, all 12 embryos were transferred to their individual wells filled with the same CsWO3 NPs solution. The criteria and method for counting the survival, hatching and malformation rates are the same as shown in the section of the intrinsic toxicity assay.

3. Results

3.1. Material characterization

To confirm the physicochemical properties of the Cs0.33WO3 materials in the forms of μ-powder and NPs, the crystal structure, atomic composition, surface morphology and contour shape were characterized. The crystalline structure of the Cs0.33WO3 μ-powder was examined by acquiring a XRD spectrum shown in Fig. 2(b), in which the characteristic peaks along the crystalline planes of (002), (102), (200), (112), (202), (212), (004), (220), (204), (312), (400) and (224) were found, consistently identical to the gold-standard of the Cs0.32WO3 material from JCPDs (No.83–1334). The atomic composition was authenticated by the EDS as shown in Fig. 2(c), in which the respective atomic proportion of cesium (Cs) and tungsten (W) are 6.88% and 21.87%, closely resembling 0.33:1 and confirming the materials’ intrinsic chemical composition. In addition, the surface microstructure of the Cs0.33WO3 μ-powder was revealed by the SEM. The SEM image presented in Fig. 2(a) not only presents the morphology of the home-made μ-powder but also verifies the molecular arrangement of the hexagonal structure.

Furthermore, Fig. 3(a-c) illustrate, from large to small NP sizes, the characterization of the NPs’ contour shape, statistical NP feature sizes, spectral absorbance and time-course temperature measurement in columns (1), (2) and (3), respectively, using the TEM, DLS equipment, VIS-NIR photospectroscopy and home-built temperature measurement apparatus. While the average feature sizes of the NPs are determined to be 140 nm, 90 nm and 60 nm, congruous with the dwindling size of the rectangle-like NPs shown in the TEM images, the NP concentration are shown to play a major role in the NIR absorbance. To better elucidate the size-dependent material properties, Fig. 3(d) depicts the profiles of the NPs’ spectral absorbance spanning from 400 nm to 1300 nm, verifying an increase in the NPs’ NIR absorbance as the NP feature size decreases as well as the compromised absorption between the NPs’ concentration and feature sizes (60 nm NPs at 0.1 mg/ml). Likewise, Fig. 3(e) presents the time-course temperature measurement as a function of the NP size, and indicates an increase in the saturation temperature as the NP feature size decreases.

3.2. Intrinsic toxicity assay of Cs0.33WO3 NPs through direct interaction

To conduct the intrinsic toxicity assay with the NPs, the zebrafish embryos were immersed in the NPs solutions of a range of concentration (color legions). The survival and cumulative hatching rates were examined and recorded at 4 observation points, 24-, 48-, 72- and 96- hpf as shown in Fig. 4. In details, For all NP sizes, the survival rates decrease

![Fig. 1. A schematic cartoon of the photothermal toxicity assay is illustrated. The maximal optical power, 0.43 Watt, was used in the experiment; 12 larvae (N = 12) were packed in a single well for conformational illumination.](image-url)
as a function of the incremental concentration, which is particularly pronounced for the two smallest NP sizes. While the role of the NP size in procrastinating the hatching rate is ambiguous, the NP concentration imposes a clear inhibition upon the early stage of embryogenesis, in which the hatching rate decreases as the NP concentration increases. Also found from the figure are the safety concentrations of the 60 nm, 90 nm and 140 nm NP solution at 0.1 mg/ml, 0.25 mg/ml and 0.25 mg/ml, respectively, at which the hatching rates are closely aligned with that of the control group. Beyond the safety concentration, the survival and hatching rates reduce substantially below 80% at 0.5 mg/ml, and log lower values as the concentration increases further. In addition, every embryo was observed from 24-hpf to 96-hpf to examine the degree of the somatic abnormalities.

Shown in Fig. 5 are the larval malformation rates of three primary stressful phenotypes, spinal curvature (SC), pericardial edema (PE) and yolk sac edema (YSE) as well as the exemplary images of the abnormalities.

As can be seen from the figure, the larvae incubated in the NPs solution rendered a various degree of somatic malformation depending on the NP concentration. For all NP sizes, as the NP concentration increases, the percentage of the abnormal larvae increases, which is especially apparent for the PE, a dominant abnormal phenotype that dwarfs the syndromes of SC and YSE. Although the malformation rate is negligible at the low concentration (< 0.5 mg/ml), it gets more accentuated once
Fig. 4. Intrinsic Toxicity Assay. The hpf-dependent survival and hatching rates of zebrafish embryos exposed to the (a, b) 60 nm, (c, d) 90 nm, and (e, f) 140 nm Cs$_{0.33}$WO$_3$ NPs alongside (g, h) their dependence (at 96 hpf) on NP concentration are presented, correspondingly. Every experimental trial of statistical significance are indicated with asterisk sign $^*$ $p < 0.05$ ($N = 3$). Legends of the color codes, (a-f) and (g-h), on the right side of each sub-figure indicate NP concentration in mg/ml and feature size, respectively.

Fig. 5. Somatic malformation of the 96-hpf larval zebrafish. The malformation rate of the larvae raised with the (a) 60 nm, (b) 90 nm and (c) 140 nm Cs$_{0.33}$WO$_3$ NPs solution, the stereoscopic images of the (d) control and (e, f, g) respective experimental groups for 140 nm, 90 nm and 60 nm, and the observed abnormalities associated with the (h) 60 nm NPs at 0.5 mg/ml, (i) 90 nm NP at 2 mg/ml, (j) 60 nm NP at 1 mg/ml, (k) 140 nm NPs at 5 mg/ml, are presented. Statistical significance of each treatment case are classified as $^*$ $p < 0.05$, $^*$ $p < 0.01$, $^{**}p < 0.001$ ($N = 3$); the NP concentration is 1 mg/ml unless otherwise indicated; SC, PE and YSE stand for spinal curvature, pericardial edema, and yolk sac edema, correspondingly; the scale bars presented in (d) is 0.5 mm, applicable to (e-k).
beyond 1 mg/ml. With a glimpse at the stereoscopic images of the control (5d) and experimental (5e-g) groups, one can notice a conformational delay in the larval growth and hatching as the NP size decreases when the same NP concentration was applied. Some exemplary images of the somatic malformation during the embryonic development are shown in Fig. 5(h-k). The swollen cardiac organs in (h) and (i) are the characteristic syndrome of PE caused by the 60 nm NPs at 0.5 mg/ml and 90 nm NPs at 2 mg/ml, and YSE and SC were caused by the 60 nm NPs at 1 mg/ml (j) and 140 nm NPs at 5 mg/ml (k). A collage of the larval images shown in the dimensions of the NP concentration and hpf for all NP size is provided in the supplemental Figs. S1-S3 where the increasing concentration increases when the same NP concentration was applied. Some exemplary insets pictures presenting the somatic malformation are also inserted. Similarly, a study on an exposure of sea urchin S. Intermedius to several kinds of NPs control (5d) and experimental (5e-g) groups, one can notice a conformity delay upon the embryonic and larval growth; and some inset pictures presenting the somatic malformation are also inserted. Similarly, a study on an exposure of sea urchin S. Intermedius to several kinds of NPs through a simple immersion also reported the embryotoxicity associated with an early onset of abnormality and death [45].

As a summary, based on the above toxicity assay and malformation assessment, the respective concentration of 60 nm, 90 nm and 140 nm NPs at 0.1 mg/ml, 0.25 mg/ml and 0.25 mg/ml are concluded as the safe doses for the following photothermal assay.

3.3. Photothermal assay of NIR-irradiated Cs$_{0.33}$WO$_3$ NPs

To interrogate the photothermal toxicity of the NIR-irradiated NPs, a control assay with the NIR-irradiation upon zebrafish embryos (N = 12) raised in the culturing water was first conducted, and found that both the survival and malformation rates are 92% and 0%, respectively, ensuring the safety of NIR optical power at 0.09 W/cm$^2$. Regarding the experimental groups, the safety concentration deduced from the intrinsic toxicity assay and two adjustable parameters for the NP-induced photothermal conversion, namely, the optical power density and duration of irradiation were implemented.

The detail of the optical power density's influence upon the larval embryogenesis and morphogenesis, is presented in Fig. S4. Note that 0.52 W (0.12 W/cm$^2$) already caused a complete wipe-out of all embryos by 96-pdf. Thus, 0.43 W (0.09 W/cm$^2$), though in association with the low survival and hatching rates (an hour irradiation), was chosen as the maximum to determine the larval viability from the photothermal toxicity. Fig. 6 depicts the temporal dependence of the survival and hatching rates of zebrafish embryos/larvae after being exposed to the NIR-irradiated NPs within 1.5 hpf for an array of duration, 0 min, 10 min, 30 min and 60 min. In comparison with the control assay (0 min), the survival rates of all NP sizes at 24 hpf drop rapidly as a function of the incremental duration of irradiation, and gradually flatten out with a slightly downward trend as the NP size decreases.

Similarly, the hatching rates at 24 hpf decreases as the NP concentration increases. Notice an interesting observation at 48 hpf that the hatching rates of the experimental groups (10 min and 30 min) are higher than those of the control groups (0 min). This phenomenon may imply that the 2-hpf (64-cell stage) zebrafish embryos immersed in the NIR-irradiated solution for 10–30 min tend to hatch early at 48 hpf. Furthermore, the hatching rates at 72 hpf begin to reach a saturation, contradicting the uprising trend of the intrinsic toxicity assay. By gleaning the results of all NP sizes as shown in Fig. 6(g, h), the dependence of the survival and hatching rates on the irradiation duration is obvious, however, is weakly compounded with the NP size.

Knowing the deleterious effects of the photothermal doses incurred upon the survival and hatching rates, it is of no surprise that the somatic morphogenesis may also get affected by the NIR-irradiated NPs. Fig. 7(a-c) illustrates the malformation rate as a function of the irradiation duration. In general, the abnormal phenotypes, spinal curvature (SC), pericardial edema (PE) and yolk sac edema (YSE), shown in Fig. 7(d-g), are present even in the case of the shortest duration of illumination, and the occurrence of SC and PE are consistently more frequent than YSE. In addition, the photothermally induced abnormalities are much more pronounced than those caused by the intrinsic toxicity assay, highly dependent on the illumination time. As can be seen from the figure, the malformation rates of all types increase as the irradiation time increases. Given an irradiation for 10 min, for instance, the SC not present in the 2-hpf zebrafish embryos/larvae after being exposed to the NIR-irradiated Cs$_{0.33}$WO$_3$ NPs within 1.5 hpf for an array of duration, 0 min, 10 min, 30 min and 60 min. In comparison with the control assay (0 min), the survival rates of all NP sizes at 24 hpf drop rapidly as a function of the incremental duration of irradiation, and gradually flatten out with a slightly downward trend as the NP size decreases.

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intrinsic toxicity assay can now be found in the photothermal assay, independent of the NP size, and the average percentage of the photothermally induced PE at the safety NP concentration are substantially increased by at least 5.5-, 7- and 17- folds for the 60 nm, 90 nm and 140 nm NPs, correspondingly.

At last, an uptake of the Cs$_{0.33}$WO$_3$ NPs by zebrafish larvae was examined by immersing the larvae in the solutions without and with the fluorescent NPs (fNPs) as the control and experimental groups ($N = 9$), respectively. These 96-hpf larvae were mounted on the cellulose matrix for observation using a home-built lightsheet fluorescence microscopy (LSFM). While Fig. 8(a) depicts a brightfield larval image with a red dot that indicates the location of image acquisition, Fig. 8(b-d) present the LSFM images of the plain cellulose matrix, and the control and experimental larvae. Readily observed in the figure, the LSFM image of the experimental group exhibits the presence of green fluorescence not observed in the LSFM images of the cellulose matrix and larval control.

4. Discussion

Bio- and ecological toxicity of nanoparticles (NPs) have become an urgent and important research topic amid the rapid commercialization of the NP-containing products for biomedical, cosmetic and industrial applications [46,47]. However, despite the grave uncertainty of NPs’ hazards over the human health and environment, the magnitude and mechanisms of bio-toxicity induced by a variety of NPs, such as Cs$_{0.33}$WO$_3$ NPs in the present study, are still largely unknown and require thorough interrogation.

Hence, in this research, the Cs$_{0.33}$WO$_3$ NP solutions were synthesized, characterized with its physiochemical properties and applied to examine, comprehensively, the NPs’ intrinsic and NIR-induced photothermal toxicity upon wild-type zebrafish embryos and larvae using a direct immersion method.

Examination of the NPs’ toxicity commences with a direct interaction with the embryos spanning 2 hpf to 96 hpf. On one hand, 50% lethal concentration ($LC_{50}$) for the 60 nm, 90 nm and 140 nm NPs range 0.1–1, 1–2 and 2–5, in mg/ml, respectively, and their safety concentration were 0.1 mg/ml, 0.25 mg/ml and 0.25 mg/ml. Herein, we found that the intrinsic toxicity of the NPs is strongly dependent on its concentration, and the influence of the smaller NP sizes (60 nm, 90 nm) becomes more deleterious only when the concentration is above 1 mg/ml. On the other hand, the hatching process is a combination of the biochemical and behavioral phenomena, and the untreated embryos normally hatch from 48 hpf to 72 hpf [43]. Generally, the hatching rates vary with the NPs' feature size and concentration, which becomes more apparent when the concentration is above 1 mg/ml. Malformity-wise, it is already known that a direct immersion of some NPs with zebrafish embryos can cause spinal curvature (SC), pericardial edema (PE) and yolk sac edema (YSE) [16–20]. In the case of the Cs$_{0.33}$WO$_3$ NPs, the malformation rate is highly dependent on the NP concentration, an increase in the NP concentration of all NP sizes incurring a higher abnormality rate. Also presented in the statistical histogram of Fig. 5 is the rate of acquiring PE that dwarfs those of YSE and SC, and even garners 3% for the control

![Fig. 7. Photothermally induced abnormality. The malformation rates of zebrafish larvae treated with the NIR-irradiated (a) 60 nm, (b) 90 nm and (c) 140 nm Cs$_{0.33}$WO$_3$ NPs within 1.5 hpf for 0 min, 10 min, 30 min and 60 min were recorded at 96 hpf, and the observed phenotypes like the spinal curvature (SC), pericardial edema (PE) and yolk sac edema (YSE), caused by (d) 140 nm NPs for 10 min, (e) 90 nm NPs for 60 min, (f) 60 nm NPs for 10 min and (g) 140 nm for 30 min are presented. The optical power density used in the assay was 0.09 Watt/cm$^2$; the scale bar indicated in (d) is 0.5 mm, applicable to (e-g).](image)

![Fig. 8. Larval uptake of Cs$_{0.33}$WO$_3$ NPs. (a) An optical brightfield image of a 96-hpf larva with a red dot illustrating the location of image acquisition by a LSFM, and the LSFM images of (b) the plain cellulose matrix, and the larvae (c) without and (d) with an uptake of the Cs$_{0.33}$WO$_3$ NPs are presented. The scale bars of the brightfield and LSFM images are 0.5 mm and 50 µm, correspondingly.](image)
group. Therefore, based on the above analysis, the safety concentration of the 60 nm, 90 nm and 140 nm NPs were determined to be 0.1 mg/ml, 0.25 mg/ml and 0.25 mg/ml, respectively, serving as the control groups for the photothermal assay.

Concerning the toxicity mechanism, a majority of the previous research attributed the main mechanisms of nanotoxicity for lethality and malformation to production of the excessive reactive oxygen species (ROS) via a variety of means [14,24–27,48]. Principally, the direct interaction of NPs with cells, cellular organelles, redox-active proteins and cell surface receptors triggering downstream signaling pathways can produce the free reactive oxygen radicals such as O₂, OOH and OH [49,50]. These ROS molecules interfere the molecular signaling, disrupt DNA and induce apoptosis. Secondly, the shredding of NPs may occur when entering from the extracellular matrix into endosome, and then into lysosome, where the gradually reducing pH values from 7.4 to 4.5 facilitates a wet-etching process of the ions off NPs, resulting in an additional ROS generation [51]. Alternatively, NPs’ physicochemical properties like the surface charge, surface-to-volume ratio, surface defects can readily react with the biological molecules like proteins and lipids through the hydrogen bonding, electro-static and van der Waals forces [52–54]. One example of nanotoxicity caused by the ROS is a disruption of sea urchin S. intermedius’ embryogenesis by silicon nanotubes, carbon nanofibers and tubes at high concentration [45].

In the case of Cs₅₃₂₇WO₃ NPs, the oxygen content of the octahedral structures of WO₃, tungsten oxide is reduced via the doping of the large tertiary Cs ions to form a stable cesium tungstate, from which the surface charge density and the surface-to-volume ratio are enhanced. These extrinsic electronic properties like the surface charge, surface-to-volume ratio, surface defects can readily react with the biological molecules like proteins and lipids through the hydrogen bonding, electro-static and van der Waals forces [52–54]. One example of nanotoxicity caused by the ROS is a disruption of sea urchin S. intermedius’ embryogenesis by silicon nanotubes, carbon nanofibers and tubes at high concentration [45].

To carry out the photothermal assay, a maximal NIR power density for safe exposure and the statistics of normal development were ensured. As presented in Fig. 6, 10 min and 30 min of illumination dwindle the survival rate at 96 hfp by approximately 20% and 50%, respectively, independent of the NP size. The NP size does not play a significant role in inoculating the embryos because the embryonic chorion serving as a barrier to the entry of the external entities ranges from 0.6 μm to 0.7 μm [58] is much larger than any of those NP sizes used in this experiment. With the NP size ruled out, since the Cs₅₃₂₇WO₃ NPs are proven to be a good conductor, and has a strong NIR absorption deduced from the polaron and localized surface plasmon resonance (LSPR) upon photon excitation, electron distribution in the crystalline structure [59], we suspect the extra mobile excited electrons on the NP’s surface may play a dominant role in inducing the photothermal toxicity.

Subsequent to the NIR-irradiation upon the NPs, three possible fates for the excited electrons may be accounted for. First, the generation of LSP that may be directly reactive to the external charged entities [59]; secondly, the interaction of the electrons with oxygen and water molecules in the formation of ROS [24]; and third, the relaxation of the energetic electrons as a single hyperthermia dose that induces the harmful oxidative stresses to biological organisms [28–30,36]. Pye et. al. confirmed that the survival rate of the 96-hpf zebrafish larvae in an environment of 34.5 °C can still retain close to 90% despite an observation of the phenotypic abnormalities [60]. Since the saturation temperature of the 60 nm, 90 nm, 140 nm Cs₅₃₂₇WO₃ NP solution under the NIR irradiation, at the safety concentration and optical power of 0.43 W (0.09 W/cm²), are 32.5 °C, 34.5 °C, 33.5 °C, (Fig. 55), respectively, the photothermal hyperthermia is, most likely, not a dominant cause for the lethality in the photothermal assay. Notice, for 10 min irradiation, the 48-dpf larvae’s higher hatching rates than those of the control groups (0 min), suggesting a slight heating effect actually facilitates the hatching process, and once beyond 10 min the hatching rate decreases monotonically as the irradiation duration and optical power density (Fig. 54) increase. Compared to the control group, the malformation rates of all phenotypes spike sharply as the irradiation duration elongates (Fig. 7), and surprisingly, the presence of SC is increasingly on par with PE as the duration increases. These NPs (at the safety concentration) irradiated with 0.43 W for 10 min warmed up the water temperature within the range from 32.5 °C to 34.5 °C, confirming a previous study that the environmental temperature between 32.5 °C and 34.5 °C can factor into the larval malformation while surviving the heat stroke [61]. Other similar studies also reported the photo-toxicity-induced abnormalities. For instance, the NIR-irradiated Cs₅₃₂₇WO₃ and UV-irradiated SiQD NPs have the same phenotypic abnormalities in the curved tail and swollen heart, and a disparity in the deformed head region. Another report indicated the near-ultraviolet-illuminated TiO₂ NPs can cause some pronounced abnormal characteristics in the swim bladder and snout regions [24,27]. From the above studies, the safety concentrations of the UV-irradiated SiQD and TiO₂ NPs are on the corresponding scales, 1 ng/ml (34 mW/cm²) and 1 μg/ml, whereas, the NIR-irradiated Cs₅₃₂₇WO₃ NPs at a fraction of 1 mg/ml allows a better tolerance for photocytotoxicity depending on the duration of illumination.

Furthermore, to verify whether there was any uptake of NPs by the zebrafish larvae, the fluorescent Cs₅₃₂₇WO₃ NPs (fNPs) were synthesized by the method implemented in our recent work [37], immersed with the zebrafish larvae (N = 9) over 96 hrs, and imaged with the LSMF. The LSFM image in Fig. 7(d) presenting a swarm of green fluorescence of the accumulated fNPs, though optically unresolvable on the scale of the particulate size since the resolution of the LSFM is only around a few micrometers, was consistently found in all larvae of the experimental group. Although a dynamic tracking of the NPs is required to unravel its actual distribution, we suspect that the fNPs were either acquired orally or adsorbed upon the larval surface. A similar finding of the uptakes of an array of NP materials in zebrafish larvae was also reported elsewhere [60]. Therefore, a conclusive remark is drawn that the larval mortality observed in the photothermal assay is most likely due to an inoculation by the LSP and consequential ROS inflicted upon the larvae rather than the NP-induced hyperthermia.

5. Conclusion

In this research, the 90 nm and 140 nm Cs₅₃₂₇WO₃ NPs were synthesized, characterized and applied, along with a commercial 60 nm NPs of the same material composition, to examining the intrinsic and photothermal toxicity of the NPs upon zebrafish embryos and larvae through a direct immersion method in a span of 96 hpf. Specifically, the intrinsic toxicity assay revealed that the survival, hatching and malformation rates of the larvae are more susceptible to the NPs’ concentration than to the NPs’ feature size, though, in general, the smaller the NPs the more negative impacts the NP casts. Regarding the photothermal assay, the photothermal effect’s negative influence upon the survival and hatching rates becomes apparent as the illumination duration increases, independent of the NP sizes, and drastically upscales the malformation rate of all abnormal phenotypes. Interestingly, it was found that a slight heating by the NPs’ photothermal hyperthermia can promote an early hatching without any prominent detrimental impact. Therefore, some
conclusive remarks are drawn that the Cs$_{0.33}$WO$_3$ NP has a light degree of intrinsic toxicity on the scale a fraction of 1 mg/ml, which can be deteriorated depending on the NP concentration and irradiation duration. Mechanistically, the ROS induced by the NPs’ intrinsic surface charges as well as the LSP may severely reduce the survival and hatching rates, and may cause a spike in the malformation rates of all abnormal phenotypes as the irradiation time increases from 10 min to 1 hr, independent of the NP size.

CRedit authorship contribution statement
P.S. Hu conceptualized the work, planned the experiment, analyzed the data, wrote the M.S. and edited the M.S.; C.A. Chen synthesized Cs$_{0.33}$WO$_3$ NPs, planned the experiment, carried out toxicity assay, image acquisition and data analysis; H.C. Hsiao and Y.H Cheng carried out toxicity assay of NIR light source; P.Y. Wu synthesized the fluorescent version of the NPs.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability
The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Appendix A. Supporting information
Supplementary data associated with this article can be found in the online version at doi:10.1016/j.toxrep.2022.05.006.

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