Published in final edited form as:

Nanotoxicology. 2016 August ; 10(6): 689–698. doi:10.3109/17435390.2015.1107147.

Toxicity assessment and bioaccumulation in zebrafish embryos exposed to carbon nanotubes suspended in Pluronic® F-108

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Abstract

Carbon nanotubes (CNTs) are often suspended in Pluronic® surfactants by sonication, which may confound toxicity studies because sonication of surfactants can create degradation products that are toxic to mammalian cells. Here, we present a toxicity assessment of Pluronic® F-108 with and without suspended CNTs using embryonic zebrafish as an *in vivo* model. Pluronic® sonolytic degradation products were toxic to zebrafish embryos just as they were to mammalian cells. When the toxic Pluronic® fragments were removed, there was little effect of pristine multi-walled CNTs (pMWNTs), carboxylated MWNTs (cMWNTs) or pristine single-walled carbon nanotubes (pSWNTs) on embryo viability and development, even at high concentrations. A gel electrophoretic method coupled with Raman imaging was developed to measure the bioaccumulation of CNTs by zebrafish embryos, and dose-dependent uptake of CNTs was observed. These data indicate that embryos accumulate pMWNTs, cMWNTs and pSWNTs yet there is very little embryo toxicity.

Keywords

Environmental toxicology; ecotoxicology; nanotoxicology; nanotubes; toxicology

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Declaration of interest

The authors thank the University of Arizona/Semiconductor Research Corporation Engineering Research Center for Environmentally Benign Semiconductor Manufacturing (Grant ERC425-048; R.D., P.P., R.W.), the National Cancer Institute (Grant R15-CA152917; R.D., P.P.), the National Institute for Environmental Health Sciences (Grant R15-ES023666; P.P., R.D.), and National Institute of Health grants ES017552-01A2 (S.H.), ES016896-01 (S.H.), P30 ES000210 (S.H.), and AFRL FA8650-05-1-5041 (S.H.) for supporting this work. The authors are also grateful to the Undergraduate Research Fund of the University of Texas at Dallas School of Natural Sciences & Mathematics for support of undergraduate students.

Introduction

Carbon nanotubes (CNTs) have potential uses in a wide variety of electronic, structural and biomedical applications with worldwide production capacity of CNTs estimated to presently exceed 5 kilotons per year (De Volder et al., 2013). There is ample evidence that CNTs may be toxic, raising concerns about what effects they may have on organisms and ecosystems [for recent reviews, see (Jackson et al., 2013; Kunzmann et al., 2011; Petersen et al., 2011; Zhao & Liu, 2012)]. Zebrafish (*Danio rerio*) embryos are an attractive whole-animal model for assessing the potential toxicity of CNTs and other nanomaterials (Lin et al., 2013; Rizzo et al., 2013). Zebrafish are prolific breeders, their embryos are transparent, develop rapidly, and offer multiple phenotypic and biochemical endpoints for assessing potential toxicity beyond general mortality (Bugel et al., 2014; Fako & Furgeson, 2009; Lin et al., 2013; Truong et al., 2011). In addition, the zebrafish genome is closely related to the human genome and many biological pathways are conserved between the species (Giannaccini et al., 2014). There have been numerous reports assessing the effects of CNTs on embryonic zebrafish development, but they often offer conflicting results and conclusions. One of the earliest studies reported that single-walled carbon nanotubes (SWNTs) did not affect embryo development when the chorion was intact, but did delay hatching, and suggested that the effect was due to metal contaminants in the SWNTs (Cheng et al., 2007). In work from the same group, less oxidized multi-walled carbon nanotubes (MWNTs) had little effect when injected directly into embryos, but shorter and more oxidized MWNTs were acutely toxic (Cheng & Cheng, 2012; Cheng et al., 2009). When present in embryo fishwater, MWNTs functionalized to be water soluble caused significant developmental effects at 60 µg/mL (Asharani et al., 2008), whereas Adenuga et al. (2013) concluded that a panel of seven different water soluble CNTs, including SWNTs and MWNTs, had no significant developmental effects on zebrafish embryos. One possible explanation for differing results was offered by Gilbertson and colleagues (2015) who found that toxicity of MWNTs to embryos scaled closely with the surface charge that became more negative as oxidation of the CNT lattice increased.

Besides surface charge, other physicochemical and experimental parameters that may influence CNT toxicity include length, diameter, rigidity, type of functionalization, the presence of toxic contaminants, the type of dispersant used, if any, and the extent of suspension or aggregation of the material (Firme & Bandaru, 2010; Jackson et al., 2013). Because pristine CNTs without chemical functionalization are highly insoluble, most studies with zebrafish embryos have used oxidized water-soluble materials. Nevertheless, even oxidized CNTs tend to aggregate in fish exposure media and may accumulate in the mouth, intestines and gills of fish that lead to adverse effects unrelated to any specific interactions of CNTs with fish at the molecular or cellular level (Firme & Bandaru, 2010; Gilbertson et al., 2015; Jackson et al., 2013). In the present article, we have explored the use of Pluronic® F-108 (PF108, also known as poloxamer 338), as a dispersant to solubilize and present CNTs to dechorionated zebrafish embryos. PF108 is biocompatible, has been often used in CNT studies with cultured cells (Cherukuri et al., 2004, 2006; Meng et al., 2013; Wang et al., 2012, 2013a), and would have several advantages for use in the zebrafish model. One advantage is that PF108 is an excellent dispersant for CNTs that helps avoid aggregation.

Another is that both pristine and functionalized CNTs can be stably suspended in PF108, which would allow for the comparison of the effects from different nanotube surface chemistries on embryo toxicity with the same surfactant platform. A third advantage is that it would permit comparison of toxicity data collected using similar CNT suspensions for both mammalian cultured cells and fish embryos to evaluate cross-species impacts and improve translation from *in vitro* to *in vivo* outcomes.

In support of toxicity data, it is important to understand the potential uptake, biodistribution and retention of nanoparticles like CNTs in aquatic organisms to assess threats they may have on ecosystems through bioaccumulation and biomagnification, which have been emphasized in recent reviews (Jackson et al., 2013; Scown et al., 2010). However, measuring environmental CNT accumulation by fish is challenging and we are aware of only two approaches in the literature, both of which require a significant investment in methodology. Maes and coworkers (2014) synthesized ^{14}C -MWNTs and noted that a small but significant amount of material was taken up by adult zebrafish and that a small fraction of the internalized radioactivity was detected in the blood and muscle tissue. This study had high statistical variation among replicates, which was presumably caused by CNT aggregation and uneven distribution in the water column. Using an optical approach, Bisesi et al. (2014) monitored the inherent near infrared fluorescence of semi-conducting SWNTs that were suspended in gum arabic and introduced into fathead minnows by single gavage. They monitored the decline of fluorescent intensity after force feeding, but found little intestinal absorption of the SWNTs from the gut.

In a previous work, we developed a simple method for extracting and quantifying CNTs from cultured mammalian cells based on the migration of CNTs in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Pantano et al., 2012; Wang et al., 2009, 2011). The method has been adapted here to measure CNT uptake by zebrafish embryos.

Four types of CNTs were used in this work: pristine and carboxylated MWNTs and pristine and carboxylated SWNTs. The physical and chemical properties of these CNT preparations have been previously characterized in addition to their effects on cultured mammalian cells (Wang et al., 2011, 2013b). All the CNTs in PF108 suspensions studied here accumulated in zebrafish embryos in a concentration-dependent manner but only the carboxylated SWNTs were toxic, similar to results found with cultured mammalian cells (Wang et al., 2011).

Methods

Materials

Dulbecco's modified Eagle medium (DMEM) was purchased from Gibco (Grand Island, NY). Fetal bovine serum (FBS) was purchased from HyClone (Logan, UT). pMWNT and cMWNT powders produced by a Fe/Co/Ni-catalyzed chemical vapor deposition process were purchased from Nanostructured & Amorphous Materials, Inc. (Houston, TX). According to the manufacturer, the pMWNT powder (lot 1236YJS-041709) was >95% in purity and contained nanotubes with outer diameters ranging from 10 to 20 nm, inner diameters of 5–10 nm, and lengths of 0.5–2 μm . The cMWNT powder (lot 1256YJF-070510) was functionalized using sulfuric acid and potassium permanganate,

contained 1.9–2.1% by weight carboxylic acid groups, and had the same purity and sizes as the pMWNTs, as described by the manufacturer. We confirmed the manufacturers' stated carbon content for both pMWNTs and cMWNTs by a combustion analysis (Braun & Pantano, 2014). The pSWNT and cSWNT powders, produced by a Ni/Y-catalyzed electric arc discharge method, were purchased from Carbon Solutions, Inc. (Riverside, CA). The manufacturer purified the pSWNT product (P2 lot 02-419) by air oxidation plus HCl treatment and the cSWNT product (P3 lot 03-A010) was functionalized with nitric acid to contain 1.0–3.0 atomic % of carboxylic acid groups. The individual nanotubes of the pSWNT and cSWNT powders were 0.5–3.0 μm in length and approximately 1.4 nm in diameter, according to the manufacturer. Both pSWNTs and cSWNTs were >90% in carbonaceous purity as measured by the manufacturer using an indirect method, solution-phase near-IR spectroscopy (Itkis et al., 2003). We also assessed the carbon content by direct combustion analysis and found that the pSWNTs and cSWNTs contained 89% and 74% carbon, respectively. *Caution:* A fine particulate respirator and other appropriate personal protective equipment should be worn when handling dry MWNT or SWNT powders. Pluronic® F-108 (PF108) was purchased from Sigma Aldrich (St. Louis, MO). For enzymatic removal of the chorionic membrane of the zebrafish, Protease from *Streptomyces griseus* (Sigma-Aldrich, cat #81750) was used. At the termination of the zebrafish experiments, the fish were anesthetized with 3-aminobenzoate ethyl ester methanesulfonate salt (tricaine, Sigma-Aldrich, cat # A-5040) in deionized water. All other chemicals were purchased from Sigma-Aldrich and were used as received.

Zebrafish culture

Zebrafish embryos (*Danio rerio*, wild type, 5D-Tropical strain) were obtained from Sinnhuber Aquatic Research Laboratory, Oregon State University, and were housed as described by Truong et al. (2011).

Mammalian cell culture and *in vitro* toxicity assay

Mouse macrophage RAW 264.7 (ATCC® TIB-71) cells were obtained from the American Type Culture Collection (Manassas, VA) and cultured in DMEM supplemented with 1.5 mg/mL sodium bicarbonate and 10% (v/v) FBS in a 37 °C incubator with 95% air and 5% CO₂. A standardized cytotoxicity assay used previously in our MWNT and SWNT toxicity work with cultured normal rat kidney (NRK) cells was adapted with minor modifications for RAW 264.7 cells. 4×10^4 RAW 264.7 cells/well were seeded in 48-well plates and incubated at 37 °C overnight before the regular cell culture media was replaced with freshly prepared control or test media and incubated for 24 h. At the end of the incubation, the cells were washed three times with fresh media, two times with phosphate buffered saline (PBS), air-dried, and then fixed using a 4% (w/v) paraformaldehyde in PBS. Cell proliferation was determined using a crystal violet assay, as described in our previous work (Wang et al., 2011).

Sonication

Sonication was done in a glass vial containing 10mL of 0.2mM (~0.3% w/v) PF108 solution alone or with the addition of 10 mg of CNT powder. The vial was secured in a hanging rack and immersed in the center of an ultrasonic bath sonicator (Elmasonic P30H, Elma

Ultrasonic, Singen, Germany) that was operated at 120W and 37 kHz frequency. The temperature of the bath was kept below 18 °C throughout the sonication period by using a cooling coil connected to a refrigerated water bath circulator (Isotemp 1006S) in a 4 °C cold room. The power delivered to the sonication bath was ~80W for our sonication system determined by measuring the change in bath temperature (starting at 22 °C) as a function of sonication time, at room temperature without a cooling coil (Murali et al., 2015; Taurozzi et al., 2011). Non-sonicated PF108 solution was labeled as PF108-0 h and PF108 sonicated for 1 h or 4 h were labeled as PF108-1 h or PF108-4 h, respectively.

Preparation of CNT-PF108 suspensions

Dry CNT powders were baked at 200 °C for 2 h to destroy potential endotoxin contaminants that could lead to ambiguous results in CNT toxicity tests. The bath sonication, centrifugation and dialysis procedures described in our previous work (Wang et al., 2013b) were used with slight modifications to disperse MWNT or SWNT powders in an aqueous 0.2mM PF108 solution. In general, 10mL of 0.2mM PF108 solution was added to a glass vial containing 10 mg pMWNT, cMWNT, pSWNT, or cSWNT powder and sonicated for 1 h at 37 kHz and 120W. After sonication, a 1mL aliquot of each CNT suspension was diluted to various concentrations and the absorbance at 500 nm was measured using a BioTek SynergyMx plate reader (Winooski, VT) to construct a calibration curve of that particular CNT suspension. The remaining suspension was centrifuged at 20 000 *g* for 5 min to remove CNT bundles, metal catalysts and other impurities. Due to the higher metal content of the SWNT powders, the supernatants of the pSWNT or cSWNT suspensions collected from the first centrifugation were centrifuged a second time at 20 000 *g* for 30 min. The supernatants collected were marked as the undialyzed “pMWNT-unD”, “cMWNT-unD”, “pSWNT-unD” or “cSWNT-unD” stock suspensions, accordingly, and stored at 4 °C. The concentration of CNTs and related carbonaceous impurities in each resulting undialyzed CNT suspension was estimated from the absorbance at 500 nm based on the extinction coefficient of the respective calibration curve derived from the same CNT powder.

Dialysis

The removal of PF108 sonolytic by-products in the undialyzed CNT-PF108 suspensions by dialysis is described in our previous work (Wang et al., 2013b). Briefly, cellulose ester dialysis tubing (Float-A-Lyzer® G2) with a molecular weight cut off of 100 000 Daltons was purchased from Spectrum Labs (Rancho Dominguez, CA). The dialysis devices were pre-washed with 10% ethanol and water according to the instructions provided by the manufacturer. Each dialysis device was filled with undialyzed CNT suspension and placed in a beaker filled with 1 L of 0.2mM fresh, non-sonicated PF108 solution. The samples were dialyzed in a 4 °C cold room and the PF108 solution was changed 6 times within 3 days. After dialysis, the samples were collected and marked as the dialyzed “pMWNT-D”, “cMWNT-D”, “pSWNT-D” or “cSWNT-D” suspensions, and stored at 4 °C. The concentration of CNTs and related carbonaceous impurities in each dialyzed suspension was determined based on the absorbance at 500 nm. Both the undialyzed and dialyzed samples were analyzed for the presence or absence of PF108 degradation products by SDS-PAGE followed by BaI₂ staining, as illustrated in Figure S1 and described in our previous work (Wang et al., 2013b).

Dynamic light scattering

The particle size distributions of polymers in the non-sonicated and sonicated PF108 solutions and CNTs in the undialyzed and dialyzed CNT-PF108 suspensions were analyzed by dynamic light scattering (DLS) using a 633 nm laser source at a fixed angle of 173° (Zetasizer Nano-ZS 3600, Malvern Instrument, Worcestershire, UK). All PF108 solutions were analyzed at a final concentration of 0.1mM in MilliQ water. Aliquots of CNTPF108 suspensions were diluted to a final concentration of 0.1mM PF108 and 50 µg/mL CNTs with MilliQ water immediately prior to analysis. 500 µL of each sample was placed in a disposable polystyrene cuvette and 10 consecutive 30-s runs were taken per measurement at 25 °C. Three independent DLS measurements were acquired per sample, and the average particle size and distribution, in terms of hydrodynamic diameter (HDD) and polydispersibility index (PDI) values, were calculated for each sample (Table S1).

Zeta potential analysis

Net surface charges of various undialyzed and dialyzed CNT-PF108 suspensions were analyzed using a Malvern Instrument Zetasizer Nano-ZS 3600. All samples were diluted to a final concentration of 0.1mM PF108 and 50 µg/mL CNTs in MilliQ water. The viscosity of the 0.1mM PF108 was determined with a viscometer to be 0.7327 cP and a transfer standard (DTS 1235, Malvern) was used to verify the correct operation of the instrument that measures zeta potential in a capillary cell, where the temperature was equilibrated to 25 °C throughout the operation.

In vivo toxicity assessments in zebrafish

Exposure of zebrafish embryos to PF108 solutions or CNT-PF108 suspensions and the subsequent evaluations of the effects on the embryos were conducted according to established protocols (Truong et al., 2011). Briefly, embryos were dechorionated at six hours post-fertilization (hpf) by Pronase enzyme digestion and at eight hpf were transferred to 96-well plates, one embryo per well. Each well was filled with 150 µL of control, PF108 or CNT-PF108 suspension sample ($n=24$). Plates were incubated at 26.5 °C under a photoperiod of 14:10 hour light:dark cycle. Effects were evaluated in binary notation as either present or not present at 24 hpf and 120 hpf. At 24 hpf, the embryos were assessed for mortality, developmental progression, notochord malformation and spontaneous movement. At 120 hpf, mortality and touch response were documented, as well as the presence of physical malformations including: pericardial edema, yolk sac edema, circulation, pigment, snout, somites, caudal fin, pectoral fin, axis, jaw, otic, eyes, trunk, brain and swim bladder. After completion of the 120 hpf mortality and morbidity observations, zebrafish in the control and CNT-treated groups were washed extensively with fresh water to remove any CNTs that may have non-specifically adhered to accessible fish surfaces. Zebrafish were vigorously swirled in MilliQ water for 30 s in a clean petri dish, transferred to another clean dish for a second 30 s wash, and then frozen and stored at -20 °C for later use. The amount of CNTs in the zebrafish were quantified, details described in the following sections.

Quantitation of CNTs in zebrafish lysates by SDS-PAGE and optical image scanning

Centrifuge tubes containing 10 frozen dechorionated zebrafish embryos per tube were thawed at room temperature and lysis buffer (100 μ L) containing 0.25M Tris-HCl (pH 6.8), 8% (w/v) SDS, and 20% (v/v) 2-mercaptoethanol was then added and fish were vortexed vigorously to dissolve embryo tissue. The centrifuge tubes were placed in a hot water bath and boiled for 2 h to ensure complete lysis. After the boiling period, the zebrafish lysates were stored at 4 °C for later use (Figure S2).

To measure CNTs in zebrafish embryo lysates, a modified SDS-PAGE protocol was used, developed previously for quantifying SWNTs extracted from cell lysates (Wang et al., 2009). Briefly, a 4% stacking gel on top of a 10% resolving gel was prepared using a Hoefer Mini Vertical Gel Caster for 10×8 cm plates with 1.5-mm thick spacers and 10-well combs. Aliquots of zebrafish lysate and known amounts of non-centrifuged CNT suspension standards were mixed with 2X SDS sample loading buffer to a final buffer concentration of 2% SDS, 5% 2-mercaptoethanol, 10% glycerol, and 62.5mM Tris-HCl and boiled for 3 min. Samples at various dilutions and volumes were subsequently loaded into the wells of the gel and an electric current was applied at a constant 100V for 2 h. CNTs in the lysates bind SDS, become negatively charged, and migrate to the buffer/gel interface where they accumulate in a sharp band because they are too large to enter the gel. Following electrophoresis, optical images of the gels were obtained using a flatbed scanner (HP Scanjet G3110, Hewlett-Packard, Houston, TX). The gels were treated with 3% H₂O₂ at room temperature overnight on an orbital rocker to bleach dark pigments from the embryos that could interfere with quantifying the dark CNT bands. Subsequently, the gels were rinsed briefly with distilled water, optical images of the H₂O₂-treated gels were acquired, and the pixel intensity of each band was quantified using ImageJ software (National Institutes of Health, Bethesda, MD). The known amount of CNTs loaded in lanes and their corresponding pixel intensities form a data set used to construct a CNT amount-versus-pixel intensity calibration curve. The linear calibration curve was used to determine the unknown amount of CNTs in zebrafish lysates based on the pixel intensities of lysate bands in the same gel. Figure 4 demonstrates the optical quantitation of pSWNTs in zebrafish embryos exposed to pSWNT-PF108 suspension at 0– 200 μ g/mL.

Validation of CNTs in zebrafish lysates by laser scanning confocal Raman microscopy

The quantitation of CNTs based on band intensities obtained by optical scanning of SDS-PAGE gels described in the previous section provides no structural information to verify that the signal arises from CNTs. Raman imaging of the bands, however, can provide both structural information, from the Raman spectral signature of the CNTs, and quantitative information from the signal intensities. The H₂O₂-treated gels that contained embryo lysates and CNT standards, as described in previous section, were preserved between two sheets of cellophane and allowed to dry into a thin film. Sections of the bands in the gel were scanned with a confocal laser Raman microscope, described in detail in the following sections and illustrated in Figure S3.

All Raman images and spectra were acquired with a WITec *Alpha300R* confocal Raman microscope system equipped with a 532 nm laser as the excitation source (WITec Focus

Innovations, Ulm, Germany), as described in our previous work (Wang et al., 2011) with slight modifications. The known Raman signal of a silicon wafer at 520.5 cm^{-1} was used to calibrate the wavenumber of the system and the laser output power density was measured using a Newport model-1918-C power meter with an 818-SL photodetector and adjusted to 10 mW/cm^2 (Newport, Irvine, CA). Raman single point spectra were acquired using a $20\times$ objective lens and an integration time of 0.1 s with 20 accumulation cycles. Figure S4 shows four representative Raman spectra acquired from embryo lysates.

To obtain a quantitative estimation of CNTs residing in a gel band, representative areas within the band were analyzed as described in Figure S3. Raman area scans were acquired over a $160\times 160\text{ }\mu\text{m}$ area using a $20\times$ objective lens. A total of 1600 spectra were acquired within a scan area at $4\text{ }\mu\text{m}$ intervals in both x- and y-directions with a 0.2 s integration time for each spectrum. Each spectrum consists of 1600 data points and was scatter plotted with charge-coupled device (CCD) counts on the y-axis and wavenumbers in cm^{-1} on the x-axis. The relative abundance of CNTs in the scanned area was indicated by the integrated Raman G-mode ($1540\text{--}1640\text{ cm}^{-1}$) intensities from each of the 1600 spectra, represented by a color scale map with yellow being the highest intensity and black the lowest. In addition, the total CNT G-mode intensity from all 1600 spectra within a scan area was calculated and the mean and standard deviation of the CNT G-mode signal in a lane was calculated from eight scan areas, a measure of the relative CNT abundance in that lane. Figure 6(A) shows four representative Raman area scan images acquired from embryo lysates. The unknown amount of CNT in an embryo lysate was determined based on its G-mode signal intensity and the calibration curve constructed from the corresponding CNT standards in the same gel. An example of pSWNT-PF108 standard calibration curve and the quantitation of three embryo lysates were shown in Figure 6(B). All Raman data analysis, including background subtraction, peak area intensity integration and scan area statistical analysis were performed using WITec Project 2.10 software (WITec Focus Innovations, Ulm, Germany).

Results

Preparation and characterization of CNTs

For *in vivo* CNT toxicity assessments using the zebrafish embryo model, four commercially available CNT products were selected: a pair of pristine and carboxylated MWNTs and a pair of pristine and carboxylated SWNTs. These CNTs represent a spectrum of commonly-used nanotube types, diameters and surface functional modifications. The pMWNTs used here were previously characterized with respect to preparing suspensions of pMWNTs in Pluronic[®] surfactants and were not toxic to cultured NRK cells (Wang et al., 2013b). We also confirmed here that pMWNTs suspended in PF108 at $200\text{ }\mu\text{g/mL}$ had little effect on the proliferation of RAW 264.7 cells, while a similar preparation of cMWNTs had only a mild effect. We had previously characterized pSWNTs and cSWNTs suspended in bovine serum albumin solution and noted that the cSWNTs were toxic to cultured NRK cells, but that the pSWNTs were not (Wang et al., 2011) (pSWNTs are referred to as C2 and cSWNTs are referred to as C3 in this publication). Similar results were found here using PF108 as surfactant, where cSWNTs were toxic and pSWNTs were not toxic to RAW 264.7 cells. Thus, the pMWNTs, cMWNTs, pSWNTs and cSWNTs studied here offered the opportunity

to assess whether their effects on mammalian cells would be replicated with an intact animal model, embryonic zebrafish.

A general workflow for making stable aqueous suspensions of CNTs in PF108 involves bath sonication, centrifugation and dialysis (Scheme 1). Previous work showed that sonication of Pluronic® dispersants generates degradation products that are toxic to mammalian cells and may interfere with toxicity tests (Wang et al., 2013b). The dialysis of CNT-PF108 suspensions against intact non-sonicated Pluronic® in Scheme 1 removes the potentially toxic sonolytic PF108 materials (Wang et al., 2013b).

Physicochemical tests were routinely performed on the intermediate and the final products of CNT-PF108 suspensions. The CNT particle size distribution in various CNT-PF108 suspensions was analyzed by DLS. As shown in Table S1, the pMWNT-PF108 and cMWNT-PF108 suspensions had mean hydrodynamic diameters (HDD) of 95 ± 4 nm and 91 ± 5 nm, respectively, with a small polydispersity index (PDI) of 0.19, suggesting that the suspended MWNT particles were homogeneous in size with no evidence of aggregation. Similarly, the mean HDD of SWNT particles in pSWNT and cSWNT suspensions were 142 ± 3 nm and 115 ± 17 nm with corresponding PDI of 0.39 and 0.33. The dialysis procedure in Scheme 1 did not induce CNT aggregation in the suspensions, indicated by the negligible differences in mean HDD and PDI values acquired before and after dialysis (Table S1). Also, the CNTs remained well-dispersed in PF108 suspensions over time in storage, as validated by DLS analysis (data not shown). The zeta potentials of dialyzed and undialyzed CNT nanoparticles were measured in 0.1mM PF108 solutions (Table S1), a model for CNTs in the zebrafish embryo media. cMWNTs and cSWNTs had mildly more negative zeta potentials than their pristine counterparts, as expected. It is also interesting that there was a trend to slightly more negative zeta potentials in dialyzed material, which might be attributed to the removal of charged small molecular weight Pluronic® sonolysis fragments that could shield the negative charges on CNTs.

Generation and removal of toxic PF108 sonolysis products upon sonicating PF108

Because Pluronic® sonolysis products are toxic to cultured mammalian cells (Wang et al., 2013b), the possibility that sonication of PF108 alone produced materials toxic to zebrafish embryos was investigated prior to assessing the embryotoxicity of sonicated CNT-PF108 suspensions. Dechorionated embryos at 8 hpf were exposed to different concentrations of PF108 sonicated for either 1 or 4 h. Mortality of the control and test groups, 24 embryos each, was recorded at 24 and 120 hpf. At 1 h of sonication, there was little mortality at 0.1mM PF108, but significant mortality at 0.2mM (Figure 1). After 4 h of sonication, mortality was 100% at both 0.1 and 0.2mM PF108 during the first 24 h of exposure. These data demonstrate that sonicating PF108 has the potential to generate degradation products that are toxic to developing zebrafish, but also suggests safe conditions for preparing suspensions of CNT-PF108; namely, 1 h of sonication with an exposure not exceeding 0.1 mM.

To further ensure that Pluronic® sonolysis products would not impact the embryo toxicity analysis, the presence of PF108 fragments in the suspensions after sonication and their subsequent removal by dialysis were confirmed using SDS-PAGE and BaI₂ staining as

previously described (Murali et al., 2015; Wang et al., 2013b). Figure S1, lanes 1–3, show PF108 sonicated for 0, 1 or 4 h and stained with BaI₂ following electrophoresis to separate intact from degraded PF108. With an increase in sonication time, the intensity of the degradation fragments, indicated by a red arrow pointing to the low molecular mass L band in the gel, increased while the intensity of the intact PF108 material, primarily in the high molecular mass H band, decreased. Figure S1, lanes 4, 6, 8 and 10, show PF108 in various CNT-PF108 suspensions before dialysis, demonstrating that the prominent PF108 fragments are present in the L band region of the gel. After dialysis (Figure S1, lanes 5, 7, 9 and 11), the PF108 degradation fragments were greatly reduced in the L band region, indicating the successful removal of PF108 degradation by-products from CNT-PF108 suspensions. In summary, sonicating CNT-PF108 for an hour or more generated PF108 degradation products that were visible in gels after electrophoresis and that were toxic to zebrafish embryos above PF108 concentrations of 0.1 mM. CNT-PF108 suspensions were nevertheless dialyzed to further ensure the removal of any toxic PF108 residual material prior to embryo toxicity studies.

Toxicity assessment of CNT-PF108 suspensions with zebrafish embryos

The effects of various CNT-PF108 suspensions on zebrafish embryos were assessed before and after dialysis. At 8 hpf, groups of 24 embryos were exposed individually to media that contained CNT-PF108 suspensions at 0, 50, 100 or 200 µg/mL CNT concentrations. The CNT-PF108 suspensions had been sonicated for 1 h and diluted into water to achieve the desired CNT concentration, while keeping the final concentration of PF108 in the suspensions at 0.1 mM, conditions developed in the previous section to minimize the toxicity of sonolytic PF108 degradation fragments, even in samples that were not dialyzed. Results were compared to controls that were exposed only to PF108 using Fisher's exact statistical test. At 120 hpf, the number of embryonic zebrafish alive and well, with compromised health, or dead in each test group or in the control group were recorded and presented as color-coded bars (green for alive and well, yellow for compromised health and red for dead), as shown in Figure 2. Zebrafish that were necrotic or lacked a heartbeat were recorded as dead, those that were alive and fully developed with no sublethal malformations were counted as alive and well, and individuals that were alive but had pericardial edema (PE), axis curvature (AXIS), yolk sac edema (YSE), and/or cranial and facial abnormalities (CF) were considered to have compromised health (Figure 3).

The results in Figure 2 for pMWNTs (Panel A) and cMWNTs (Panel B) revealed little or no effects on the embryos under the test conditions. Representative images of embryos further document the absence of developmental abnormalities with exposure to either pMWNTs or cMWNTs (Figure 3). There was only 4% mortality with undialyzed pSWNTs until the highest concentration of 200 µg/mL, which resulted in 38% mortality, but mortality dropped to 8% at this concentration after dialysis (Figure 2 Panel C and images in Figure 3). In contrast, there was significant mortality in undialyzed samples of cSWNTs at 100 µg/mL and higher, whereas with dialyzed samples, mortality was rare, but developmental abnormalities were obvious (Figure 2 Panel D and Figure 3). The toxicity of pSWNTs and cSWNTs used in these studies has been well-characterized with mammalian cells where cSWNTs, but not pSWNTs, were generally toxic (Wang et al., 2011). However, the toxicity

of the cSWNTs was not due to intact nanotubes but was correlated with the presence of non-tubular amorphous carbon fragments apparently generated by the oxidizing conditions used to carboxylate the CNTs (Wang et al., 2011). Thus, it is possible that the embryo toxicity of the cSWNTs could also be attributed to the generation of a toxic by-product during the oxidation process.

The results in Figures 2 and 3 suggest that there is very little effect of CNTs well-suspended in PF108 on the development of zebrafish embryos, even at high CNT concentrations. One explanation for this could be that the embryos did not take up the CNTs, so there is little opportunity for the CNTs to adversely affect the embryos. To directly address this concern, we developed a method to measure the accumulation of CNTs by embryos that enables a direct comparison of CNT effects with the amount of CNTs associated with the embryos, as described next.

Preparation of zebrafish embryo lysates and quantitation of CNTs by SDS-PAGE

Embryos exposed to dialyzed CNT-PF108 suspensions at different concentrations and unexposed control embryos were lysed in a buffer containing SDS and then processed for SDS-PAGE. Embryos exposed to 0, 50, 100 or 200 $\mu\text{g/mL}$ of pSWNT-PF108 suspensions were used to illustrate the development of the approach. Pigments in the embryos imparted an obvious brown color to the lysates (Figure S2), which were readily visible in SDS-PAGE gels and interfered with detecting CNTs in the lysates after scanning the gels with a flat-bed optical scanner (Figure 4A, top gel, lanes 1–4). Soaking the gels in 3% H_2O_2 bleached the pigments, but not the CNTs (Figure 4A, bottom gel, lanes 1–9). After scanning the gel with an optical flat-bed scanner, the intensities of the CNT bands from the lysates (Figure 4A, bottom gel, lanes 1–4) and the CNT standards (Figure 4A, bottom gel, lanes 5–9) were readily quantified with ImageJ software. As shown in Figure 4B), a calibration curve was constructed based on the known amount of pSWNTs loaded in lanes 5–9 and their corresponding pixel intensities. The linear calibration curve was then used to estimate the amounts of pSWNT present in zebrafish lysate samples. Note that negligible pixel intensity above background was detected in the lysate not exposed to CNTs (Figure 4A, bottom gel, lane 1), suggesting that bands in lanes 2–4 represented above-background CNTs extracted from the embryos and that the intensities of these bands was a function of exposure concentration.

Figure 5 summarizes the quantitation data for the accumulation of CNTs by embryos exposed to 0, 50, 100 or 200 $\mu\text{g/mL}$ of dialyzed CNTs suspended in PF108 from 8 to 120 hpf. The accumulation of pMWNTs and cMWNTs (Figure 5A) is a function of concentration and is in the range of 10–15 ng/fish at an exposure concentration of 200 $\mu\text{g/mL}$. Similar results were found for both pSWNTs and cSWNTs (Figure 5B), except that cSWNTs showed reduced accumulation compared to the other CNTs. This is most likely because the health of the embryos was compromised (Figure 2D and Figure 3B) by the sublethal toxicity of the cSWNTs to the point that the active accumulation of CNTs from the environment was reduced. Weak respiration leading to less exchange through the gills, for example, could reduce CNT uptake.

Identifying and quantifying CNTs in zebrafish lysates using laser scanning confocal Raman microscopy

The basis of CNT quantitation described in the previous section is the acquisition of pixel intensities from bands in an SDS gel using an optical flat-bed scanner; however, this method provides no information about the structure of the material in the bands. An alternative approach is to analyze material in the bands by Raman scattering, which can provide structural information, based on the distinctive Raman signature of CNTs, and also provide quantitative information on the amount of material present. To validate the presence of CNT in zebrafish lysates, the same gel described in Figure 4(A) containing pSWNTs was preserved between two sheets of cellophane and allowed to dry into a thin film. A laser scanning confocal Raman microscope was used to analyze bands in the gel as described in the “Methods” section, including Figures S3 and S4. Figure S3 shows bands in the SDS gel, and an expanded view of one band to illustrate how the Raman scattering data were acquired. Sample areas were defined for scanning at eight locations evenly spaced within the band. Figure S3 also shows an expanded view of one of the eight $160 \times 160 \mu\text{m}$ areas that were scanned for the band in lane 4. The complete Raman spectra from each of the 1600 pixels in the area were collected and analyzed for the presence or absence of CNTs. Four representative spectra for the bands in lanes 1–4 of the gel are presented in Figure S4. CNTs have a characteristic peak at 1595 cm^{-1} (termed the G-mode) corresponding to tangential vibrations of the carbon lattice, and this peak is present in the Raman spectra acquired in lanes 2, 3 and 4 but absent in lane 1 (Figure S4), direct evidence that CNTs are present in embryos exposed to pSWNT-PF108 suspensions.

In addition, the area under the G-mode peak is proportional to the amount of material and Figure 6(A) shows the intensities of the integrated G-mode peak values for one of the eight $160 \times 160 \mu\text{m}$ areas of each band in the gel plotted on a heat scale with yellow being the most intense. The integrated total CCD counts for each of the areas are also provided in Figure 6(A). A dose-dependent accumulation of pSWNTs in zebrafish embryos is evident. The mean and standard deviation of the pSWNT G-mode signal were calculated from eight scan areas in each lane, as described in Figure S3. Figure 6(B) plots the accumulated data for the standards (lanes 5–10 in Figure 4A) and from this standard curve the amount of CNTs in lysates (lanes 1–4 in Figure 4A) is estimated. For pSWNT exposure concentrations of 50, 100 and $200 \mu\text{g/mL}$, the corresponding CNT amounts were 1.5 ± 0.6 , 4.1 ± 0.6 and $6.0 \pm 0.9 \text{ ng/fish}$, respectively. These data are in reasonable agreement (within a factor of about 2) with the quantitation data obtained for pSWNTs in Figure 5(B) and provide a clear demonstration that CNT accumulation by zebrafish embryos is a function of exposure concentration.

In summary, the data in Figure 5 show the dose-dependent accumulation of CNTs by zebrafish embryos. Independent analysis of pSWNT accumulation by Raman spectroscopy in Figure 6 (and supporting figures) directly verified the presence of CNTs in the SDS gel bands for this sample, and validated the quantitative data obtained by the optical scanning method used in Figure 5.

Discussion

One objective of the present work was to evaluate the use of the Pluronic®-based surfactant PF108 for suspending CNTs in toxicity studies using the zebrafish embryo model. PF108 is a very effective dispersant for CNTs, allowing the presentation of bioavailable unbundled CNTs to embryos. Bioavailability is promoted in natural ecosystems by organic matter and surfactants, such as humic acid that are known to help disperse CNTs (Hyung et al., 2007; Kennedy et al., 2008, 2009). There is a large body of work in which Pluronic® surfactants have been used to study the toxicity of CNTs in mammalian cell culture systems and the availability of data with fish using the same surfactant enables comparison of results across animal models and *in vitro* to *in vivo*. We are aware of only two reports in which Pluronic®-based surfactants have been used in CNT toxicity studies with zebrafish. Filho et al. (2014) prepared a MWNT network using Pluronic® F-127 and assessed the effect on adult zebrafish. They purposely did not prepare well suspended MWNTs as their objective was to study MWNT networks, and they found no genotoxic effects of the MWNTs. Pan et al. (2011) suspended amide functionalized SWNTs in Pluronic® F-68 by sonication overnight and observed dose-dependent toxicity to zebrafish embryos. However, it is not clear to what extent the results may have been influenced by the generation of potentially toxic Pluronic® degradation products upon extended sonication (Wang et al., 2013b). In the work reported here, sonication of PF108 did produce surfactant solutions that were toxic to zebrafish and care was taken to remove the toxic material by dialysis prior to CNT toxicity testing, avoiding potential artifacts. Previous work with mammalian cells showed that the toxicity of sonicated Pluronic® surfactants was due, at least in part, to reactive oxygen species generated during sonication of the surfactant, suggesting that a similar toxic mechanism likely underlies the lethality of sonicated surfactant with embryos studied here (Wang et al., 2013b).

Of the four CNTs tested, the pMWNT and cMWNT suspensions in 0.1mM PF108 had little effect on embryo development, whether or not the suspensions had been dialyzed to remove low levels of potentially toxic PF108 sonolysis products. The results with pMWNTs are significant because, to our knowledge, this is the first report of toxicity studies where embryos have been presented with well-solubilized pMWNTs at such high concentrations. The results with cMWNTs are consistent with data in the literature that oxidized MWNTs with low levels of carboxylation are also not toxic to zebrafish embryos (Adenuga et al., 2013; Gilbertson et al., 2015). The undialyzed pSWNT suspensions caused significant mortality but only at the highest concentration of 200 µg/mL, whereas there was very little effect in the dialyzed samples. In contrast, cSWNTs affected multiple parameters of embryo development. With the undialyzed cSWNT samples there was significant mortality at 100 µg/mL, while the dialyzed samples had adverse effects on development at all concentrations tested, but little mortality. Since the PF108 degradation products present in the 0.1mM PF108-1 h solution were not toxic to embryos in the absence of CNTs (Figure 1), the difference observed between the undialyzed and dialyzed SWNT suspensions, regardless of carboxylation, suggests that PF108 degradation products may become toxic in the presence of SWNTs, perhaps synergistically with material derived from the SWNT samples. This

finding emphasizes the importance of removing potentially toxic Pluronic[®] sonolysis products by including the dialysis step after sonication, as shown in Scheme 1.

In our previous work, we found that the cSWNTs were toxic to mammalian normal rat kidney (NRK) epithelial cells, whereas pSWNTs and pMWNTs were not (Wang et al., 2011). Here, we confirmed this toxicity pattern using mouse macrophage RAW 264.7 cells, where only cSWNTs exerted significant reduction in cell proliferation at all concentrations tested. These observations lend confidence to the results since similar results are observed in both cultured mammalian cells and the whole animal zebrafish embryo model (Wang et al., 2011, 2013b). The toxicity of the cSWNTs with mammalian cells in prior work correlated with the presence of small oxidized carbon fragments that could be separated from the cSWNTs, suggesting that the cSWNTs themselves were not toxic (Wang et al., 2011). We hypothesize that these oxidized fragments may be why the cSWNTs were also toxic to embryonic zebrafish and work to better understand the mechanism of toxicity with both mammalian cells and zebrafish is in progress.

The absence of significant toxicity with CNT exposure inevitably raises the question of whether the CNTs were bioavailable and accumulated by the organisms exposed. To directly address this concern, we adapted the SDS-PAGE procedure for extracting and measuring CNTs in mammalian cells to zebrafish embryos. Many studies report on the toxicity of exposure concentrations; however, the SDS-PAGE method provides a way to assess uptake, and therefore bioaccumulation, after a water-borne nanomaterial exposure. The dynamic nature of nanomaterials in aquatic environments can drastically affect bioavailability to test organisms and our method provides a toxic response matched with an internal dosage of material, further supporting the non-toxic nature of the CNTs. Based on quantitation by optical scanning and Raman imaging, there was a dose-dependent accumulation of all CNTs by the embryos. The CNT uptake results acquired using the faster and easier optical scanning methods were confirmed by the presence of the signature CNT G-mode by the Raman imaging method. This is the first time to our knowledge that quantitative Raman analysis has been used to address the uptake of CNTs by zebrafish. Accumulation of pMWNTs, cMWNTs and pSWNTs by embryos was significant but nevertheless not toxic, even after increasing the vulnerability of the embryos by removal of the chorionic barrier before exposure. The notable observation is that the accumulation of cSWNTs by embryos after dialysis was reduced compared to the other CNT products. This can be explained because all of the embryos exposed to dialyzed cSWNTs were unhealthy with a range of abnormalities that could reduce uptake; for example, weak respiration would reduce exchange through the gills resulting in less CNT accumulation. The correlation between the toxicity of the cSWNT sample with low uptake also suggests that the association of the CNTs with fish is not entirely the result of passive surface binding: If it were, then dead or dying fish, which still have surfaces, would register CNTs adhering to accessible surfaces just like healthy fish, but they did not. The available data do not provide information about where the CNTs accumulated in the developing zebrafish, but reasonable possibilities are the gills, mouth or gut. While the current study shows increasing CNT bioaccumulation by zebrafish embryos after an acute exposure has little toxic effect, the approach is applicable in future research to assess the potential CNT toxicity to aquatic organisms after long-term chronic exposure.

Conclusions

Sonication of CNTs with Pluronic[®] surfactants produces Pluronic[®] sonolysis by-products that are toxic to zebrafish embryos, similar to what has been demonstrated for mammalian cells (Wang et al., 2013b). When the toxic Pluronic[®] fragments were removed, there was little effect of pMWNTs, cMWNTs or pSWNTs on embryo viability and development, even at 200 µg/mL. Interestingly, cSWNTs were toxic, likely the result of carbonaceous oxidation products in the cSWNT preparations, as noted previously with mammalian cells (Wang et al., 2011). The accumulation of CNTs by embryos was measured using an SDS-PAGE method adapted to zebrafish embryos, and the dose-dependent uptake of the CNTs was observed. The overall conclusion of this work is that, except for cSWNTs that contain carbon oxidation products hypothesized to be toxic, CNTs accumulate in zebrafish embryos over a 5-day period but are nevertheless not overtly toxic.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

The authors thank the core facility at the Sinnhuber Aquatic Research Laboratory (Corvallis, OR) for supplying the zebrafish embryos (National Institute for Environmental Health and Sciences, Grant P30-ES000210).

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Figure 1.

Zebrafish embryotoxicity of PF108 as a function of sonication time and concentration.

Percent mortality in dechorionated zebrafish embryos (n=24 per group) exposed to 0.1 or 0.2mM PF108 after sonication for 0, 1, or 4 hours. Control embryos not exposed to PF108 were alive and well throughout the experiment. Solid bars represent mortality at 24 hpf and patterned bars represent mortality at 120 hpf.

Figure 2.

Effect of CNT type, concentration, and dialysis on zebrafish embryotoxicity of CNT-PF108 suspensions. All CNT-PF108 suspensions were prepared by 1 h sonication in 0.2mM PF108, and centrifuged with or without subsequent dialysis to remove PF108 degradation by-products, as described in the Methods section. The undialyzed and dialyzed CNT-PF108 suspensions were first diluted in 0.2mM sonicated (PF108-1 h) or fresh non-sonicated (PF108-0 h) PF108 solutions, respectively, prior to 1:1 dilution with MilliQ water to achieve a final 0.1mM PF108 and CNT concentration of 50, 100, or 200 µg/mL. Groups of 24 dechorionated zebrafish embryos were exposed to CNT-PF108 suspensions at increasing concentrations at 8 hpf. The control group was incubated in DI water that contained no PF108 or CNTs. The number of embryos that were alive and well (green), with compromised health (yellow), or dead (red) at 120 hpf after exposure to (A) pMWNTs (B) cMWNTs (C) pSWNTs or (D) cSWNTs suspensions at 50, 100, and 200 µg/mL before or after dialysis. Green indicates fish that were alive with no sublethal malformations, yellow indicates fish that were alive, but had a combination of pericardial edema, axis curvature, and yolk sac edema, and red indicates fish that were dead.

(A)

Sample	Sonication (h)	Conc. (mM)	120 hpf	Developmental Abnormality
PF108	0	0.1		None
	1	0.1

(B)

Sample	Dialysis	Conc. (µg/ML)	120 hpf	Developmental Abnormality
pMWNT	-	200		None
	+	200
cMWNT	-	200
	+	200
pSWNT	-	200
	+	200
cSWNT	-	100		Severe PE, YSE, CF
	-	200		Severe PE, YSE, Severe AXIS, CF
	+	50		Minor PE
	+	100		PE, YSE, CF
	+	200		PE, YSE, CF

Figure 3.

Impact of PF108 and CNT-PF108 suspensions on zebrafish embryonic development. Representative images of zebrafish exposed to (A) 0.1mM of sonicated or non-sonicated PF108, and (B) various undialyzed or dialyzed CNT-PF108 suspensions at 50, 100, or 200 µg/mL at 120 hpf. Panel A shows fish exposed to PF108 alone that were asymptomatic whereas panel B shows fish exposed to various CNT-PF108 suspensions that were either asymptomatic or with compromised health. Sublethal malformations listed under developmental abnormalities include pericardial edema (PE), yolk sac edema (YSE), axis curvature (AXIS), and cranial and facial abnormalities (CF).

Figure 4.

Quantitation of CNTs extracted from zebrafish by SDS-PAGE/ H_2O_2 treatment and optical image scanning. (A) A representative gel was loaded with zebrafish lysates in lanes 1 – 4 (each lysate prepared from 10 embryos exposed to dialyzed pSWNT suspensions at 0, 50, 100, or 200 $\mu\text{g/mL}$ pSWNT concentrations during the interval from 8 to 120 hpf), with known standards of pSWNTs in lanes 5 – 9 (50, 75, 100, 125, and 150 ng, respectively), and with a blank in lane 10 that contains no CNTs or lysate. After electrophoresis, an optical image of the gel on the top without H_2O_2 (labeled “No H_2O_2 ”) treatment was acquired with a flatbed scanner. The optical image of the same gel on the bottom (labeled “ H_2O_2 treated”) was acquired after the gel was treated with 3% H_2O_2 overnight to bleach pigments in the zebrafish lysates. (B) The circles (●) show the linear relationship between pixel intensities summed from the pSWNT bands of the pSWNT-PF108 standards (shown in panel A, bottom gel image, lanes 5 – 10) and the known amount of pSWNTs loaded in the corresponding lanes. The calibration curve, shown as best-fit linear equation, was used to estimate the unknown amounts of CNTs in the lysate bands (lanes 1 – 4) in the same gel image. The diamond-shaped data points (◇) show the calculated CNT amounts and corresponding pixel intensities summed for the lysate bands in lanes 2 – 4. Negligible pixel intensity above background level was detected in lane 1.

Figure 5.

Accumulation of CNTs in zebrafish exposed to various concentrations of dialyzed pristine and carboxylated CNT-PF108 suspensions. Zebrafish lysate samples were prepared from dechorionated zebrafish embryos exposed to (A) dialyzed pristine or carboxylated MWNT-PF108 suspensions at 0, 50, 100, or 200 µg/mL MWNT concentrations, or (B) dialyzed pristine or carboxylated SWNT-PF108 suspensions at 0, 50, 100, or 200 µg/mL SWNT concentrations during the interval from 8 to 120 hpf. Each lysate sample was prepared from 10 embryos at 120 hpf and the amount of CNTs in the lysate was quantified by SDS-PAGE after H_2O_2 treatment of the gel, as described in Methods and illustrated in Figure 4 for pSWNTs. Each data point is the mean from at least three independent trials, and the error bars show the standard deviations.

Figure 6.

Quantitation of CNTs in zebrafish lysates validated by laser scanning confocal Raman microscopy of SDS gels. (A) Representative Raman scan area images acquired from the CNT bands in lanes 1 – 4 of the H_2O_2 treated gel shown in Figure 4A. A total of 1600 Raman spectra were acquired within a $25\,600\,\mu m^2$ scan area and the integrated intensity, in CCD counts, of the CNT-specific G-mode signal between $1540 - 1640\,cm^{-1}$ of each spectrum was quantified, background subtracted, and plotted on a map using a color intensity scale ($0 - 2 \times 10^5$ CCD counts) where yellow is the brightest and black is the lowest. The scale bars are $30\,\mu m$. The total G-mode signal intensity from all the 1600 spectra in each scan area was calculated and listed below the corresponding image. Identical Raman analysis was performed on all 10 lanes, 8 scan areas per lane, using the same laser power density and integration time, as described in Figure S3. The average and standard deviation of the total G-mode Raman intensity per scan area was calculated from all 8 scan areas acquired from the same lane. (B) The circular data points (●) show the average total G-mode Raman signal intensity per scan area acquired from lanes 5 – 10 that contained pSWNT standards of known amounts as described in Figure 4A. A calibration curve shown as best-fit linear equation was used to estimate the amounts of CNTs in the lysates loaded in lanes 1 – 4 of the same gel. The diamond-shaped data points (◇) show the calculated CNT amounts and the average integrated Raman G-mode intensities of the lysate bands in lanes 2 – 4 with the corresponding amounts of pSWNT extracted from these zebrafish lysates.

Scheme 1.

Sonication, centrifugation and dialysis procedures to prepare CNT-PF108 suspensions.