



Published in final edited form as:

Carbon N Y. 2007 August ; 45(9): 1891–1898.

***In vivo* evaluation of carbon fullerene toxicity using embryonic zebrafish**

Crystal Y. Usenko, Stacey L. Harper, and Robert L. Tanguay*

Department of Environmental and Molecular Toxicology and the Marine and Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR, 97331.

Abstract

There is a pressing need to develop rapid whole animal-based testing assays to assess the potential toxicity of engineered nanomaterials. To meet this challenge, the embryonic zebrafish model was employed to determine the toxicity of fullerenes. Embryonic zebrafish were exposed to graded concentrations of fullerenes [C₆₀, C₇₀, and C₆₀(OH)₂₄] during early embryogenesis and the resulting morphological and cellular responses were defined. Exposure to 200 µg/L C₆₀ and C₇₀ induced a significant increase in malformations, pericardial edema, and mortality; while the response to C₆₀(OH)₂₄ exposure was less pronounced at concentrations an order of magnitude higher. Exposure to C₆₀ induced both necrotic and apoptotic cellular death throughout the embryo. While C₆₀(OH)₂₄ induced an increase in embryonic cellular death, it did not induce apoptosis. Our findings concur with results obtained in other models indicating that C₆₀(OH)₂₄ is significantly less toxic than C₆₀. These studies also suggest that the embryonic zebrafish model is well-suited for the rapid assessment of nanomaterial toxicity.

1. Introduction

Unique physicochemical properties of nanomaterials make them attractive for a wide range of novel applications in the electronics, healthcare, cosmetics, technologies and engineering industries [1–6]. The rapid discovery and development of new nanomaterials will undoubtedly increase the potential for human and environmental exposures [7]. Lack of toxicological data on nano-sized materials makes it difficult to determine if there is a risk associated with nanomaterial exposure. Timely evaluation of nanomaterial toxicity will provide this critical data, improve public trust of the nanotechnology industry, assist regulators in determining environmental and health risks of commercial nanomaterials, and provide industry with information to direct the development of safer nanomaterials and products [8–10]. Thus, there is an immediate need to develop rapid, relevant and efficient testing strategies to assess emerging materials of concern.

Numerous biological models have been used for toxicological assessments. *In vitro* techniques, such as cell culture, are often preferred because they are rapid, efficient and cost effective. While these studies are useful, direct translation to a human health risk is often difficult to infer. *In vivo* (whole animal) studies, on the other hand, can provide improved prediction of the biological response in intact systems. Since *in vivo* studies often employ rodent models;

*Corresponding Author: Robert L. Tanguay, Ph.D., Department of Environmental and Molecular Toxicology, Environmental Health Sciences Center, Marine and Freshwater Biomedical Sciences Center, Oregon State University, Corvallis, OR, 97331-7301, 541-737-6514 (voice), 541-737-7966 (fax), robert.tanguay@oregonstate.edu.

Publisher's Disclaimer: This is a PDF file of an unedited manuscript that has been accepted for publication. As a service to our customers we are providing this early version of the manuscript. The manuscript will undergo copyediting, typesetting, and review of the resulting proof before it is published in its final citable form. Please note that during the production process errors may be discovered which could affect the content, and all legal disclaimers that apply to the journal pertain.

assessments are generally expensive, time-consuming and require extensive facilities for housing experimental animals. Cost, labor, time and infrastructure requirements can be significantly reduced by replacing the traditional rodent model with the embryonic zebrafish model. Embryonic zebrafish are a well-established model for studying basic developmental biological processes (reviewed in 11) and for toxicological assessments [12–14]. Zebrafish are vertebrates that share many cellular, anatomical and physiological characteristics with other vertebrates. Their small size, rapid development and short life cycle make zebrafish logistically attractive for rapidly evaluating integrated system effects [15,16].

Research presented herein examines the utility of embryonic zebrafish as a model for rapid assessment of nanomaterial toxicity. For proof-of-concept, three carbon fullerenes varying in size and surface functionalization [C_{60} , C_{70} , and $C_{60}(OH)_{24}$] were tested in the system and the results were compared to available nanotoxicity data obtained using different methods, test systems and routes of exposure. Carbon fullerenes were chosen because much nanotechnology attention, particularly nanotoxicological evaluations, has focused on carbon-based nanomaterials. C_{60} , otherwise known as “bucky balls” or “Buckminsterfullerenes”, have been proposed for use in fuel cells, groundwater remediation, cosmetics and drug delivery. C_{70} , a common by-product of C_{60} synthesis, is likely to be found in products containing C_{60} unless extensive purification steps are taken. The lack of solubility of C_{60} in water has limited its research in biological systems [17]. However, surface functionalization with hydroxyl groups produces $C_{60}(OH)_{24}$, which is readily soluble in water. Similarly to C_{60} , hydroxylated C_{60} have been proposed for use in groundwater remediation and drug delivery [2,17,18]. Numerous reports suggest that differences in size and surface functionalization among the three fullerenes tested are reflected in their relative toxic potential [19–21].

2. Methods

2.1 Preparation of Solutions

Fullerenes C_{60} and C_{70} were obtained from Sigma Aldrich, WI (98+% and 99% purity respectively) and hydroxylated C_{60} was obtained from MER Corporation, AZ (99.98% purity). The purity of the materials used in these studies was not tested and assumed to be as stated by the manufacturer (2% and 1% higher order fullerene impurities in C_{60} and C_{70} , respectively; 0.02% water and sodium impurities in $C_{60}(OH)_{24}$). Due to the insolubility of C_{60} and C_{70} in water, each was sonicated to form a uniform suspension in 100% dimethyl sulfoxide (DMSO). Stock solution of 50 ppm (C_{60} and C_{70}) and 500 ppm [$C_{60}(OH)_{24}$] were based on the maximum achievable concentration of nanomaterials to dissolve in DMSO after sonication. The highest exposure concentration was made by adding 1% of the DMSO-fullerene solution to fish water. Previous evaluations in our laboratory have demonstrated no adverse biological effect of DMSO at this concentration; however, use of a solvent will likely increase the uptake of material into the animal. It is important to point out that our studies were specifically designed to evaluate interactions between the nanomaterial and the biological system, not to mimic, for example, an environmental exposure scenario. Dilutions were made at 100 ppb increments with concentrations ranging between 100 and 500 ppb for C_{60} and C_{70} and 500 to 5000 ppb for $C_{60}(OH)_{24}$. C_{60} and C_{70} solutions were sonicated for 1.5 hours to ensure a uniform concentration and size distribution. Only 5 minutes of sonication was necessary to uniformly distribute $C_{60}(OH)_{24}$ in solution. Particle size distributions obtained using photon correlation spectroscopy on an N4 Plus submicron particle sizer (Beckman Coulter, Fullerton, CA) are indicated in Figure 1.

2.2 Exposure Protocol

Embryonic zebrafish were obtained from an AB strain of zebrafish (*Danio rerio*) reared in the Sinnhuber Aquatic Research Laboratory (SARL) at Oregon State University. Adults were kept

at standard laboratory conditions of 28°C on a 14h light/10h dark photoperiod in fish water (FW) consisting of reverse osmosis water supplemented with a commercially available salt solution (0.6% Instant Ocean®). Zebrafish were group spawned and embryos were collected and staged as described by Kimmel et al [22]. To increase bioavailability, the chorion, an acellular envelope surrounding the embryo, was removed via enzymatic reaction with pronase prior to exposure. Briefly, embryos at 24 hours post fertilization (hpf) were placed in 25 ml of FW with 50 µl of 50 mg/ml pronase for 10 minutes. Embryos were then rinsed with pure FW for 10 minutes prior to the initiation of nanomaterial exposure. Dechorionated embryos were immediately transferred to individual wells of a 96-well plate with 100 µl of prepared nanomaterial solution. Control animals were exposed to vehicle (1% DMSO) only. Non-treated animals (embryos grown in pure FW with the chorion intact) were retained to ensure embryo quality was good. The static nanomaterial exposure continued in sealed plates until 96 hpf, and the exposure solutions were changed every 24 hours. At 96 hpf, the embryos were rinsed and raised in pure FW. Each individual embryo was scored for morphological malformations and developmental delays daily for five days. The cumulative mortality rates were graphed and analyzed to estimate the lethal concentration to cause 50% embryonic mortality (LC₅₀).

2.3 Cellular Death Assays

To determine if *in vivo* fullerene exposures induced inappropriate cellular death, two independent cellular death assays were exploited. Cell death was detected in live embryos using acridine orange staining, a nucleic acid selective metachromatic dye that interacts with DNA and RNA by intercalation or electrostatic attractions. Acridine orange stains cells with disturbed plasma membrane permeability so it preferentially stains necrotic or very late apoptotic cells. Dechorionated embryos were exposed to carbon fullerenes at 24 hpf and were analyzed for cellular death at 36 hpf. The 36 hpf time point of assessment was selected because at this developmental stage, there were no overt morphological signs of toxicity. Embryos were rinsed with FW and then incubated in 100 µl of 5µg/ml acridine orange for one hour in the dark at 28 °C. Embryos were rinsed again with FW, mounted in low melt agarose (1% w/v, Promega, Madison, WI) and imaged using an Axiovert 200M Zeiss microscope (Carl Zeiss International, Germany) with a 546 nm filter and AxioVision software (Carl Zeiss International, Germany). For each captured image, the initial focal plane was the otolith. A 50 member z-series was collected and merged into one plane corresponding to approximately 300 micron thick section of the embryo. Whole-embryo fluorescence was measured and quantified using ImagePro Plus software (Media Cybernetics, Inc., Silver Spring, MD).

To more specifically quantify cellular death due to apoptosis, the terminal deoxynucleotidyl transferase (TdT)-mediated dUTP nick end labeling (TUNEL) assay was performed on whole mounted 36 hpf embryos exposed beginning at 24 hpf. The TUNEL assay fluorescently labels the blunt ends of double-stranded DNA breaks which are indicative of programmed cell death (apoptosis). Embryos were fixed overnight in 4% paraformaldehyde and stored in phosphate buffered saline (PBS) + 0.01% sodium azide at 4°C until analysis. Embryos were incubated in proteinase K (1 mg/ml) for 30 minutes at 37°C to permeabilize the tissue. The permeabilized embryos were rinsed in PBS and incubated in 25 µl of 1X TTase buffer (Roche Diagnostics Corp., Indianapolis, IN), 25 mM CoCl₂, and dH₂O (sterile H₂O) for 15 minutes at 37°C. Embryos were then transferred to a reaction solution (10 µl 5X TTase buffer, 4 µl 25 mM CoCl₂, 0.2 µl FL-dUTP, 0.25 µl TTase enzyme and 35.6 µl dH₂O) on ice, in the dark, for 60 minutes and incubated an additional 60 minutes in the dark at 37°C. Embryos were washed 3 times in PBS, mounted in 50% glycerol and analyzed on the fluorescent microscope (488 nm) as described for the acridine orange assay.

2.4 Statistics

All statistics were compiled using SigmaStat and plotted using Sigma Plot (SPSS Inc, Chicago, IL). Fischer's exact test was used to determine significant difference between control and treated groups ($p < 0.05$). All exposure groups consisted of 24 individually exposed embryos ($N=24$) unless otherwise noted with 80% confidence of significant difference. LC_{50} was calculated using probit and sigmoidal regression analyses. Significant difference from control for cell death assays was determined using one-way ANOVA ($p < 0.05$).

3. Results

3.1 In Vivo Toxic Potential of Carbon Fullerenes

The advantage of evaluating the consequence of nanomaterial exposure during development is that perturbations of the developmental program can lead to a number of easily detected responses. Again, the goal of these studies was to determine if the zebrafish embryonic responses could be used to determine, and eventually predict, the structural properties of nanomaterials that produce toxicity. In our system, an adverse biological response to nanomaterial exposure can result in delayed development, morphological malformations (i.e. body axis, eye, snout, jaw, otic vesicle, notochord, heart, brain, somite, fins) and/or behavioral abnormalities (i.e. hyperactivity, hypoactivity, paralysis). Embryos exposed to graded concentration of C_{60} , C_{70} , and $C_{60}(OH)_{24}$ were scored daily for these effects. Concentrations above 200 ppb C_{60} and C_{70} resulted in 100% mortality during the first 48 hours (Figure 2 a,b). $C_{60}(OH)_{24}$ exposure did not result in significant mortality until the exposure concentration was above 4000 ppb (Figure 2c). The estimated LC_{50} for C_{60}/C_{70} - and $C_{60}(OH)_{24}$ -exposures were approximately 200 ppb and 4000 ppb, respectively. Sublethal morphological and developmental effects are presented in Figure 2 as cumulative values for the 5 day scoring period, not accounting for mortality that may have occurred before or after scoring the effects. Representative pictures of the malformations resulting from exposure are shown in Figure 2 (d,i). Embryonic exposure to 200 ppb of C_{60} and C_{70} resulted in delayed development (approximately 12–20 hours according to staging of embryogenesis) and a specific caudal fin malformation (Figure 2 d,e). Exposure to C_{60} also resulted in significant pericardial edema and yolk sac edema at 200 ppb (Figure 2 h,i). Neither C_{60} nor C_{70} induced sublethal responses at concentrations higher than 200 ppb due to the high mortality observed at these concentrations within the first 24 hours of exposure. Concentrations less than 2500 ppb of $C_{60}(OH)_{24}$ did not elicit a significant response; whereas, concentrations over 2500 ppb induced pericardial edema, yolk sac edema and pectoral fin malformations (Figures 2 f,g). Exposure to 5000 ppb $C_{60}(OH)_{24}$ resulted in an overall swelling of embryos and delayed development (approximately 15–20 hours).

3.2 Fullerene-induced Cellular Death

Cellular death assays were performed to determine if exposure to fullerenes would lead to an increase in cellular death in specific cells or tissues, prior to the overt signs of toxicity reported in Figure 2. C_{60} -exposed embryos exhibited a concentration-dependent increase in overall cellular death, significant at 100 ppb and higher (Figure 3). Cell death was detected throughout the head region (Figures 3 a–d) and down the notochord to the region of the caudal fin malformations (Figures 3 e–h). Conversely, embryos exposed to $C_{60}(OH)_{24}$ exhibited significant overall cell death only in the head region and only at a concentration of 5000 ppb (data not shown). For C_{60} exposures, acridine orange staining for cellular death was a more sensitive endpoint than any morphological malformations.

Acridine orange binds all cells undergoing cellular death, both necrosis and apoptosis, and the TUNEL assay is more specific for detecting cells undergoing programmed cellular death. Similar to the results of the acridine orange staining for overall cell death, there was a

concentration-dependent increase in apoptotic cellular death detected in the head (Figures 4 a–d) and trunk region (Figures 4 e–h) of C₆₀-exposed embryos. The total fluorescence of TUNEL positive (TUNEL⁺) cells was approximately half of the total fluorescence measured using acridine orange staining suggesting that at least a portion of overall C₆₀-induced cell death was attributed to induction of apoptotic pathways (compare Figures 3i,4i). In embryos exposed to C₆₀(OH)₂₄ there was no significant difference in apoptosis compared to control embryos, even at 5000 ppb C₆₀(OH)₂₄ (data not shown).

4. Discussion

The toxicological effects of carbon fullerenes were assessed *in vivo* using the zebrafish embryo as a model organism. Research presented herein clearly demonstrates the usefulness of this model as an effective platform to rapidly assess novel nanomaterial toxicity. Several attributes make embryonic zebrafish tractable for such studies. The embryos develop rapidly with most body organs formed within 48 hours; thus, thorough toxicological evaluations can be completed within just a few days. Due to the transparent nature of the embryos, numerous effects can be assessed non-invasively over the course of the experiment. Cellular death assays, the most sensitive endpoint defined in these studies, were completed within two days. Females produce hundreds of eggs weekly so large sample sizes are easily achieved for statistically powerful dose-response studies. This abundant supply of embryos also makes it possible to simultaneously assess the toxicity of a large number of materials in a short period of time.

There are additional advantages to using zebrafish as part of a comprehensive approach to nanomaterial risk assessment. Although beyond the scope of this paper, many routes of exposure (i.e. ingestion, injection and dermal) could also be assessed individually or in combination. Since zebrafish are amenable to genetic manipulations, biological targets and modes of action can be determined. Because embryos are transparent, tissue dose and distribution could potentially be determined using fluorescently labeled nanomaterials and laser scanning confocal microscopy. In addition, zebrafish attain sexual maturity by 90 days post-fertilization (dpf) making chronic studies feasible. Because zebrafish adults grow to an average size of 3–4 cm and are easy to maintain at high densities, the infrastructure and maintenance costs required for housing the large numbers of animals required for screening are relatively low. Such features are favorable for adapting this model system to high-throughput assays for nanomaterial toxicity evaluations.

The potential toxicity of fullerenes was evaluated during early vertebrate development for two important reasons. First, fundamental processes of development are remarkably conserved across species. Second, vertebrates at the earliest life stages are often more responsive to chemical insult (NRC, 2000). The probable molecular explanation for increased embryonic susceptibility is that during this period, the full repertoire of molecular signaling is necessary and active. It has been postulated that overall there are only 17 general molecular signaling pathways in vertebrates and that each of these is active during early development. Importantly, each of these pathways is essential for other cells in tissues later in life (NRC, 2000). Since development is highly coordinated requiring cell-to-cell communications, if nanomaterials perturb these interactions, then development would be expected to be disrupted.

Much of data, thus far, on the effects of fullerene exposure had been gained from *in vitro* methods (with few exceptions: Mori et al. 2006; Oberdorster 2004; Oberdorster et al. 2005; Tsuchiya et al. 1996b; Zhu et al. 2006) using C₆₀ or a derivative of C₆₀, such as hydroxylated C₆₀. There is almost no information regarding the toxicity of C₇₀. *In vitro* data may be of limited use in predicting *in vivo* responses especially since those results may be dependent on the cell culture system chosen for the experiment. For example, the cytotoxicity of C₆₀ to human dermal fibroblasts, human liver carcinoma cells (HepG2) and neuronal human astrocytes was found

to be dependent on cell type [23]. However, Isakovic and others found the toxicity of C_{60} and $C_{60}(OH)_n$ to be neither species nor cell specific, nor selective for primary or transformed cell lines [24]. Independent of evaluation method/system, studies on C_{60} and C_{60} derivatives consistently report on differences in physicochemical properties and biological effects [17, 25].

In our studies, embryo exposure to fullerenes elicited increased mortality, sublethal malformations and increased cellular death. Studies in an embryonic mammalian model (mouse) also report severe morphological malformations and increased mortality when exposed to C_{60} *in utero* [26]. All carbon fullerenes tested in our system produced deleterious effects to the developing fin regions indicative of signaling perturbation during early development. C_{60} and C_{70} elicited similar responses, indicating the small difference in size does not significantly affect the toxic potential of these materials. Functionalization of the C_{60} material with hydroxyl groups; however, decreased the toxic potential in our system. These results are similar to studies conducted in other systems [23,27–29]. In cell culture studies of C_{60} and $C_{60}(OH)_{24}$, Sayes (2004) reported that underivatized fullerenes were at least three orders of magnitude more toxic than their hydroxylated counterpart. In addition, the cytotoxic action of underivatized C_{60} was much faster and more effective than its hydroxylated derivative [24]. Results obtained from our studies are consistent with the predominant belief that hydroxylated- C_{60} is less toxic than underivatized C_{60} and that the toxicity of C_{60} can be diminished through manipulation of surface functionalization [30].

In addition to gross pathologies, the mechanism of cellular death induction is an important consideration because of its correlation with overall tissue damage. Necrosis tends to cause extensive tissue damage resulting in an inflammatory response *in vivo*; while apoptosis does not cause tissue damage since macrophages effectively remove apoptotic signaling cells. Prior cell culture studies have suggested that pristine and hydroxylated- C_{60} have distinct mechanisms for the induction of cellular death [24]. The cellular death assays in this study also demonstrated a difference in mechanism of cell death induction between C_{60} and $C_{60}(OH)_{24}$. Although C_{60} elicited a concentration-dependent response in both total cell death and apoptosis, $C_{60}(OH)_{24}$ did not induce an apoptotic response. We suspect that $C_{60}(OH)_{24}$ interacts with water and permeabilizes membranes upon uptake. If this is the mechanism by which the animals swelled in response to $C_{60}(OH)_{24}$ exposure, then apoptosis would not be expected to occur. Alternatively, the swelling observed in $C_{60}(OH)_{24}$ exposed embryos could be due to significant uptake of $C_{60}(OH)_{24}$ into the animal followed by a simple osmotic response. These findings are not consistent with cell culture studies that found hydroxylated- C_{60} to induce cell death with characteristics of apoptosis and C_{60} to induce cell death independent of apoptotic cell signaling [24]. These pathway level effects may be more cell or tissue specific, since fullerene-induced cell death (both necrosis and apoptosis) has been demonstrated in various cell culture lines with different effects dependent on the type of material, surface functionalization and cell culture system [19,24,31,32]. These discrepancies show a need for further evaluations into the mechanisms through which fullerenes initiate cellular death resulting in toxic effects.

The cellular death measured in this study could result from oxidative stress, the most often proposed mechanism of action for fullerene toxicity, or due to direct interaction of the fullerene with cells through an unknown mechanism. Oxidative stress can lead to a variety of downstream effects including lipid peroxidation, DNA and protein adduction and cellular death. Since there are limited tests that directly measure reactive oxygen species (ROS), researchers rely on detection of lipid peroxidation products and cell death as markers of oxidative stress. With few exceptions, underivatized C_{60} are reportedly toxic due to pro-oxidant behavior that results in cytotoxicity [24]. *In vitro*, C_{60} synergized the effects of oxidative stress-inducing agents and elicited cytotoxic action through ROS-mediated cell membrane lipid

peroxidation [23,24]. *In vivo*, C₆₀ induced oxidative stress and lipid peroxidation in the brain of juvenile largemouth bass [28]. However, a recent article by Gharbi and others has questioned whether derivations of C₆₀ really are less toxic than their pristine counterparts [33]. Their study reported that C₆₀ acted as a powerful antioxidant *in vivo* in rats with no acute or subacute toxicities [33]. Methods currently being developed to fluorescently detect ROS *in vivo*, will be applied to the embryonic zebrafish model in order to localize the stress in these transparent animals.

5. Summary and Conclusions

The effects of carbon fullerenes were assessed *in vivo* using the zebrafish embryo as a model organism for nanotoxicology evaluations. Research presented herein clearly demonstrates the usefulness of this model as an effective tool for rapidly assessing nanomaterial toxicity and investigating the details of exposure-related effects. The time involved for animal maintenance, experimental preparation and completion of these studies has been vastly improved over traditional *in vivo* studies. Our dynamic whole animal assay can be used to reveal the toxic potential of novel nanomaterials at the genetic, cellular and physiological level. Since these processes are highly conserved between zebrafish and humans, especially early in development, information gained by exploiting the unique advantages of one vertebrate model system can be immediately applied to predict effects in other systems. Furthermore, information gained using this model system can be used as rapid feedback for engineers designing novel nanomaterials, such that they can take into consideration potential toxicity to favor the development of materials with minimal toxicity.

Acknowledgements

We would like to thank Jane La Du for her technical assistance. These studies were partially supported by the Oregon State University Research Office, The Safer Nanomaterials and Nanomanufacturing division of the Oregon Nanoscience and Microtechnologies Institute and NIEHS grants ES03850 and ES07060.

References

1. Lecoanet HF, Bottero JY, Wiesner MR. Laboratory assessment of the mobility of nanomaterials in porous media. *Environmental Science & Technology* 2004;38(19):5164–5169. [PubMed: 15506213]
2. Lecoanet HF, Wiesner MR. Velocity effects on fullerene and oxide nanoparticle deposition in porous media. *Environmental Science & Technology* 2004;38(16):4377–4382. [PubMed: 15382867]
3. Lecoanet H, Wiesner MR. Assessment of the mobility of nanomaterials in groundwater aquifers. *Abstracts of Papers of the American Chemical Society* 2004;227:U1275–U1275.
4. Okamoto Y. Ab initio investigation of hydrogenation of C-60. *Journal of Physical Chemistry A* 2001;105(32):7634–7637.
5. Sun O, Wang Q, Jena P, Kawazoe Y. Clustering of Ti on a C-60 surface and its effect on hydrogen storage. *Journal of the American Chemical Society* 2005;127(42):14582–14583. [PubMed: 16231905]
6. Forrest DR. *Molecular Nanotechnology*. IEEE 2001:11–20.
7. Thomas K, Sayre P. Research strategies for safety evaluation of nanomaterials, Part I: evaluating the human health implications of exposure to nanoscale materials. *Toxicol Sci* 2005;87(2):316–21. [PubMed: 16049265]
8. Guzman KAD, Taylor MR, Banfield JF. Environmental risks of nanotechnology: National nanotechnology initiative funding, 2000–2004. *Environmental Science & Technology* 2006;40(5):1401–1407. [PubMed: 16568748]
9. Hurt RH, Monthieux M, Kane A. Toxicology of carbon nanomaterials: status, trends, and perspectives on the special issue. *Carbon* 2006;44:1028–1033.
10. Hoet P, Bruske-Hohlfeld I, Salata O. Nanoparticles- known and unknown health risks. *Journal of Nanobiotechnology* 2004;2:12–37. [PubMed: 15588280]

11. Wixon J. Featured organism: Danio rerio, the zebrafish. *Yeast* 2000;17(3):225–31. [PubMed: 11025533]
12. Hill AJ, Teraoka H, Heideman W, Peterson RE. Zebrafish as a model vertebrate for investigating chemical toxicity. *Toxicol Sci* 2005;86(1):6–19. [PubMed: 15703261]
13. Ton C, Lin Y, Willett C. Zebrafish as a model for developmental neurotoxicity testing. *Birth Defects Res A Clin Mol Teratol* 2006;76(7):553–67. [PubMed: 16933308]
14. Tilton F, La Du JK, Vue M, Alzarban N, Tanguay RL. Dithiocarbamates have a common toxic effect on zebrafish body axis formation. *Toxicol Appl Pharmacol* 2006;216(1):55–68. [PubMed: 16797628]
15. Rubinstein AL. Zebrafish: from disease modeling to drug discovery. *Curr Opin Drug Discov Devel* 2003;6(2):218–23.
16. Dodd A, Curtis PM, Williams LC, Love DR. Zebrafish: bridging the gap between development and disease. *Hum Mol Genet* 2000;9(16):2443–9. [PubMed: 11005800]
17. Nakamura E, Isobe H. Functionalized fullerenes in water. The first 10 years of their chemistry, biology, and nanoscience. *Accounts of Chemical Research* 2003;36(11):807–815. [PubMed: 14622027]
18. Anderson R, Barron AR. Reaction of hydroxyfullerene with metal salts: a route to remediation and immobilization. *Journal of American Chemical Society* 2005;127:10458–10459.
19. Sayes CM, Fortner JD, Guo W, Lyon D, Boyd AM, Ausman KD, et al. The differential cytotoxicity of water-soluble fullerenes. *Nano Lett* 2004;4(10):1881–1887.
20. Sayes CM, Liang F, Hudson JL, Mendez J, Guo W, Beach JM, et al. Functionalization density dependence of single-walled carbon nanotubes cytotoxicity in vitro. *Toxicol Lett* 2006;161(2):135–42. [PubMed: 16229976]
21. Lockman P, Koziara J, Mumper R, Allen D. Nanoparticle surface charges alter blood-brain barrier integrity and permeability. *Journal of Drug Target* 2004;12:635–641.
22. Kimmel C, Ballard W, Kimmel S, Ullmann B, Schilling T. Stages of embryonic development of the zebrafish. *Developmental Dynamics* 1995;203:253–310. [PubMed: 8589427]
23. Sayes CM, Gobin AM, Ausman KD, Mendez J, West JL, Colvin VL. Nano-C60 cytotoxicity is due to lipid peroxidation. *Biomaterials* 2005;26(36):7587–95. [PubMed: 16005959]
24. Isakovic A, Markovic Z, Todorovic-Markovic B, Nikolic N, Vranjes-Djuric S, Mirkovic M, et al. Distinct cytotoxic mechanisms of pristine versus hydroxylated fullerene. *Toxicological Sciences* 2006;91(1):173–183. [PubMed: 16476688]
25. Bosi S, Da Ros T, Spalluto G, Prato M. Fullerene derivatives: an attractive tool for biological applications. *European Journal of Medical Chemistry* 2003;38:913–923.
26. Tsuchiya T, Oguri I, Yamakoshi Y, Miyata N. Novel harmful effects of [60]fullerene on mouse embryos *in vitro* and *in vivo*. *FEBS Letters* 1996;393:139–145. [PubMed: 8804443]
27. Lovern SB, Klaper R. *Daphnia magna* mortality when exposed to titanium dioxide and fullerene (C₆₀) nanoparticles. *Environmental Toxicology & Chemistry* 2006;25(4):1132–1137. [PubMed: 16629153]
28. Oberdorster E. Manufactured nanomaterials (fullerenes, C₆₀) induce oxidative stress in the brain of juvenile largemouth bass. *Environmental Health Perspectives* 2004;112:1058–1062.
29. Zhu S, Oberdorster E, Haasch ML. Toxicity of an engineered nanoparticle (fullerene, C₆₀) in two aquatic species, *Daphnia* and fathead minnow. *Marine Environmental Research*. 2006in press
30. Jensen AW, Wilson SR, Schuster DI. Biological applications of fullerenes. *Bioorganic & Medicinal Chemistry* 1996;4(6):767–779. [PubMed: 8818226]
31. Dugan LL, Gabrielezen JK, Yu SP, Lin TS, Choi DW. Buckminsterfullerenol free radical scavengers reduce excitotoxic and apoptotic death of cultured cortical neurons. *Neurobiology of Disease* 1996;3:129–135. [PubMed: 9173920]
32. Edinger AL, Thompson CB. Death by design: apoptosis, necrosis and autophagy. *Current Opinion in Cell Biology* 2004;16:663–669. [PubMed: 15530778]
33. Gharbi N, Pressac M, Hadchouel M, Szwarc H, Wilson SR, Moussa F. [60]fullerene is a powerful antioxidant *in vivo* with no acute or subacute toxicity. *Nano Lett* 2005;5(12):2578–85. [PubMed: 16351219]

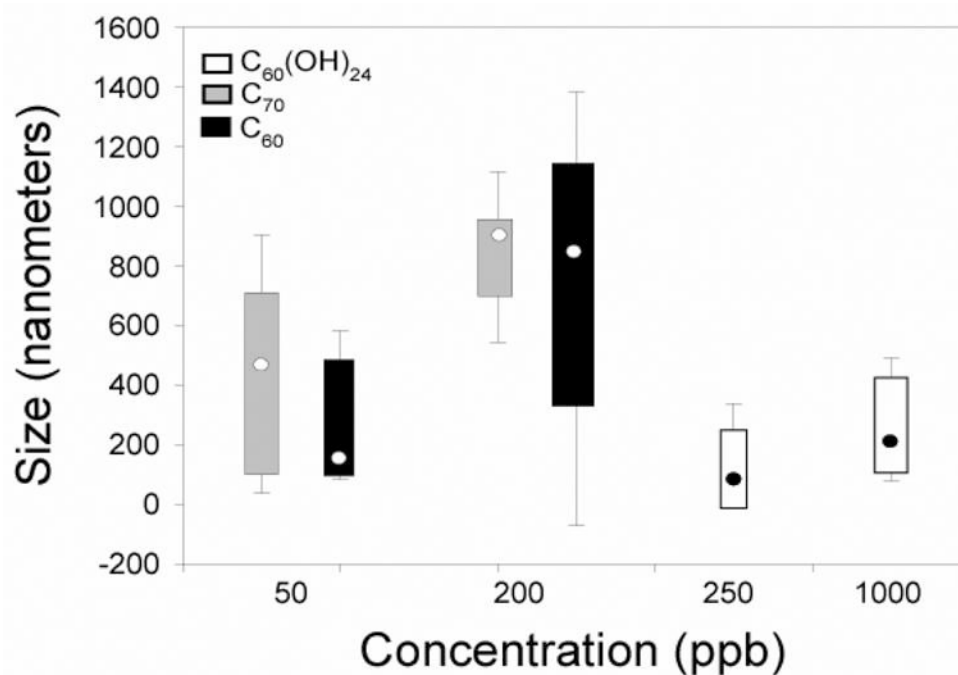


Figure 1. Photon correlation spectroscopy of C₆₀ (black) and C₇₀ (gray) at 50 and 200 ppb, and C₆₀(OH)₂₄ (white) at 250 and 1000 ppb. Mode size of particles (highest number of observations) for each concentration is indicated by (•). Boxes represent particle size distribution with upper and lower standard deviation determined by size distribution processor analysis.

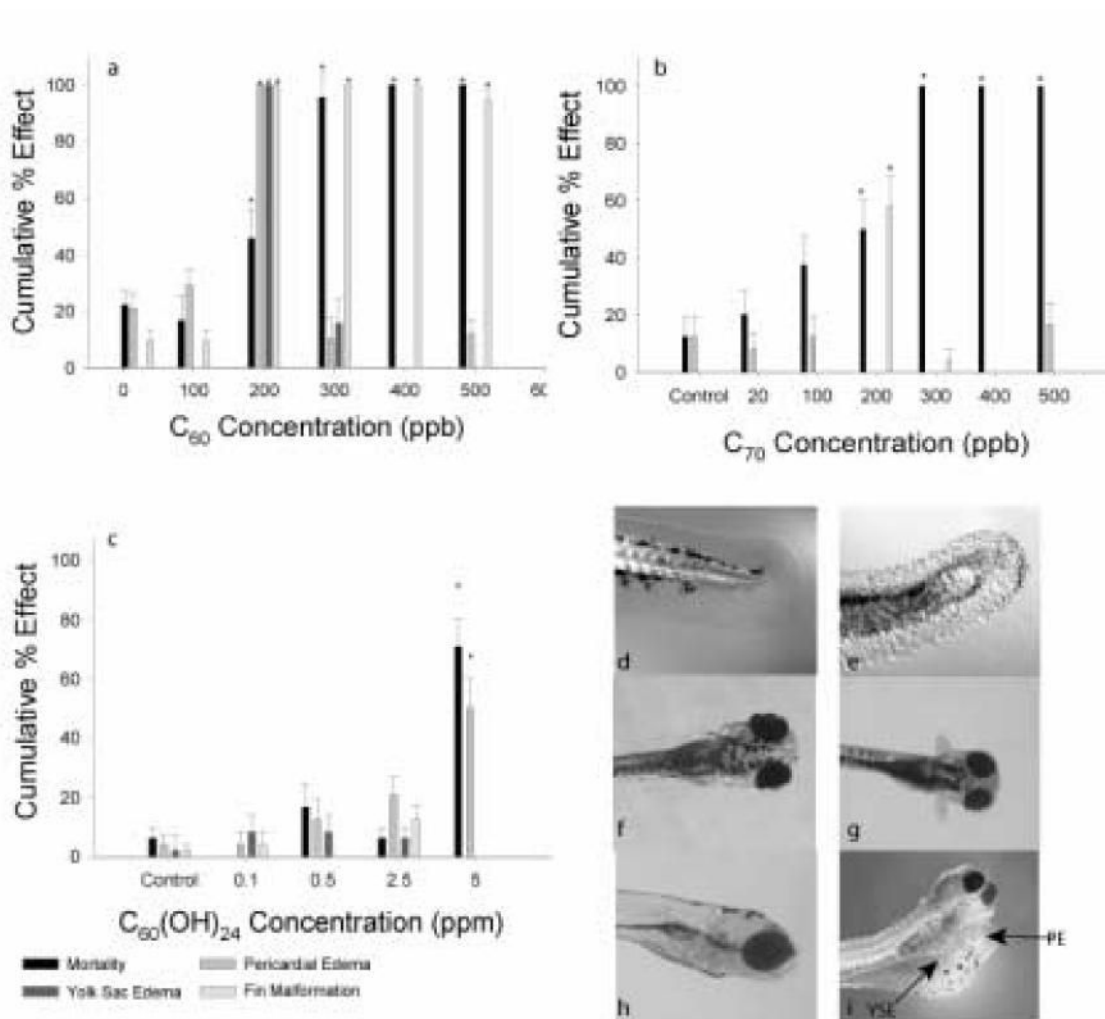


Figure 2.

Concentration-responses observed for embryonic zebrafish exposed to (a) C₆₀, (b) C₇₀, and (c) C₆₀(OH)₂₄ from 24 hpf to 96 hpf; evaluated daily until 6 dpf. Values represent the % showing the effect by day 6 (Cumulative % Effect): mortality, pericardial edema, yolk sac edema, and fin malformations. Representative images of the caudal fins for (d) control and (e) 200 ppb C₆₀-exposed animals are given. Representative images of the pectoral fin for (f) control and (g) 3500 ppb C₆₀(OH)₂₄-exposed animals. Representative images of (h) 1% DMSO control head at 6 dpf and (i) 200 ppb C₆₀-exposed head at 6 dpf; arrows designate pericardial edema (PE) and yolk sac edema (YSE). Significance was determined using Fisher's Exact test (*p<0.05) compared to 1% DMSO control (N=24).

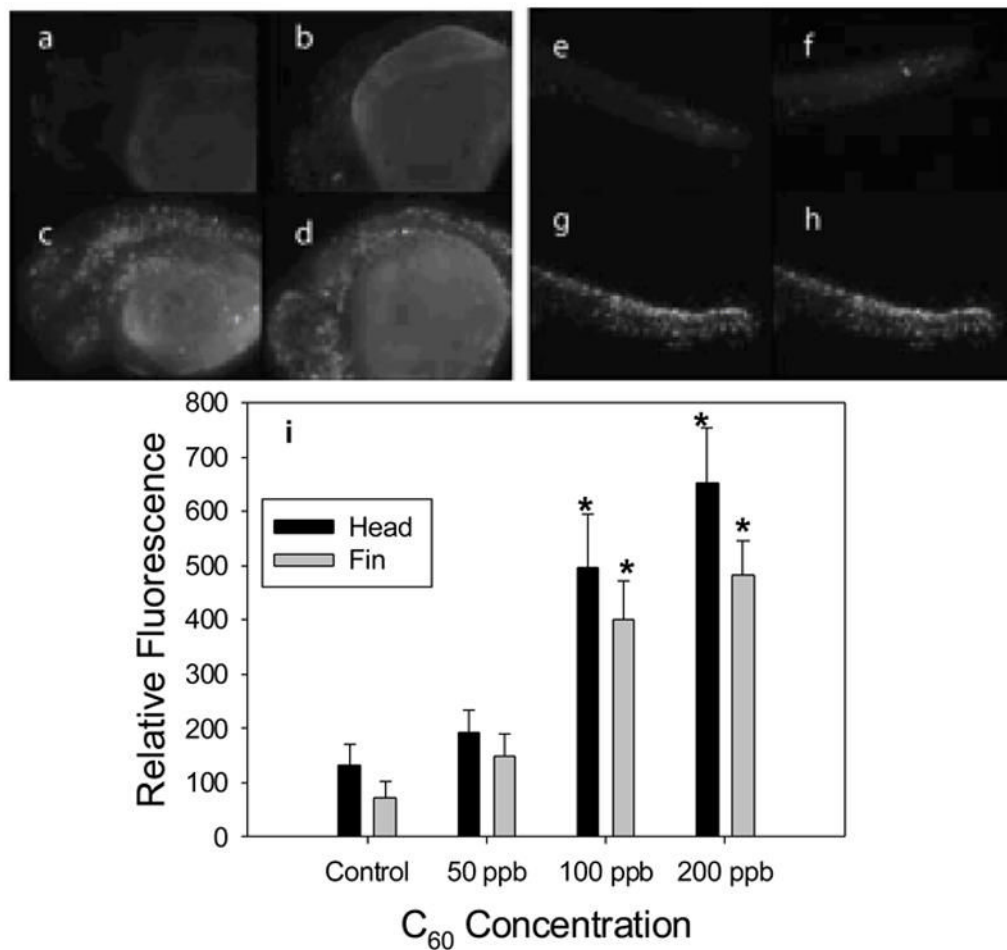


Figure 3. C₆₀-exposure leads to increased cellular death in the live zebrafish embryo

Cellular death was determined using acridine orange staining of C₆₀-exposed embryos at 36 hpf, after a 12 hour C₆₀ exposure. Fluorescence emitted from cells undergoing cellular death indicated by white signal on a black background, shown for the head region for (a) 1% DMSO control, (b) 50 ppb C₆₀-exposed (c) 100 ppb C₆₀-exposed (d) 200 ppb C₆₀-exposed; and the caudal fin for (e) 1% DMSO control, (f) 50 ppb C₆₀-exposed, (g) 100 ppb C₆₀-exposed, and (h) 200 ppb C₆₀-exposed embryos. (i) Concentration-response curves for cell death measured as relative fluorescence in the head (●) and trunk regions (°) of the embryos. Cellular death was significantly different than controls at 100 and 200 ppb determined using one-way ANOVA (*p<0.05), N=12

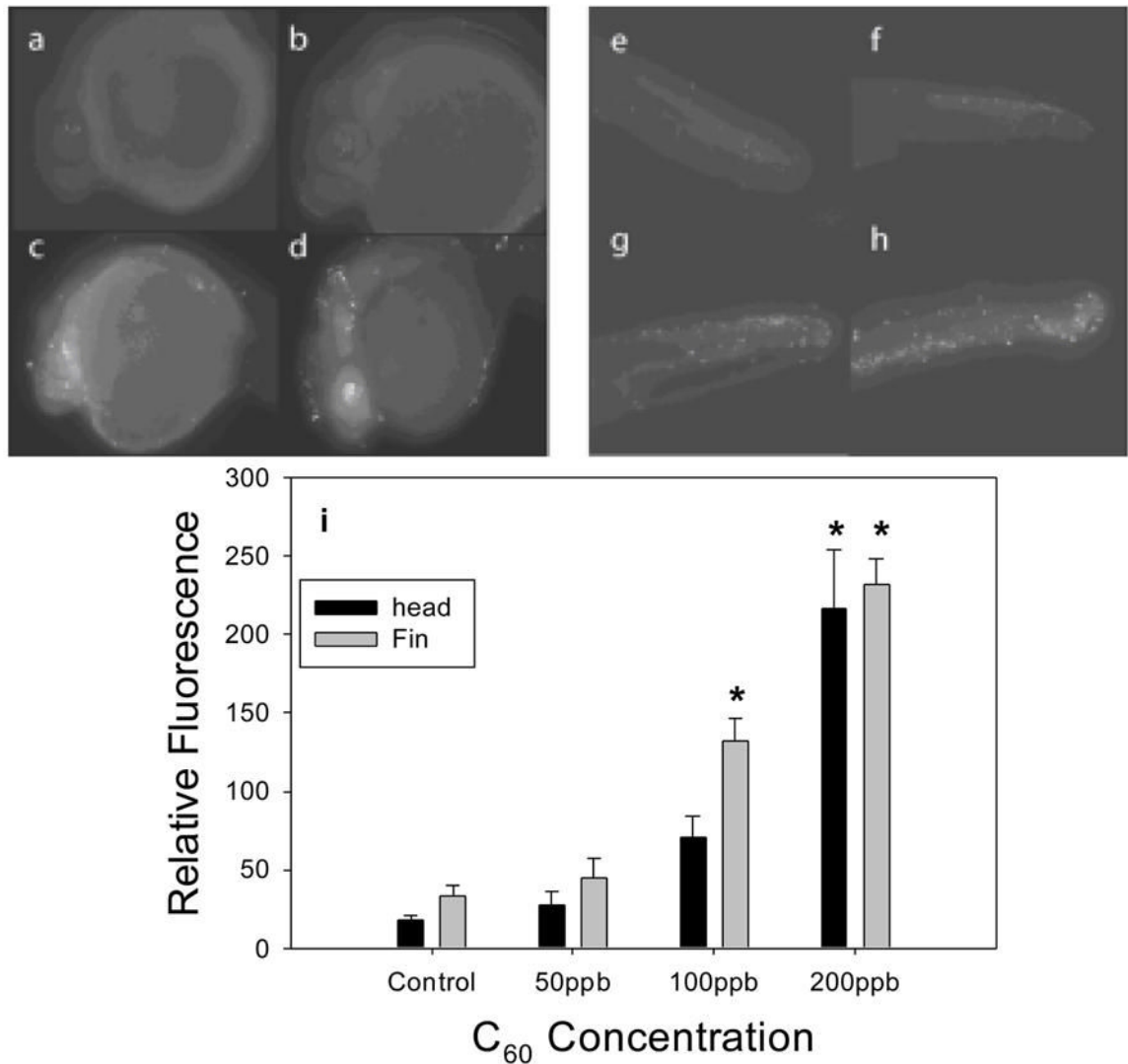


Figure 4. Apoptotic cell death was measured in embryos at 36 hpf, after 12 hours of exposure. White spots indicating a TUNEL⁺ response (apoptotic signaling) shown for the head region (a) 1% DMSO control, (b) 50 ppb C₆₀-exposed (c) 100 ppb C₆₀-exposed (d) 200 ppb C₆₀-exposed and the caudal fin region (e) 1% DMSO control, (f) 50 ppb C₆₀-exposed, (g) 100 ppb C₆₀-exposed, and (h) 200 ppb C₆₀-exposed embryos. (i) Concentration-response curves for cell death measured as relative fluorescence in the head (black) and trunk (grey) regions of the embryos. Apoptotic cellular death was significantly different than controls at 100 and 200 ppb determined using one-way ANOVA (*p<0.05), N=12.