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# Quantification of Fullerenes by LC/ESI-MS and Its Application to in Vivo Toxicity Assays

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With production and use of carbon nanoparticles increasing, it is imperative that the toxicity of these materials be determined; yet such testing requires specific and selective analytical methodologies that do not yet exist. Quantitative liquid–liquid extraction was coupled with liquid chromatography/electrospray ionization mass spectrometry for the quantitative determination of fullerenes from $C_{60}$ to $C_{98}$. Isotopically enriched, $^{13}C_{60}$, was used as an internal standard. The method was applied to determine the loss of $C_{60}$ from exposure water solution and uptake of $C_{60}$ by embryonic zebrafish. The average recovery of $C_{60}$ from zebrafish embryo extracts and 1% DMSO in aqueous-exposure solutions was 90 and 93%, respectively, and precision, as indicated by the relative standard deviation, was 2 and 7%, respectively. The method quantification limit was 0.40 $\mu$g/L and the detection limit was 0.02 $\mu$g/L. During the toxicological assay, loss of $C_{60}$ due to sorption to test vials resulted in the reduction of exposure-solution concentrations over 6 h to less than 50% of the initial concentration. Time-course experiments indicated embryo uptake increased over course of the 12-h exposure. A lethal concentration that caused 50% mortality was determined to be 130 $\mu$g/L and was associated with a zebrafish embryo concentration, $LD_{50}$, of 0.079 $\mu$g/g of embryo.

With production projected to reach $1 trillion in revenue and a projected creation of 7 million jobs worldwide by 2015,[1] interest is increasing in nanomaterials. Some of the potential areas of application of nanomaterials include medical equipment, drug delivery, fuel cells, and personal care products.[2] The projected large production volume and widespread use has led to growing concern over the potential for nanomaterials to adversely affect environmental and human health.[3,4]

One group of nanomaterials that has received much attention is the fullerenes.[5–7] With increasing production and use of fullerenes, it is imperative to determine the possible environmental and human health affects of these materials since adverse outcomes[8–12] as well as protective effects are observed upon exposure to $C_{60}$.[13,14]

Many biological models, including cells in culture, aquatic organisms including embryonic zebrafish (*Danio rerio*), and whole-animal tests such as rodents, currently are used to determine potential toxicological effects of chemicals. Cell cultures are advantageous because they are of low cost and require little time; however, inferring implications for human health risks are often difficult.[15] With rodent studies, it is easier to infer human health risks, but such models are expensive and require extensive facilities for animal care, and longer assessment time periods.[15] Zebrafish are an attractive alternative since they have many cellular, anatomical, and physiological characteristics similar to that of higher vertebrates; however, they have shorter life cycles and females produce hundreds of eggs a week, which allows for large sample sizes and robust statistics.[16,17] In vivo testing with embryonic zebrafish is advantageous because there is a full repertoire of signaling pathways expressed in numerous cell types during development, thereby increasing the likelihood of identifying toxicological targets.[18,19]

There is a lack of quantitative information on the toxicology of $C_{60}$ due, in part, to the lack of quantitative methods for the

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quantification of fullerenes in aqueous and biological matrixes. While there are several reports for the nonquantitative determinations of fullerenes in environmental matrixes,[20-24] the number of reports on the quantitative analysis of $C_{60}$ is limited, especially in biological systems. Toluene extraction followed by liquid chromatography–mass spectrometry with quantification by external standard calibration is reported for quantification of $C_{60}$ in blood,[25] tissues,[26] and protein-containing media.[27] To the best of our knowledge, isotopically labeled internal standard quantification has not yet been developed for any fullerene.

The purpose of this research was to develop and validate an analytical method to quantify a suite of fullerenes and then apply the analytical method to determine the behavior of a single fullerene, $C_{60}$, during a toxicological assay using zebrafish embryos and aqueous-exposure solutions. Method performance characteristics including accuracy, precision, and quantification limits were determined from a series of spike and recovery experiments. The developed method was then applied to determine the time course of $C_{60}$ uptake by embryonic zebrafish and the actual body burden of $C_{60}$ in embryonic zebrafish. In addition, the reduction in $C_{60}$ remaining in the aqueous-exposure solution due to sorption of $C_{60}$ to a variety of test materials was quantified.

## EXPERIMENTAL METHODS

**Materials.** Authentic standards of $C_{60}$ (+99%), a higher-order mixture of fullerenes $C_{70}-C_{98}$, and $^{13}C_{60}$ (+99% with 20−30% $^{13}C$ enrichment) were purchased from Materials and Electrochemical Research Corp. (MER Corp., Tucson, AZ). In addition, 5,6-$C_{70}$ (99%) was purchased from Aldrich (Milwaukee, WI). Toluene (HPLC grade) and methanol (Optima grade) were purchased from Fisher Scientific (Fair Lawn, NJ), and DMSO was purchased from J.T. Baker (Phillisburg, NJ). An artificial freshwater salt, Instant Ocean, was purchased from Aquarium Systems, Inc. (Mentor, OH) for use in creating the aqueous-exposure solutions.

**Preparation of Standards and Aqueous-Exposure Solutions.** Fullerene standards for the quantification of $C_{60}$, $C_{70}$, and the higher-order mixture of fullerenes were prepared by adding 2 mg of each individual fullerene standard or the higher-order mixture into 10 mL of toluene and sonicating for 1 h. Standards were stored in amber vials at 4 °C and allowed to come to room temperature before use. To ensure the integrity of the standards, steps such as not using the light in the fume hood, were taken to avoid exposure to light. For analysis, standards in toluene were added to methanol to achieve a 80:20 methanol/toluene solution and analyzed within 24 h of preparation.

Suspensions of $C_{60}$ in DMSO were prepared by adding 0.5 mg of $C_{60}$ to 10 mL of DMSO, and the resulting suspension was sonicated for 1 week to achieve a nominal concentration (e.g., the concentration estimate from the mass of $C_{60}$ weighed) of 0.05 mg/mL. Suspensions of $C_{60}$ in DMSO were stored in the dark at room temperature and sonicated for 1.5 h prior to each use.

Aqueous-exposure solutions were prepared by making dilutions of the $C_{60}$ standard in DMSO into a commercially available freshwater salt solution (0.6% Instant Ocean). The resulting solutions had an conductivity of 500 $\mu$S/cm and contains <1% DMSO.[15]

**Developmental Toxicity Assay.** Zebrafish were reared as described by Usenko et al.[15] Once the chorion, the outer membrane surrounding the embryo, was removed from the zebrafish embryos, 100 zebrafish embryos at 36-h postfertilization were pooled into 24-well styrene–divinylbenzene plates. The zebrafish embryos were then exposed to aqueous-exposure solutions with nominal $C_{60}$ concentrations of 100, 200, and 400 $\mu$g/L $C_{60}$ for 2, 6, and 12 h; each sampling time point and concentration was performed in triplicate. After exposure, the dead and live embryos were separated into polypropylene centrifuge tubes; the live embryos were euthanized with tricaine. To prepare for extraction, euthanized embryos were thoroughly rinsed with blank (e.g., no $C_{60}$) aqueous-exposure solution. Blank samples were analyzed during the course of the exposure study and consisted of 100 embryonic zebrafish, prepared as described above, but only exposed to blank aqueous-exposure solution (e.g., no $C_{60}$). Control samples were prepared and analyzed along with exposure samples at each concentration and each time point. Control samples consisted solely of aqueous-exposure water containing $C_{60}$ but no zebrafish. In addition, cell death was measured by an acridine orange assay as described in Supporting Information (SI).

**Aqueous-Exposure Solution and Embryonic Zebrafish Extraction.** For the analysis of aqueous-exposure solutions, a 0.5-mL sample was pipetted into a 2-mL autosampler vial to which 0.5 mL of toluene and 10 ng of $^{13}C_{60}$ were added. The sample was vortexed for 1 min and then centrifuged for 10 min. A 0.2-mL aliquot of the toluene phase was then added to 0.8 mL of methanol and the resultant mixture analyzed by LC (ESI)-MS within 24 h.

The methodology for extracting $C_{60}$ from zebrafish embryos was similar to that described by Xia et al.[27] and Moussa et al.[26] Embryos were homogenized by sonication on a Sonic Dismembrator (Fisher Scientific, Fair Lawn, NJ). The homogenate was digested with 0.3 mL of glacial acetic acid and vortexed with a Vortex Genie Mixer (Scientific Products, Evanston, IL). To the homogenate, 10 ng of $^{13}C_{60}$ was added as internal standard. Toluene (0.5 mL) was then added to the embryo homogenate and extracted by vortexing for 1 min. The extract was then centrifuged for 10 min on an Eppendorf centrifuge 5415 C (Brinkman Instruments, Inc., Westbury, NY). A 0.2-mL aliquot of the toluene phase was placed in a 2-mL autosampler vial to which 0.8 mL of methanol was added and the resultant mixture analyzed by liquid chromatography with electrospray mass spectrometry (LC/ESI-MS) within 24 h.

Experiments to define the accuracy and precision of the method as indicated by recovery and the relative standard deviation of replicate extractions from embryo homogenate and aqueous-exposure solutions are described in SI. In addition, experiments were conducted to determine the loss of $C_{60}$ due to

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sorption on to different test vial materials (see SI for details) since loss onto styrene−divinylbenzene plates could not be determined directly since the plates were dissolved by toluene.

**Liquid Chromatography/Electrospray Mass Spectrometry.** LC/ESI-MS detection was used to identify and quantify $C_{60}$−$C_{98}$ fullerenes. All separations were performed on an Agilent 1100 LC (Santa Clara, CA). Samples were loaded onto a 900-$\mu$L stainless steel injection loop that was connected to a 4 mm × 2 mm guard column (Phenomenex, Torrance, CA) followed by a 150 mm × 2 mm Targa $C_{18}$ column (Higgins Analytical, Mountain View, CA). Separations were performed at 30 °C with a toluene/methanol (55:45) isocratic mobile phase. The divert valve was enabled for the first 6 min of the analysis.

The LC was attached to a Waters Quatro Micro mass spectrometer (Beverly, MA) that was equipped with an ESI source. The mass spectrometer was operated in negative ion mode with single ion monitoring. The cone voltage was set to 250 V, and the capillary voltage was set to 4 kV. The source temperature and desolvation capillary were set to 125 and 250 °C, respectively. The nebulizer and desolvation gases were set to flow rates of 20 and 600 L/h, respectively.

**Fullerene Quantification.** Fullerenes in embryo homogenates and aqueous-exposure solutions were quantified using solvent-based calibration curves (e.g., no matrix) since standard addition experiments (see SI for details), indicated that embryo homogenate and aqueous-exposure solution matrixes did not adversely affect quantification when solvent-based calibration curves were used. Quantification was based on peak area ratios of analyte to that of the $^{13}C_{60}$ internal standard. Calibration curves for $C_{60}$ and $C_{70}$ ranged from 0.1 to 150 $\mu$g/L. Calibration curves for fullerenes larger than $C_{70}$ were constructed from the higher-order fullerene mixture and ranged from 10 to 100 $\mu$g/L total fullerene mixture, which gave individual fullerene concentrations ranging from 0.08 to 47 $\mu$g/L. All calibration curves were plotted with $1/X$ weighting with $r^2 > 0.99$. Residuals were <15% for $C_{60}$ and $C_{70}$ while residuals were <25% for the higher-order fullerenes; calibration standards outside these limits were excluded from the calibration.

For quality assurance, blanks and check standards were analyzed within each sample set and comprised greater than 30% of all samples. Blanks consisted of 80:20 methanol/toluene with no analyte or internal standard and check standards consisted of calibration standards. Experiments aimed at determining the instrumental and whole-method detection and quantification limits are described in SI.

## RESULTS

**Liquid Chromatography/Mass Spectrometry.** The most abundant ions formed under ESI-MS conditions were molecular ions [M$^{\bullet-}$] including $m/z$ 720 ($C_{60}$) and 728−740 for the $^{13}C_{60}$ internal standard, which is 20−30% enriched in $^{13}$C. Molecular ions were produced for $C_{70}$−$C_{98}$, (e.g., $m/z$ 840 for $C_{70}$; $m/z$ 912 for $C_{76}$; and $m/z$ 1176 for $C_{98}$), which is consistent with previous reports.[25,26,28−32] In addition, fullerene oxide ions (e.g., $C_{60}$O, $m/z$
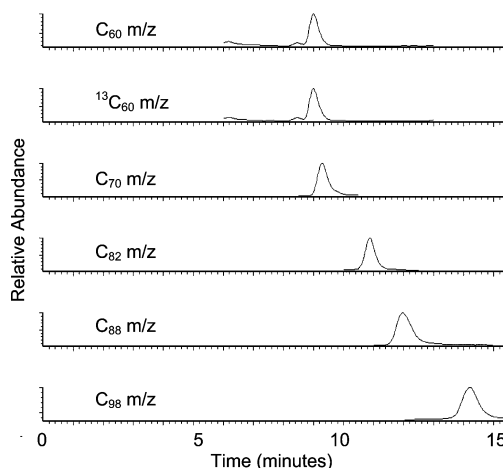


**Figure 1.** Selected LC/ESI-MS chromatograms in methanol/toluene (80:20) unless otherwise noted; including $C_{60}$ (2 $\mu$g/L in zebrafish homogenate matrix), $^{13}C_{60}$ (10 $\mu$g/L in zebrafish homogenate matrix), $C_{70}$ (10 $\mu$g/L), $C_{82}$ (3.4 $\mu$g/L), $C_{88}$ (2.5 $\mu$g/L), and $C_{98}$ (0.4 $\mu$g/L). Additional fullerenes in higher-order mixture not shown.

736) were observed in low abundance; however, methanol and toluene adducts were not observed as has been reported for APCI.[28,29] Single ion monitoring was used for the quantification as no interference was observed in blank embryo homogenate extracts and no fragmentation was observed upon collision-induced dissociation. For the internal standard, $^{13}C_{60}$, the ion selected for quantification was $m/z$ 732 because it gave the most reproducible response. Although the $^{13}C_{60}$ internal standard contains $C_{60}$, the addition of 10 ng of $^{13}C_{60}$ in a final extract volume of 1 mL (0.01 ng/L) did not produce a measurable response for $C_{60}$ at $m/z$ 720. Others have used $C_{70}$ as an internal standard;[26] however, the 20−30% $^{13}$C-enriched $C_{60}$ internal standard is a better and cost-effective internal standard since, at the mass used (10 ng), no signal is obtained for $C_{60}$ in blank analyses. A 85% $^{13}$C-enriched $C_{60}$[33] is also available from MER but at greater cost. Therefore, 10 ng of 20−30% $^{13}$C-enriched $C_{60}$ was the internal standard mass used for all subsequent analyses.

The mobile phase of 55:45 toluene/methanol provided chromatographic focusing of the fullerene analytes from the large injected volume (500 $\mu$L). The large-volume injection did not result in carryover of $C_{60}$ from sample to sample, as $C_{60}$ was not observed in solvent blanks analyzed throughout each sample set. Chromatographic analysis indicated coelution of $C_{60}$ with the $^{13}C_{60}$ internal standard with the retention times of the larger fullerenes increasing in order of increasing carbon number (Figure 1). Although fullerenes larger than $C_{98}$ are reported by others,[29] fullerenes larger than $C_{98}$ were not observed in the higher-order fullerene mixture.

**Quantification of Higher-Order Fullerenes.** An approach was developed to quantify the higher-order fullerenes using the higher-order fullerene mixture since authentic standards of individual fullerenes of >$C_{70}$ were prohibitively expensive (see SI for details). Good agreement with the percent composition reported for $C_{84}$ was obtained, but the percent compositions of

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**Table 1. Whole-Method Accuracy[a]**

| spiked concn (μg/L) | exposure water (% ± 95% CI) (RSD) | embryo homogenate (% ± 95% CI) (RSD) |
|---|---|---|
| 9 | b | 90 ± 3 (2) |
| 35 | 110 ± 10 (4) | b |
| 250 | 93 ± 7 (7) | b |

[a] As indicated by the average recovery (±95% CI) and precision, as indicated by RSD, determined from spike and recovery experiments with exposure-aqueous solutions (n = 3) and embryo homogenate (n = 4). [b] Experiment not performed.

$C_{76}$ (4.2%) and $C_{78}$ (8.6%) were low compared to manufacture specifications, which indicated that these two components are each present in the mixture at 20% (Table 1 SI).[33] The lack of agreement between the measured values and that of the manufacturer for $C_{76}$ and $C_{78}$ may be due to the use of LC/UV for characterizing the mixture, which does not distinguish between individual fullerenes based on mass. In contrast to the manufacturer specifications, which indicate that the mixture contains 20% of the other fullerenes (e.g., $C_{70}$, $C_{80}$, $C_{82}$, and $\leq C_{96}$), analysis by LC/ESI-MS indicated that these components were ~40% of the mixture (Table 1 SI). With the defined composition of the higher-order fullerene standard, calibration curves were then constructed from the known masses of the higher-order fullerene mixture and the $^{13}C_{60}$ internal standard. Linear calibration curves (e.g., $r^2 >$ 0.9999) for $C_{76}$–$C_{98}$ demonstrates for the first time the utility of the LC/ESI-MS method for quantify higher-order fullerenes. However, for the remaining study, the focus was placed on quantifying $C_{60}$ in the developmental toxicology assays.

**Fullerene Standards in Toluene and DMSO.** The "nominal" concentration (e.g., based on the fullerene mass weighed) of 0.20 mg/mL in toluene used in this study as an analytical standard is 1 order of magnitude below the reported equilibrium solubility of $C_{60}$ in toluene[34] and a factor of 5 less than the reported solubility of $C_{70}$ in toluene.[35] Therefore, the $C_{60}$ in the toluene standard is likely "truly dissolved". To the best of our knowledge, the solubility of fullerenes larger than $C_{70}$ in toluene has not been reported.

DMSO is of interest as a solvent because it is nontoxic at low doses and is therefore commonly used in toxicological studies.[36] Although nominal concentrations of 0.05 mg/mL $C_{60}$ in DMSO were routinely prepared for this study, when the DMSO solutions were allowed to stand for several hours, $C_{60}$ aggregates settled out of solution, which indicates that the $C_{60}$ is not truly dissolved. This observation is consistent with previous work in which we demonstrated that $C_{60}$ aggregates (100–450 nm) form when the DMSO standard of $C_{60}$ was added to artificial freshwater solution.[15] Dilutions into toluene of the 0.05 mg/mL $C_{60}$ standard in DMSO gave an average measured $C_{60}$ concentration of 0.033 ± 0.007 mg/mL (±95% CI), which is only 66% of the nominal concentration.

**Whole-Method Validation and Instrumental Repeatability.** Spike and recovery experiments with aqueous-exposure solution (n = 3) at $C_{60}$ concentrations of 35 and 250 μg/L gave average

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recoveries (±95% CI) of 110 ± 10 and 93 ± 7%, respectively (Table 1). Precision, as indicated by relative standard deviation (RSD), was 4 and 7%, respectively (Table 1). Average recoveries of 9 μg/L $C_{60}$ in embryo homogenate (n = 4) were 90 ± 3% with a RSD of 2% (Table 1).

Intraday and interday instrumental repeatability was assessed by analysis of standard over the course of the study. The repeatability of the instrument over the course of 1 day for n = 10 analyses of standards ranging from 1 to 250 μg/L was 3.9% (RSD) while the repeatability based on n = 40 analyses of standards of 1–250 μg/L was 8.5% (RSD) over the course of multiple days.

**Instrumental and Whole-Method Detection and Quantification Limits.** The instrumental detection limits for $C_{60}$, as defined by the concentration that gave a S/N of 3:1, was 0.0004 μg/L. The instrumental quantification limit, 0.002 μg/L, was determined as the concentration that gave a S/N of greater than 10:1.

Whole-method detection limits were equivalent for embryo homogenate and aqueous-exposure solution at 0.020 μg/L while whole-method quantification limits were 0.040 μg/L, which is 2 orders of magnitude less than that achieved by Xia et al.[27] The low detection limit achieved in this study is due to the large-volume injection (500 μL) method.

**Quantifying $C_{60}$ in Zebrafish Developmental Toxicology Assay.** Aqueous-exposure solution and zebrafish embryo concentrations of $C_{60}$ were measured over a 12-h time course for experiments conducted at three different $C_{60}$ concentrations. Toxicological end points including embryonic zebrafish mortality and cellular death were assessed at each time point.

To be consistent with previous work,[15] the three nominal concentrations of aqueous-exposure solutions were used including 100, 200, and 400 μg/L $C_{60}$ (based on mass of $C_{60}$ weighed). However, extraction of the solutions followed by LC/ESI-MS quantification revealed that the actual concentrations were 70 ± 3, 130 ± 6, and 250 ± 8 μg/L, respectively, which are 30–38% below the nominal concentration. This finding is consistent with the 33% difference between the nominal and measured $C_{60}$ concentration for standards prepared in DMSO. Therefore, "nominal" concentrations that are based on weighed masses of $C_{60}$ should be explicitly verified by analytical measurements since measured concentrations were significantly and consistently lower.

Concentrations of $C_{60}$ in wells that received an initial concentration of 70 ± 3 μg/L $C_{60}$ (±95% CI) decreased significantly within 2 h (Figure 2a). Further decreases occurred by 6 h with only 40% remaining in solution (27 ± 9.7 μg/L $C_{60}$) in wells with and without (control) embryos; no additional loss occurred after 6 h (Figure 2a). The difference in $C_{60}$ concentrations remaining in solution for wells with and without (control) zebrafish embryos was not statistically significant, which indicates that the presence of 100 zebrafish embryos did not significantly affect the $C_{60}$ remaining in solution.

Uptake of $C_{60}$ by zebrafish embryos from aqueous-exposure solutions containing an initial 70 μg/L $C_{60}$ reached a maximum concentration (±95% CI) of 0.013 ± 0.018 μg $C_{60}$/g embryonic zebrafish at 6 h (Figure 2b). This uptake of $C_{60}$ accounts for only 0.24 ± 0.34% of the total initial mass of $C_{60}$ in the aqueous-exposure solution. Therefore, the apparent loss from solution (Figure 2a) is due to loss of $C_{60}$ to the polystyrene–divinylbenzene plates. In order to better understand the loss of $C_{60}$ from solution, sorption
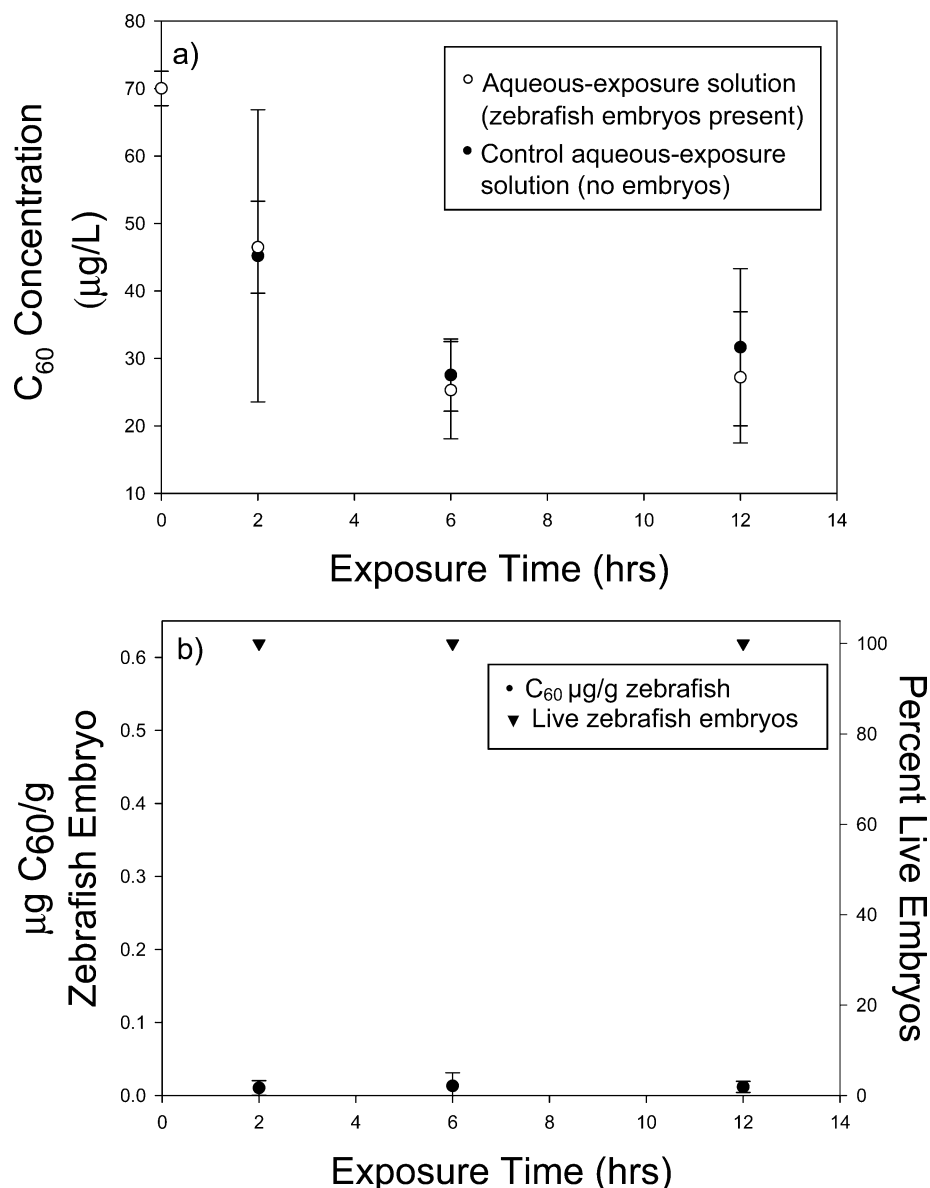
**Figure 2.** Concentration-time course for experiments conducted with an initial measured concentration of 70 $\mu$g/L C$_{60}$ ($\pm$95% CI) in (a) aqueous-exposure solution with and without (control) embryonic zebrafish and in (b) embryonic zebrafish along with numbers of live embryos remaining.

of C$_{60}$ to a variety of test vial materials was determined (Table S2); see SI for further discussion. However, sorption of C$_{60}$ to polystyrene–divinylbenzene plates could be determined as toluene dissolves polystyrene–divinylbenzene plates. It is important to note that the LC/ESI-MS method does not provide any measure of in vivo agglomeration size, which is an area where further research is needed.

At 70 $\mu$g/L C$_{60}$, 100% of the embryos remained alive (Figure 2b). In previous work, exposure of embryonic zebrafish to a nominal concentration of 100 $\mu$g/L (measured in this study as 70 $\mu$g/L) for 5 days did not result in overt signs of toxicity.[15] In addition, cellular death was used as a sensitive indicator of embryonic zebrafish exposure to C$_{60}$. Dead and dying cells are efficiently labeled in live animals by intercalation of acridine orange and are visualized as fluorescent cells on a dark background (Figure 3a) and can be readily quantified (Figure 3b). Zebrafish embryos exposed for 2 and 6 h showed increased cell death relative to controls (Figure 3a and b). No statistically significant

increase in the number of dead and dying cells was observed from 6 to 12 h (Figures 3b). The lack of increase in cell death could be due to the overwhelming fluorescence from deceased cells after 6 h, making it difficult to discern differences between the two time points.

For aqueous-exposure solutions containing an initial C$_{60}$ concentration of 130 $\pm$ 6 $\mu$g/L, the concentration decreased over the course of 12 h in wells with and without (control) embryonic zebrafish (Figure 4a). The decrease in aqueous-exposure solution concentration from 130 to 90 $\pm$ 36 $\mu$g/L by 2 h and to 12 $\pm$ 7 $\mu$g/L (with embryos) and to 40 $\pm$ 7 $\mu$g/L (without embryos) by 12 h indicates losses of up to 90% due to sorption onto plate materials.

The uptake of C$_{60}$ by embryonic zebrafish exposed to an initial concentration of 130 $\pm$ 6 $\mu$g/L C$_{60}$ increased over the course of 12 h (Figure 4b). From 2 to 12 h, the average embryonic zebrafish uptake ($\pm$95% CI) of C$_{60}$ increased from 0.015 $\pm$ 0.0083 to 0.10 $\pm$ 0.058 $\mu$g C$_{60}$/g embryonic zebrafish. The statistically significant
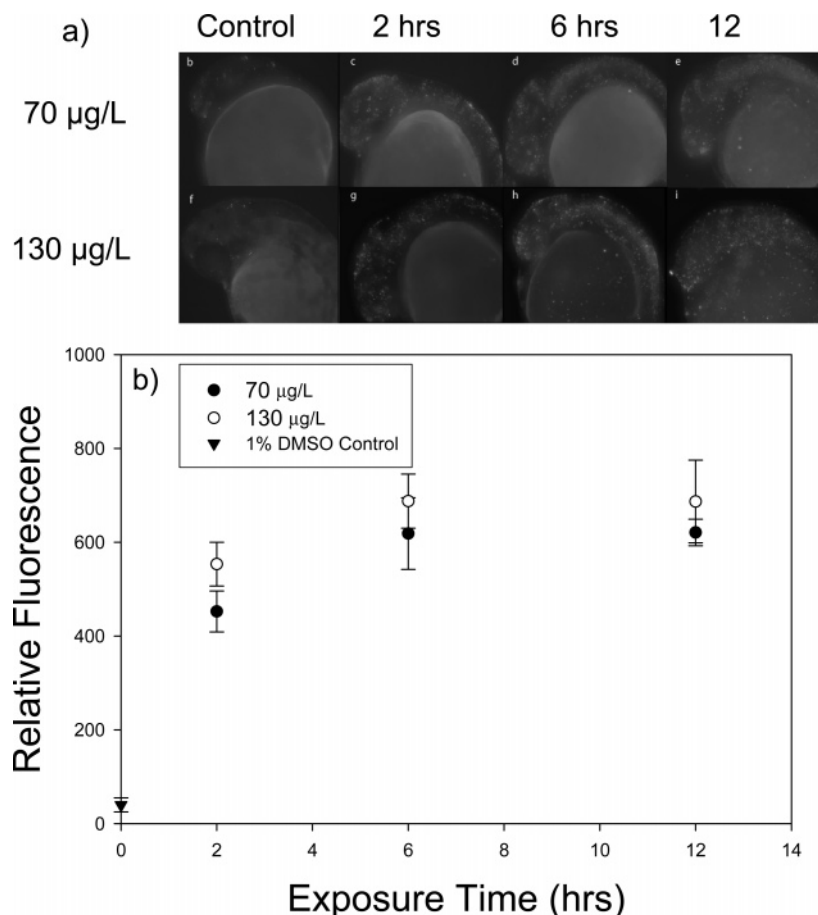
**Figure 3.** Cell death with increasing lengths of exposure (2−12 h) to initial concentrations of 70 and 130 μg/L $C_{60}$ (a). Aqueous-exposure solution concentrations of 250 μg/L at 6 and 12 h resulted in 100% mortality; therefore, cell death could not be not determined. White spots that fluorescence in indicate from cells undergoing cellular death (b).

difference in $C_{60}$ concentration for the 130 μg/L $C_{60}$ exposure water with and without zebrafish at 12 h (Figure 4a) is not due to uptake of $C_{60}$ by the embryonic zebrafish since embryo uptake accounts for only 0.25 ± 0.078% of the $C_{60}$ mass in the aqueous-exposure solution. The discrepancy between aqueous-exposure solution concentrations for wells with and without (control) embryonic zebrafish for 130 μg/L $C_{60}$ is likely due to differential sorption in the exposure wells.

Over the time course of the 130 μg/L $C_{60}$ exposures, the number of live embryos decreased from 100% at 2 h to only 40% at 6 and 12 h (Figure 4b). The increased mortality of embryos exposed for 6 and 12 h occurs in wells containing 40 μg/L $C_{60}$ (Figure 4a), which is ~10 μg/L higher than for the experiments conducted with an initial concentration of 70 μg/L (Figure 2b) in which there was no mortality (Figure 2b). Therefore the mortality of the embryos can be attributed to the initial 130 μg/L $C_{60}$ and not the 40 μg/L $C_{60}$ at 6 and 12 h. Because exposures conducted at an initial concentration of 130 ± 6 μg/L $C_{60}$ caused >50% mortality in test embryos at 6 and 12 h, the corresponding concentrations were averaged to give an estimate of 39 μg/L as the $LC_{50}$ for $C_{60}$ in zebrafish embryos (Figure 4b). While this value appears in contrast to the previously reported $LC_{50}$ of 200 μg/L,[15] when this nominal concentration is measured by LC/ESI-MS, the actual concentration was 130 μg/L. The corresponding $LD_{50}$, which is the average concentration in the embryo at 6 and 12 h, that is associated with 50% mortality, is 0.079 ± 0.063 μg $C_{60}$/g

embryonic zebrafish (Figure 4b). To the best of our knowledge, this is the first $LD_{50}$ reported for $C_{60}$ in an aquatic model. The ability to measure the $LD_{50}$ for $C_{60}$ and the associated dose of $C_{60}$ will give policy makers one more tool for making well-informed risk assessment decisions.

In addition to embryo mortality, cell death also was significantly increased from 2 to 6 h in embryos exposed to an initial $C_{60}$ concentration of 130 μg/L (Figure 3a and b). There was no increase in cell death from 6 to 12 h (Figure 3b), which is consistent with the lack of additional uptake of $C_{60}$ from 6 to 12 h (Figure 4a). However, as stated above, the lack of an apparent increase in cell death could also be due to the high fluorescence from deceased cells after 6 h, making it difficult to discern differences between the 6- and 12-h time points. The developmental outcomes from exposure to 130 μg/L, which corresponds to a nominal concentration of 200 μg/L, were delayed development, fin malformations, pericardial edema, and mortality.[15] Similar toxicological effects were observed by Zhu et al.,[37] who reported that zebrafish exposed to a single nominal concentration of 1500 μg/L $C_{60}$ experienced delayed embryo and larval development, decreased survival and hatching rates, and developed pericardial edema.

For aqueous-exposure solutions that were initially 250 ± 8 μg/L $C_{60}$, a decrease in $C_{60}$ concentration from wells with and without

(37) Zhu, X.; Zhu, L.; Li, Y.; Duan, Z.; Chen, W.; Alvarez, P. J. J. *Environ. Toxicol. Chem.* **2007**, *26*, 976−979.
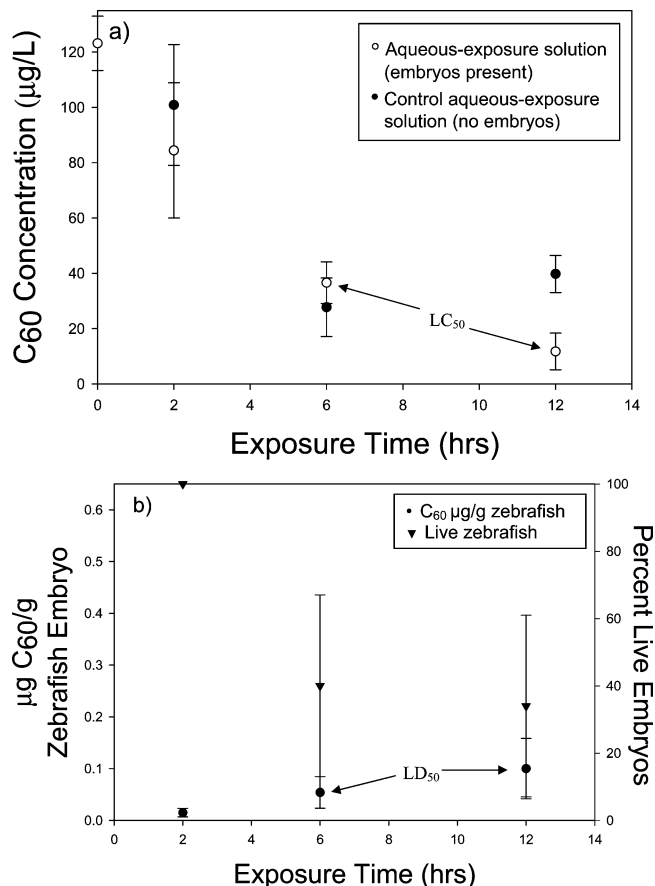
**Figure 4.** Concentration time course for experiments conducted with an initial measured concentration of 130 $\mu$g/L $C_{60}$ ($\pm$95% CI) in (a) aqueous-exposure solution with and without (control) embryonic zebrafish and in (b) embryonic zebrafish along with numbers of live embryos remaining.
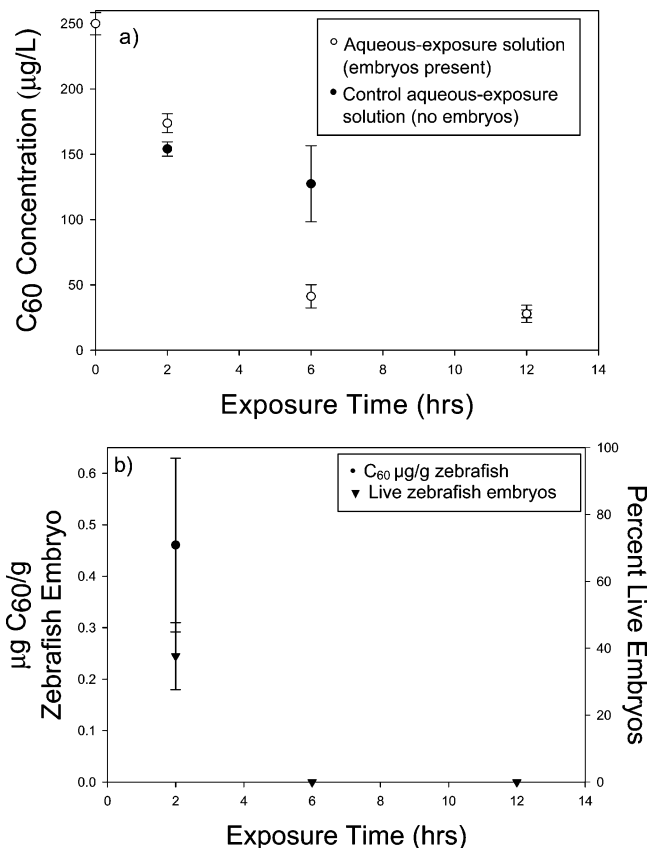


**Figure 5.** Concentration time course for experiments conducted with an initial measured concentration of 250 $\mu$g/L $C_{60}$ ($\pm$95% CI) in (a) aqueous-exposure solution with and without (control) embryonic zebrafish and in (b) embryonic zebrafish along with numbers of live embryos remaining.

(control) zebrafish embryos was also observed over the course of 12 h (Figure 5a). Except for the time point at 6 h, there was not a statistically significant difference in the $C_{60}$ remaining in solution between wells with and without (control) zebrafish embryos. After 12 h, statistically equivalent concentrations of ~28 $\mu$g/L $C_{60}$ indicated that only 11% of the $C_{60}$ remained in the aqueous phase while 89% of the $C_{60}$ was lost to the plate material. In addition, the final $C_{60}$ concentration of 28 $\pm$ 3 $\mu$g/L is equal to the concentrations remaining in solution from experiments conducted with initial concentrations of 70 (Figure 2a) and 130 $\mu$g/L (Figure 4a).

Uptake of $C_{60}$ by zebrafish embryos (0.46 $\pm$ 0.17 $\mu$g$C_{60}$/g) from aqueous-exposure solutions containing an initial concentration of 250 $\mu$g/L $C_{60}$ was significantly higher than for other exposure concentrations (Figures 5b). After 2 h of exposure, only 40% of the embryos remained alive (Figure 5b), and at 6 h, there was 100% mortality. Due to the 100% mortality, uptake and cell death data are not reported for the 6- and 12-h exposures. The 100% mortality is consistent with previous exposures at a nominal concentration of 400 $\mu$g/L $C_{60}$, which corresponds to 250 $\mu$g/L as measured by LC/ESI-MS.[15]

It is likely that the initially high exposures to concentrations of 250 $\mu$g/L $C_{60}$ results in 100% mortality rather than the concentration measured at the 6-h time point, 41 $\pm$ 4.0 $\mu$g/L $C_{60}$. For example, no mortality was observed for concentrations of <70 $\mu$g/L (Figure 2a and b). In contrast, exposures to $C_{60}$ concentrations between 90 (2 h) and 70 $\mu$g/L (6 h) caused 60% mortality (Figure 4a and b). Finally, although the concentrations of $C_{60}$ decreased to levels not associated with mortality, <50 $\mu$g/L (Figures 2 and 4), 100% mortality results when embryos were exposed to concentrations from 250 to 130 $\mu$g/L (Figure 5a). Clearly, is it imperative that the time course of fullerene concentrations be measured, to establish the range in concentrations to which the organism is actually exposed.

### SUPPORTING INFORMATION AVAILABLE

Additional information as noted in text. This material is available free of charge via the Internet at http://pubs.acs.org.