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Comparative toxicity of C₆₀ aggregates towards mammalian cells: role of the tetrahydrofuran (THF) decomposition

Michael Kovichich^{1,‡}, Benjamin Espinasse^{2,‡}, Melanie Auffan^{2,‡}, Ernest M. Hotze^{2,‡}, Lauren Wessel², Tian Xia¹, Andre E. Nel^{1,3,4}, and Mark R. Wiesner^{1,*}

¹Department of Medicine, Division of NanoMedicine, UCLA School of Medicine, 10833 Le Conte Ave, Los Angeles, CA90095

²Department of Civil and Environmental Engineering, Duke University, 121 Hudson Hall, Durham, NC27708

³The Southern California Particle Center, University of California, 10833 Le Conte Ave, Los Angeles, CA90095

⁴California NanoSystems Institute, UCLA, 10833 Le Conte Ave, Los Angeles, CA90095

Abstract

C₆₀ fullerene is a promising material due to its unique physiochemical properties. However, previous studies have reported that colloidal aggregates of C₆₀ (nC₆₀) produce toxicity in fish and human cell cultures. The preparation method of the nC₆₀ raises questions as to whether the observed effects stem from the fullerenes themselves or from the organic solvents used during the preparation of the suspensions. In this paper we set out to elucidate the mechanism by which tetrahydrofuran (THF) treatment to enhance the preparation of nC₆₀ leads to cytotoxicity in a mouse macrophage cell line. Our results demonstrate that THF/nC₆₀ but not fullerol or aqueous nC₆₀ generates cellular toxicity through a pathway that involves increased intracellular flux and mitochondrial perturbation in RAW 264.7 cells. Interestingly, the supernatant of the THF/nC₆₀ suspension rather than the colloidal fullerene aggregates mimics the cytotoxic effects due to the presence of γ -butyrolactone and formic acid. Thus, the role of nC₆₀ in the cellular responses is likely not due to the direct effect of the nC₆₀ material surface on the cells but related to the conversion of THF into a toxic by-product during the preparation of the suspension.

Keywords

Fullerene; nC₆₀; THF; nanoparticles; toxicology

INTRODUCTION

The quantities of nanomaterials produced per year are large and increasing rapidly. Among them, the fullerene is being manufactured on a large-scale and is a promising material due to its high electron affinity and reactivity with nucleophiles. Fullerenes may serve as photosensitizers, producing reactive oxygen species (ROS) (1), but are also widely reported

*Corresponding author: Mark R. WIESNER, Duke University, Durham, NC27708, Phone/Fax: +19196605292/5219, wiesner@duke.edu.

‡Contributed equally to this work

SUPPORTING INFORMATION AVAILABLE

UV-vis, GC-MS data and experimental details. This information is available free of charge via the Internet at <http://pubs.acs.org>.

to be anti-oxidants or radical sponges (2). These properties have been explored for possible medical applications such as DNA cleavage (3), tumor destruction (4), antiviral action (5).

Observations such as DNA cleavage and tumor destruction also suggest that, with adequate exposure, these nanoparticles could have deleterious consequences on biological processes in humans and the environment. Unfortunately, little is known about their hazard potential. Two previous studies concluded that colloidal aggregates of C₆₀ (nC₆₀) produced oxidative damage in the brains of largemouth bass (6) and relatively high toxicity in tissue culture experiments, using human cells (7). A mechanism of ROS production and subsequent attack of C₆₀ on cell membrane lipids was invoked to explain these results. However, the preparation of the nC₆₀ raises questions as to whether the observed effects stem from the fullerenes themselves or from the organic solvents used in preparing the suspension. We have previously shown that the colloidal aggregates of fullerenes used in these studies, THF/nC₆₀, likely contained significant quantities of the residual solvent, tetrahydrofuran (THF) (8). Another study (9) compared toxicity of colloidal C₆₀ prepared with and without polar solvents and concluded that suspensions prepared without using polar organic solvents not only lack toxicity in rodents but they also protected the livers of the exposed animals in a dose-dependent manner against free-radical damage. Another study in larval zebra fish (10) attributed the toxicity of THF/nC₆₀ to THF decomposition products rather than the colloidal fullerene aggregates. This is also in keeping with the demonstration by (11) that γ -irradiation rendered THF/nC₆₀ innocuous, actually converting the suspension to a cytoprotective agent. It has also recently been reported (12) that the formation of THF-peroxide from THF/nC₆₀ may react with indigo dye leading to oxidative stress in *Escherichia coli*. However, this previous work does not exclude possible toxicity of nC₆₀ itself and recommends prudence when interpreting toxicity data.

Against this background, the goal of the present study was to assess the biological impact of THF/C₆₀ aggregates from the perspective of possible cytotoxic products being generated by the fullerene/solvent or THF/solvent interaction. We compare THF/nC₆₀ aggregates with fullerol and water-made nC₆₀ called aq/nC₆₀ in a mouse macrophage cell line, which represents a cell type targeted by nanoparticles at the point of entry into the body of a number of mammalian species. We found that fullerene particles alone were not toxic under the metrics of cytotoxicity employed in this study and that the apparent toxicity of THF/nC₆₀ resides in the soluble components in the particle suspensions. THF itself at the concentrations used to prepare THF/nC₆₀ was not toxic, but two THF degradation by-products identified in solution could produce the toxicity of the THF/nC₆₀ suspension. These data show that the toxicity observed may be misinterpreted as originating from nC₆₀. Indirect mechanisms of toxicity are valid from a perspective of the necessity to treat these materials with solvents and other suspending media and we provide a practical approach to discern between nanoparticle toxicity and the potential toxicity of the medium into which the nanoparticles are transferred.

MATERIALS AND METHODS

Experimental design

Our aim was to analyze whether the nC₆₀ or the synthesis residues (THF and its degradation by-products) was contributing to the toxic effect previously observed with the THF/nC₆₀. We conducted a three-steps experiment. First, the toxicity of THF/nC₆₀ suspension was assessed and compared to the toxicity of aq/nC₆₀. Second, toxicity assays were performed on the supernatant of the THF/nC₆₀ stock suspension (liquid phase) and the centrifugate/dialysate resuspended in water (solid phase). All the toxicity associated with the THF/nC₆₀ suspension comes from this supernatant; its chemical composition was analyzed. Third, the

toxicity of the main components of this supernatant (γ -butyrolactone and formic acid) was assessed compare to the toxicity of the THF alone.

Preparation of C₆₀ aggregates

Fullerols (C₆₀(OH)₁₈) (MER[®], USA) were introduced to water using a sonication bath and filtered on 0.2 μ m membrane. For the aq/nC₆₀, C₆₀ powder (MER[®]) was crushed, introduced to water, and sonicated during 15h under vacuum. For the THF/ nC₆₀, C₆₀ powder (MER[®]) were prepared following the method outlined by (13) and altered by (14).

The concentration of nC₆₀ in the aq/nC₆₀ and fullerols suspensions was determined by Total Organic Carbon TOC (Shimadzu 5050A, USA) and adjusted to 20 mg/L. As carbon compounds related to THF are present in the THF/nC₆₀ suspension, its concentration was determined UV-vis spectrophotometry (Hitachi U2810, USA) (see Supporting information).

Gas chromatography-mass spectrometry GC/MS (Shimadzu QP5050A, USA) was used to identify organic residues present in the THF/nC₆₀ suspension according to (15, 16). Acquisition was done in a selected ion-monitoring (SIM) mode (detector voltage: 1.2 kV, solvent cut time: 3 min). The concentration of formic acid was determined by ionic chromatography IC (Dionex DX120, USA) equipped with a column pack (Ionpack AS14A, Dionex), an AG14 guard column, an auto-sampler (JASCO 851-AS). The concentration of γ -butyrolactone was determined using a gas chromatography-flame ionization detector GC/FID (17A, Shimadzu, USA). The flame ionization process was performed with a DBI-30W J&W Scientific fused silica capillary column (see Supporting Information).

Physico-chemical characterization

Particle size distribution was carried out via dynamic light scattering (DLS) using a CGS 3 (ALV-GmbH, Germany) equipped with a helium-neon laser (633.4 nm). Suspensions were analyzed at 25°C in 5 mm diameter cells with the photomultiplier setting to a scattering angle of 90°. The zeta potential was determined using a Zetasizer Nano ZS (Malvern, UK) equipped with a 633-nm helium-neon laser. The size and zeta potential measurements were performed at 25°C, without addition of salt, and at pH 6.6 \pm 0.4 for the three fullerene-based nanoparticles. The measurements of aggregation states in culture media were performed after centrifugation of the Dulbecco's Modified Eagle Medium (DMEM, Invitrogen, USA) at 50 000 rpm during 30 minutes.

The superoxide anions were targeted and measured via the reduction of XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) (17, 18) when combined with superoxide dismutase (SOD) as a control for non-superoxide reductions (19). The reduction of XTT induces an increase in optical density at 470 nm used to quantify the amount of superoxide present. 5 μ M of each type of nanoparticles was tested for superoxide production in the presence of 5.6 mM NADH (see Supporting Information).

Cell culture and treatment

RAW 264.7 cells were cultured in a 5% CO₂ in DMEM containing 5% Fetal Bovine Serum (Atlanta Biologicals, USA), 5000 U/ml penicillin, 500 mg/L streptomycin, and 2 mM L-glutamine (Invitrogen, USA). Cells were cultured in 48-well plates at a density of 10⁵ cells/well and exposed to nanoparticles in 0.4 mL of medium at 37°C for the indicated time periods. All the nanoparticles solutions were prepared fresh from stock solutions. The fullerene dose used in this study (20 mg/L) is within the range of amounts used in past studies to demonstrate fullerene toxicity (between 10 and 50 mg/L for (7)).

Cellular staining with fluorescent probes and flow cytometry

Cells were stained with the fluorescent dyes diluted in DMEM. The following dye combinations were added for the indicated time periods at 37 °C in the dark: (i) 47.5 mg/L propidium iodide (PI) (Sigma, USA) for 15min in 200 μ L DMEM to assess cell death; (ii) 2.5 μ M dichlorofluorescein diacetate (DCF) for 15min to assess H₂O₂ production; (iii) 5 μ M Fluo-4 for 30min to assess cytoplasmic free calcium; (iv) 4 μ M Rhod-2 for 30min to assess the mitochondrial free calcium (Molecular Probes, USA). Flow cytometry was performed using a FACScan (Becton Dickinson, USA) equipped with a single 488 nm argon laser. DCF and Fluo-4 stained cells are analyzed on the FL-1 channel. Rhod-2 stained cells are analyzed on the FL-2 channel. Data are expressed as fold increase in mean fluorescence intensity normalized to control cells. PI stained cells are analyzed on channel FL-2. Data are expressed as percent positive cells, which are a distinct population from PI negative cells. An unpaired Student's *t*-test was used to assess the statistical significance.

RESULTS

Physico-chemical behavior of fullerene-based nanoparticles in the nutritive medium

The three C₆₀ aggregates have similar zeta potential values of -35.5 ± 1.5 mV. This leads to relatively stable C₆₀ suspensions with mean hydrodynamic diameters of 45–80 nm (Figure 1) and a specific surface area estimated between 46–88 m²/g. When these stock solutions are diluted in DMEM for 16h, rapid aggregation occurs. The THF/nC₆₀ and aq/nC₆₀ suspensions had smaller mean aggregate hydrodynamic diameters of 400–450 nm compare to the ~800 nm of the fullerols. Colloidal destabilization is mainly related to the neutral pH, the elevated ionic strength of the DMEM, and the presence of serum. Because previous work reported that the toxicity of nC₆₀ was related to the production of ROS by the fullerenes, we followed superoxide generation by THF/nC₆₀, fullerol and aq/nC₆₀. No significant difference in abiotic generation of superoxide was seen between the control (DMEM with fetal bovine serum), the three fullerene-based nanoparticles in the dark (Figure 2). Thus, neither form of nC₆₀ produced significant quantities of ROS, while fullerol produces singlet oxygen under UV light, particularly when an appropriate electron donor such as NADH is present (20). These results demonstrate that abiotic ROS production cannot account for THF/nC₆₀ toxicity.

Assessment of cellular induction of H₂O₂ production by fullerene-based nanoparticles

It is possible that nanoparticles that are incapable of abiotic ROS production may promote cells to produce ROS as a result of a functionalized surface, *e.g.* cationic attachment (21). ROS production (H₂O₂) in response to exposure to C₆₀ aggregates was assessed in a macrophages cell line through the use of the fluorescent dye DCF (Figure 3A). While in the presence of aq/nC₆₀ and fullerol there were insignificant ($p > 0.05$) increases in the fluorescence intensity, THF/nC₆₀ suspensions induced a small but significant increase after 4h. This H₂O₂ production is unlikely from fullerene origin and suggests that the THF/nC₆₀ suspension might contain non-particulate toxic components that could be responsible for cellular ROS production.

Cellular and mitochondrial calcium levels and cellular cytotoxicity

Nanoparticle-induced ROS production is capable of generating cellular oxidant injury. An increase in the intracellular calcium concentration or [Ca²⁺]_i constitutes a major cellular response to oxidative stress. Ca²⁺ can be derived from numerous sources, including cellular organelles such as mitochondria and the endoplasmic reticulum (22). Cellular staining with Fluo-4 showed a 6.5-fold increase in [Ca²⁺]_i in response to THF/nC₆₀, whereas no increase was observed with aq/nC₆₀ or fullerols (Figure 3B). This rise in [Ca²⁺]_i can trigger

additional cellular responses, including Ca^{2+} sequestration by mitochondria. This serves to buffer rapid increases in $[\text{Ca}^{2+}]_i$ up to a threshold, beyond which the Ca^{2+} triggers opening of the mitochondrial permeability transition pore. Mitochondrial Ca^{2+} levels, $[\text{Ca}^{2+}]_m$, were assessed by cellular staining with Rhod-2 (Figure 3C). THF/ nC_{60} exposure induced a significant (2-fold) increase in Rhod-2 fluorescence and in cytotoxicity as determined by propidium iodide (PI) staining (Figure 3D). In contrast, aq/nC_{60} and fullerol did not produce a significant increase in $[\text{Ca}^{2+}]_m$ while fullerol had a small but significant effect on nuclear PI uptake (Figure 3D). These data demonstrate that THF/ nC_{60} suspensions induce an increase in mitochondrial Ca^{2+} levels that was associated with a cytotoxic outcome. Since THF/ nC_{60} is incapable of spontaneous ROS production (Figure 2), the increase in DCF fluorescence is likely represents a cellular response to a chemical component in the suspension.

Comparative toxicity of the nanoparticulate and supernatant phases of the THF/ nC_{60} suspension

The role played by possible chemical impurities present in the solution following the synthesis of THF/ nC_{60} was assessed by comparing the toxicity of the solution phase after the removal of the nC_{60} to the toxicity of the whole THF/ nC_{60} suspension. To remove the C_{60} aggregates, the stock solutions of aq/nC_{60} , fullerol and THF/ nC_{60} were either centrifuged at 16,000 rpm or dialyzed across a membrane with a cut-off of 4 nm. Cytotoxic assays were performed on the whole suspension, supernatant, dialysate and the solid phase C_{60} aggregates. No significant toxicity was observed for the as-synthesized aq/nC_{60} and fullerol suspension as well as the centrifuged pellet resuspended in water nor their supernatants (Figure 4). This differs from the almost 100% toxicity of the whole THF/ nC_{60} suspension (LS) as well as its centrifuged supernatant (L). In contrast, the resuspended THF/ nC_{60} pellet was devoid of toxicity after resuspension and addition to the cell culture (Figure 4). These data are in favor of a toxic component in the supernatant. This point was further illustrated by analyzing the THF/ nC_{60} dialysate, which generated the same level of toxicity as the centrifugation supernatant (Figure 4). This dialysate was proven to be devoid of particles since no characteristic fullerene peak was observed by UV-vis spectroscopy (see Supporting information, Figure S1). These results confirm that the toxic component does not migrate with the THF/ nC_{60} aggregates and is likely is due to residual THF or its by-products.

Identification of the toxic chemical components in the THF/ nC_{60} supernatant

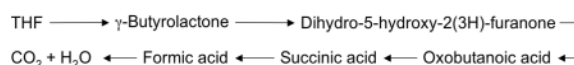
An estimated $26 \pm 2\%$ (20 mg/L) of the TOC content of the THF/ nC_{60} stock suspension is attributed to the nC_{60} with $74 \pm 2\%$ (56 mg/L) of the signal coming from dissolved carbon compounds. No THF is detected in the THF/ nC_{60} stock solution by GC/FID. A search for suspected by-products of THF degradation focused on succinic acid, acetic acid, γ -butyrolactone, glycolic acid, and formic acid since these are the possible intermediates for the photocatalytic degradation of THF (23). GC/MS detected the presence formic acid (6% of the TOC or 8 mg/L) and the γ -butyrolactone (22% of the TOC or 22 mg/L) in the THF/ nC_{60} (see Supporting information, Figure S2).

The kinetic of the THF degradation were studied using fresh suspensions made with aq/nC_{60} and fullerol to which THF were added. After 3h in presence of nC_{60} , THF was no longer detectable and γ -butyrolactone appeared in the suspensions (Figure 5A). But this THF degradation does not required the presence of nC_{60} . Indeed, this reaction occurs when a pure solution of THF (without nC_{60}) is maintained (during < 2h) in an open, stirred-environment that promotes contact with oxygen (Figure 5B).

Cellular toxicity was assessed in the presence of pure and fresh THF, γ -butyrolactone or formic acid. A solution containing 0.15vol. THF in DMEM did not induce toxicity (Figure 6A). This is ~15 times higher than the total carbon concentration in the THF/nC₆₀ stock suspension. THF alone showed evidence of toxicity only when concentrations exceed 0.35vol. Similar toxicity assays performed on the two by-products confirmed that they are highly toxic at the amounts present in the THF/nC₆₀ stock suspension (Figure 6B and C). γ -butyrolactone but not the formic acid stimulated an increase [Ca²⁺]_i (Figure 6D). These results show that at least two THF by-products can explain the cellular toxicity induced by THF/nC₆₀ suspension.

DISCUSSION

A significant finding is that the cellular toxicity observed for the THF/nC₆₀ resides primarily in the supernatant of the nC₆₀ suspensions. We arrive at this finding based on results obtained after separating the fullerene aggregates from the supernatant by either centrifugation or dialysis. THF/nC₆₀-induced cytotoxicity appears to be mediated by the oxygenation of THF into at least two toxic by-products (γ -butyrolactone and formic acid) in amounts sufficient to induce cell death. Among these breakdown products, γ -butyrolactone was capable of inducing intracellular Ca²⁺ release and an increase in mitochondrial Ca²⁺ that contribute to cellular toxicity by mitochondrial perturbation. One mechanism to explain the degradation of THF is that it is initially oxygenated to γ -butyrolactone, in turn degraded to several compounds such as the observed formic acid as proposed by (23):



(12) also observed the formation γ -butyrolactone in the THF/nC₆₀ suspension. Our results complete this study since this toxic compound comes from the oxygenation of the THF rather than its interaction with the nC₆₀. This is reinforced by (10) showing that the by-products of THF oxidation could be responsible of the toxicity of THF/nC₆₀ suspensions in larval zebra fish. In contrast to our study, (10) did not observe formic acid as a by-product in their suspensions. However their THF/nC₆₀ were prepared in the dark under an N₂ atmosphere while our THF/nC₆₀ were prepared under ambient light and O₂.

Earlier studies on nC₆₀ toxicity attributed abiotic ROS production as the source of injury (7). However the lack of ROS production by aq/nC₆₀ and THF/nC₆₀ suspensions under abiotic conditions calls these results into question and is further bolstered by the finding that fullerol, which was capable of abiotic ROS production, was devoid of toxicity. The small but significant amount of H₂O₂ production that we observed with the THF/nC₆₀ suspension is likely from cellular origin and could reflect mitochondrial, either by the toxic THF by-products or as a concomitant of cell death. Thus, instead of a fullerene-induced oxidative stress mechanism, in our experimental conditions most of the toxicity resides in the use of THF, which leads to toxic breakdown products as described above.

This study resolves much of the controversy surrounding THF/nC₆₀ toxicity in highlighting the toxic effect that THF produces in eukaryotic cell cultures. This is distinct from a lower-level toxicity observed for aq/nC₆₀ in microbial cell cultures and in some human cell cultures (7). Thus, the true toxicity of C₆₀ is certainly far less than previously reported and in no case can conclusions be drawn on the toxicity of nC₆₀ based on studies using THF/nC₆₀. The methodology proposed in this study deals with short-term assays on cultured cells, but can be applied to different organisms, or other solvents. For instance, previous studies (24, 25) have used DMSO to dispersed nC₆₀ and have observed that DMSO/nC₆₀ induces changes in the gene expression in zebrafish embryos. Even if the DMSO does not

induce toxicity at the concentrations studied (24) the same recommendation of prudence in interpreting data from studies with THF/nC₆₀ applies to the DMSO/nC₆₀ studies given the possibility of toxic DMSO by-products (26–28).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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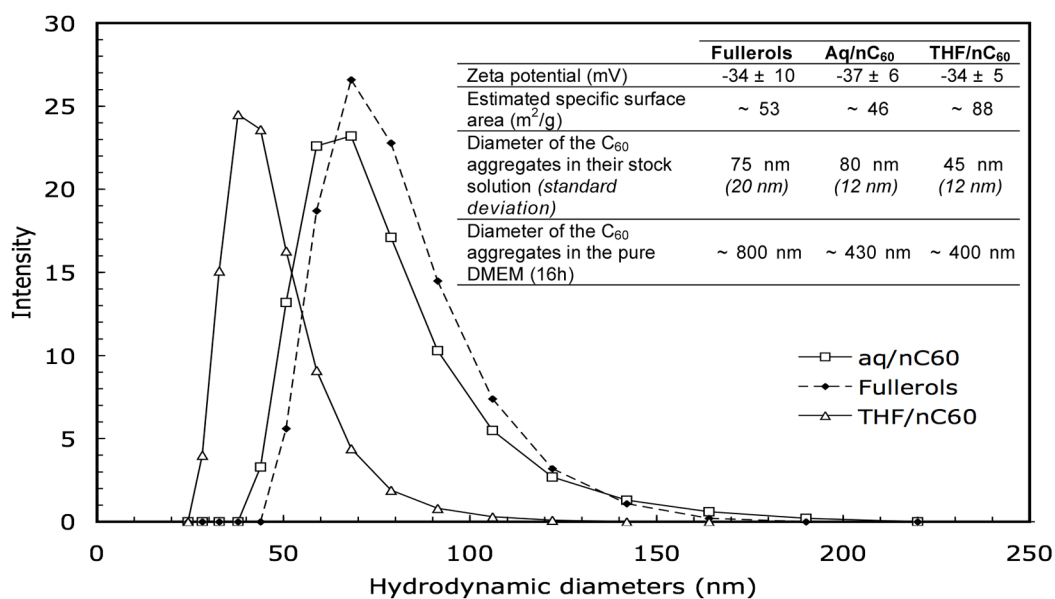


Figure 1. Distribution of the hydrodynamic diameters of the aq/C₆₀, THF/nC₆₀ and fulleroles in their initial stock suspensions. Addition to DMEM resulted in rapid aggregation (inset).

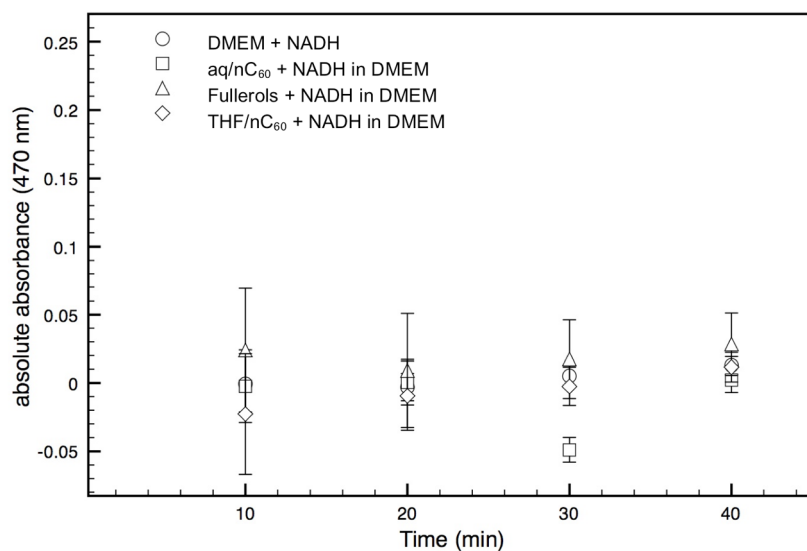


Figure 2. Reactive Oxygen Species generation by the C₆₀ aggregates (5 μM) in complemented DMEM. NADH (5.6 mM) is present as an electron donor. Low absorbance of XTT indicates a lack of significant superoxide activity in these suspensions. Error bars represent 95% confidence interval where n=3 for each point.

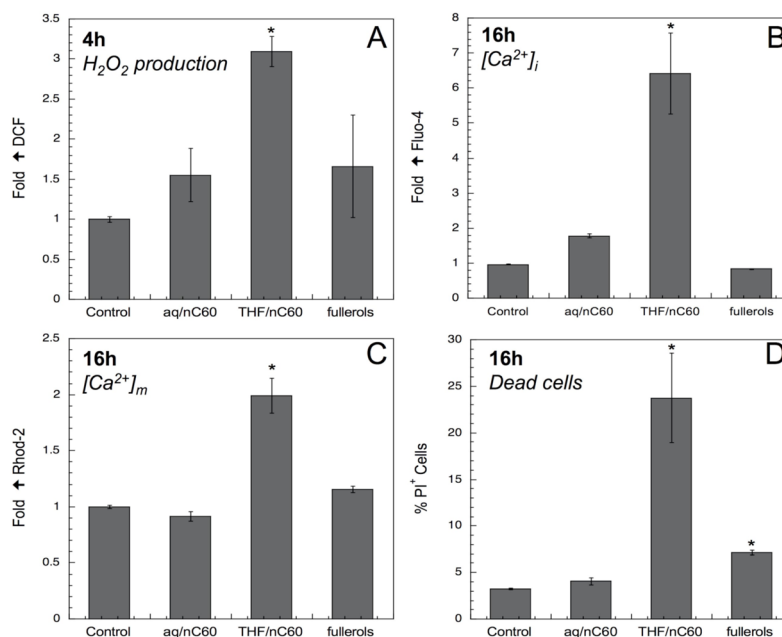


Figure 3. H_2O_2 production in RAW 264.7 in presence of aq/nC₆₀, THF/nC₆₀ and fullerol (A). Effect of nanoparticles on intracellular $[Ca^{2+}]_i$ (B) and mitochondrial $[Ca^{2+}]_m$ (C) calcium levels. Changes in cellular toxicity (PI uptake) during fullerene-based nanoparticles exposure (D). *:p<0.05.

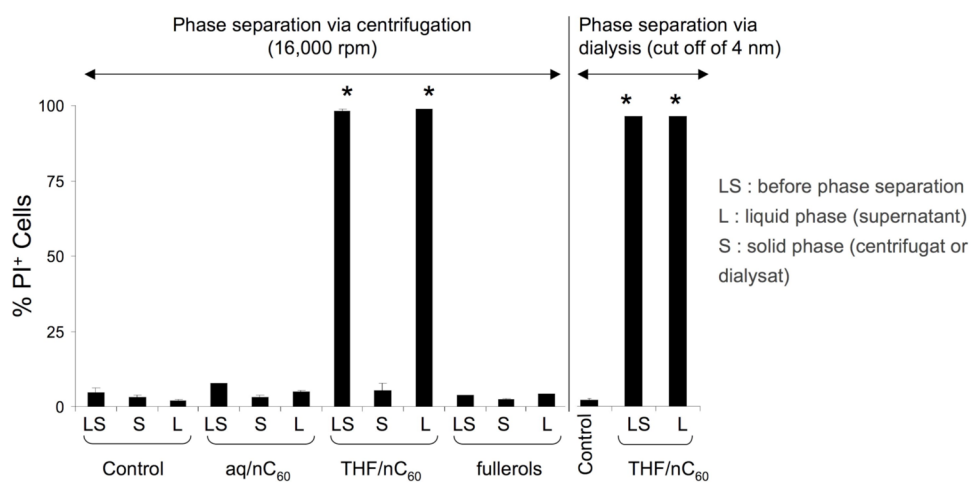


Figure 4. Comparative toxicity (PI) of the supernatant (liquid phase containing the dissolved residue from the synthesis) and the solid phase containing C₆₀ aggregates. The phase separation is performed by centrifugation at 16,000 rpm for all particles. Dialysis was performed with cut-off of 4 nm dialysis tubes for THF/nC₆₀. The toxicity observed in presence of THF/nC₆₀ comes from the supernatant.

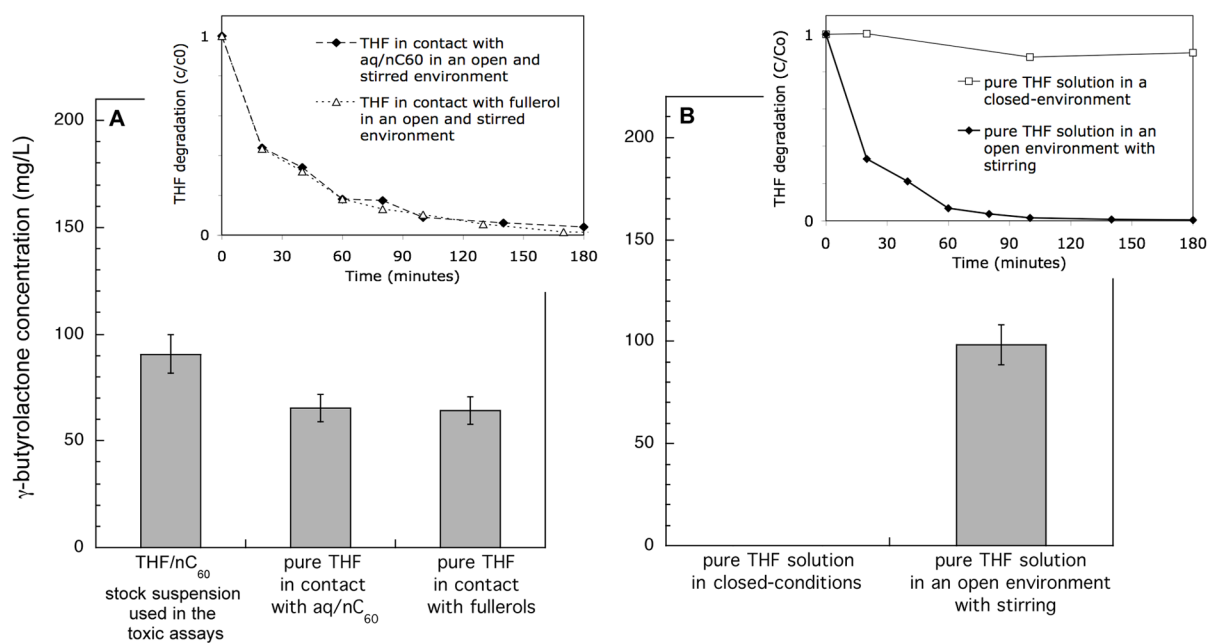


Figure 5. THF degradation (insets) and γ -butyrolactone appearance after 180 minutes (main graphs) in the dark. (A) In presence of nC_{60} (aq/ nC_{60} or fullerols). The suspension are stirred in an open environment. (B) In absence of nC_{60} in closed-conditions or in an open environment.

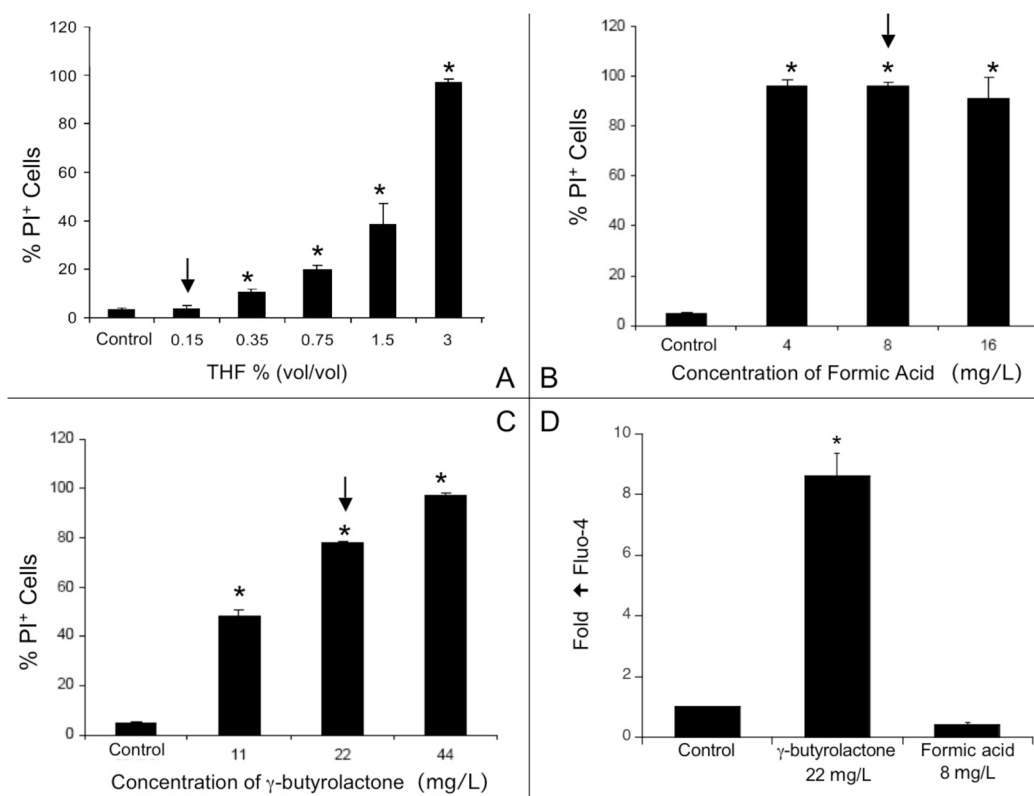


Figure 6.

(A) Toxicity (PI) of a pure THF solution towards RAW 264.7 cells. The arrow indicates a concentration in THF ~15 times larger than the total organic carbon concentration of the THF/nC₆₀ stock suspension. (B) Toxicity (PI) of formic acid and (C) gamma-butyrolactone. The arrows indicate the concentration measured in the THF/nC₆₀ stock suspension. (D) Effects of formic acid and gamma-butyrolactone on intracellular [Ca²⁺]_i calcium levels. *: p<0.05.