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The Differential Cytotoxicity of Water-Soluble Fullerenes

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ABSTRACT

We show that the cytotoxicity of water-soluble fullerene species is a sensitive function of surface derivatization; in two different human cell lines, the lethal dose of fullerene changed over 7 orders of magnitude with relatively minor alterations in fullerene structure. In particular, an aggregated form of C_{60}, the least derivatized of the four materials, was substantially more toxic than highly soluble derivatives such as C_3, $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$, and $C_{60}(OH)_{24}$. Oxidative damage to the cell membranes was observed in all cases where fullerene exposure led to cell death. We show that under ambient conditions in water fullerenes can generate superoxide anions and postulate that these oxygen radicals are responsible for membrane damage and subsequent cell death. This work demonstrates both a strategy for enhancing the toxicity of fullerenes for certain applications such as cancer therapeutics or bactericides, as well as a remediation for the possible unwarranted biological effects of pristine fullerenes.

Introduction. Water soluble fullerene derivatives are essential for many emerging biomedical technologies which exploit the unique chemical properties and physical structure of C_{60}.[1−3] Their toxicity, both in tissue culture and in vivo, is an important characteristic for defining and constraining these applications.[4−18] In some cases, the phototoxicity of fullerene molecules has been identified as a feature useful for therapeutics.[16,17,19] Other work has sought to minimize the toxicity of water-soluble fullerenes so as to permit their use in drug delivery applications.[1−3] Water-soluble fullerene species are also important for understanding the eventual fate and environmental implications of fullerenes used in consumer products.[20] In this case, underivatized fullerene materials upon contact with water form sparingly soluble fullerene colloids, termed in this work "nano-C_{60}". Because of both the environmental and biological significance of fullerenes in water, this paper examines the comparative cytotoxicity of several important types of water-soluble fullerenes using human liver carcinoma cells and dermal fibroblasts.

Our attention was drawn to this issue by the recent interest in the environmental effects of nanoscale aggregates of C_{60}, which we refer to here as "nano-C_{60}". This form results when pristine C_{60}, from either the solid state or organic solution, is placed into contact with neutral water.[21−23] Rather than completely precipitating, some C_{60} will form suspended and water-stable aggregates up to 100 ppm concentrations.[24−28] Several groups have proposed this method for water solubilizing fullerenes, but the low yield of this process has limited the study and application of nano-C_{60}.[26] However, the unintentional generation of these aggregates in aqueous environments is a possibility, particularly if C_{60} finds widespread use in consumer products such as coatings and fuel cells. Thus their toxicological and ecotoxicological effects are of great importance. Just recently these fullerene aggregates were found to elevate lipid oxidation levels in the brains of fish.[29]

Here, we report for the first time the effects these fullerene aggregates, or nano-C_{60}, have on human cells in culture, and find that even at very low concentrations this species is cytotoxic (Figure 1). While the concentration of nano-C_{60} is low (50 mg/L or 50 ppm) upon formation in water, the ppm levels required for cytotoxicity tests are entirely accessible.[26−28] For comparison, we have also included in these studies biological characterization of three other highly soluble fullerene derivatives. These derivatives represent a range of possible systems where covalent bonds made to the pristine cage of C_{60} result in substances with varying levels of water

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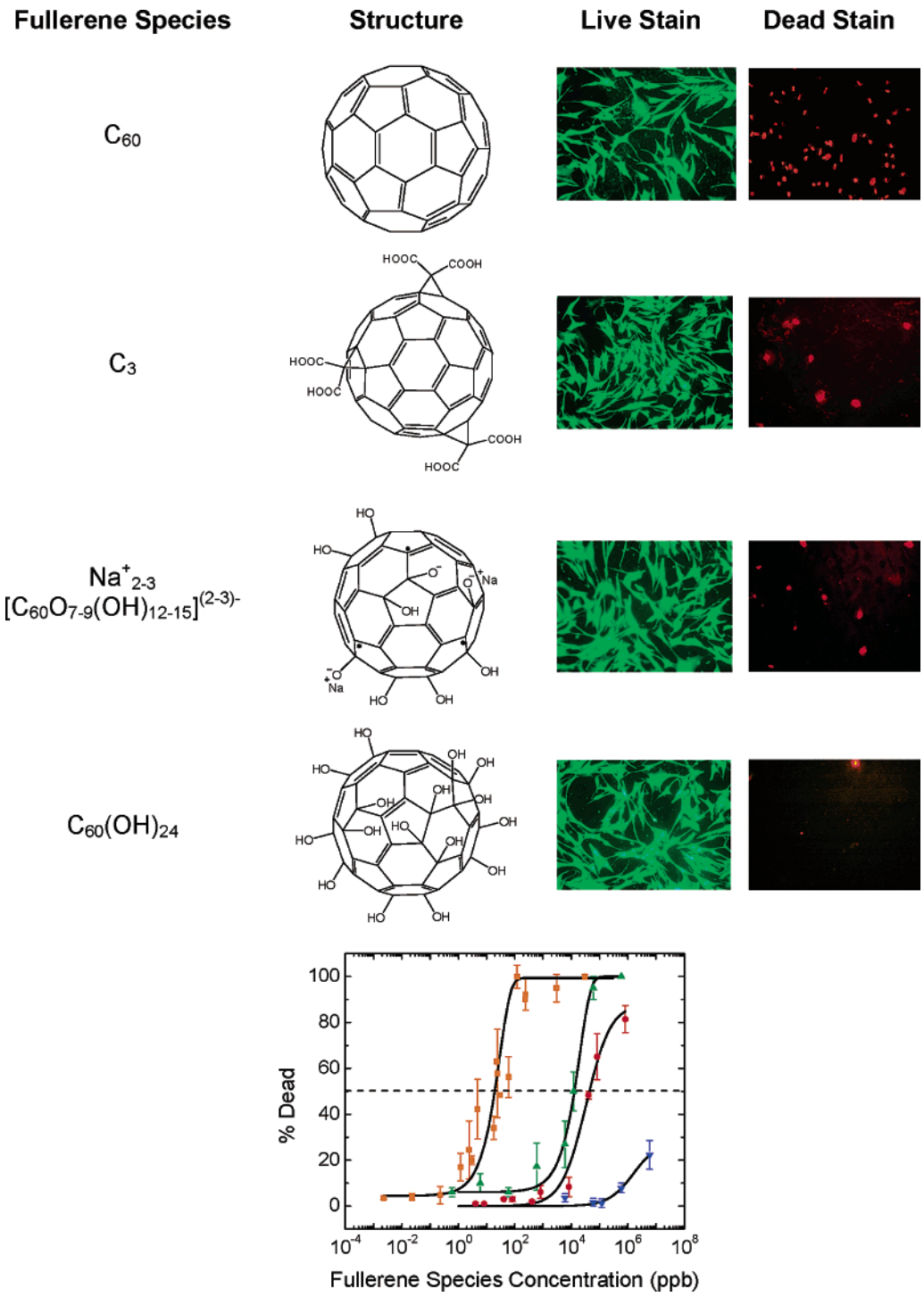

Figure 1. Differences in the structure and cellular activity of nano-C_{60}, C_3, $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$, and $C_{60}(OH)_{24}$. The structure of each fullerene species is shown in the table, as well as the live and dead stains. (Bottom) The differential cytotoxicity of nano-C_{60} (■) as compared to C_3 (▲), $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$ (●), and $C_{60}(OH)_{24}$ (▼) in human dermal fibroblasts. Cells were exposed to toxicant for 48 h.

solubility (13 000−100 000 mg/L).[30−36] For the same cell lines, and under the same conditions, we find the high cytotoxicity of nano-C_{60} is not a universal property of C_{60} materials. Instead, the cytotoxicity of C_{60} derivatives systematically correlates with their chemical functionality in both human skin (HDF) and liver carcinoma (HepG2) cells. We show that for cells exposed to nano-C_{60} cell death occurs because of lipid oxidation caused by the generation of oxygen radicals; more highly derivatized C_{60} systems are not as facile at generating these species and thus have lower cellular toxicity. This mechanism suggests a basis for the first structure−function relationship for the toxicity of C_{60} based materials.

We hypothesize that sparingly soluble fullerenes will cause oxidative damage to cellular membranes even at relatively low concentrations, and that the resulting toxicity will

diminish as the fullerene cage becomes more fully derivatized and water soluble. Eastoe and Guldi reported oxidative damage to the surfactant of a micelle stabilized in toluene containing pristine C_{60}. They suggested that the high electron affinity of C_{60} coupled with available oxygen and water resulted in radical generation.[30,37,38] In organic media, the low-lying and degenerate LUMOs of C_{60} permit its active participation in many free-radical and electron-transfer processes.[37,39−42] Other studies of water solubilized fullerenes have also found these materials will, under some circumstances, generate oxygen radicals.[38,43,44] In one study, the free radical generated in the presence of atmospheric oxygen and C_{60} is the superoxide anion, the anion primarily responsible for peroxidation of the lipid bilayer.[43]

Given their rich radical chemistry, it is not surprising that previous studies have found some specific fullerene derivatives to be toxic to cells in culture.[10−14,36,45−52] Typical lethal concentration (LC_{50}) values reported for 48 h in vitro exposures with these systems range from 100 ppm for a malonic acid fullerene derivative (C_3) in human cervix cells to 1 ppm for a polymer-wrapped fullerol species exposed to mouse fibroblasts.[13,14,17] These data taken together suggest that fullerene cytotoxicity is highly variable and dependent in some cases on the light exposure of cells. Direct comparison of this existing data, however, is not possible as cell lines and methodologies are not consistent across various studies.

Fullerene Derivatives. Sublimed C_{60} and $C_{60}(OH)_{24}$ were purchased from MER at 99.95% and 99.8% purity, respectively. C_3 and $Na^+_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$ were received from Dr. Lon Wilson, Rice University, and their characteristics have been previously reported.[53−55,56] Preparation of the Na^+−fullerenol sample began with C_{60} dissolved and sonicated in toluene (1.25 μM). A basic aqueous solution (NaOH) and tetrabutylammonium hydroxide (TBAH) were added at 13.8 mM and 1.0 mL, respectively, and a water-soluble species was isolated after 48 h. The Na^+−fullerenol sample was purified via crystallization and characterized by absorbance spectroscopy.

Of particular interest here is the water-soluble form of C_{60} that results when pristine materials are added to water.[26−28] By means of solvent extraction, sonication, or simply by stirring over time, C_{60} can be brought into water to produce a yellow suspension with concentrations up to 100 mg/L. Figure 2 shows the absorption spectrum and liquid chromatographic materials used in this study. They are composed primarily (>99%) of underivatized fullerenes, aggregated into negatively charged crystalline particles with a mean size of ~60 nm. Also in Figure 2 is a representative cryogenic transmission electron micrograph of flash-frozen nano-C_{60} suspensions. This image reveals the aggregates are faceted particles, and electron diffraction confirms that the materials possess an HCP crystal structure consistent with bulk fullerene crystals.[57] With an average aggregate size of 60 nm, only 1 in 10^6 C_{60} molecules are present at the surface of the nanoparticles. Thus, the vast majority of the material remains completely underivatized as indicated by the HPLC chromatogram of the aggregates re-extracted into toluene. The elution time is identical to that of pristine C_{60} (Figure 2) and only very small features of derivatized materials are seen just above the noise.

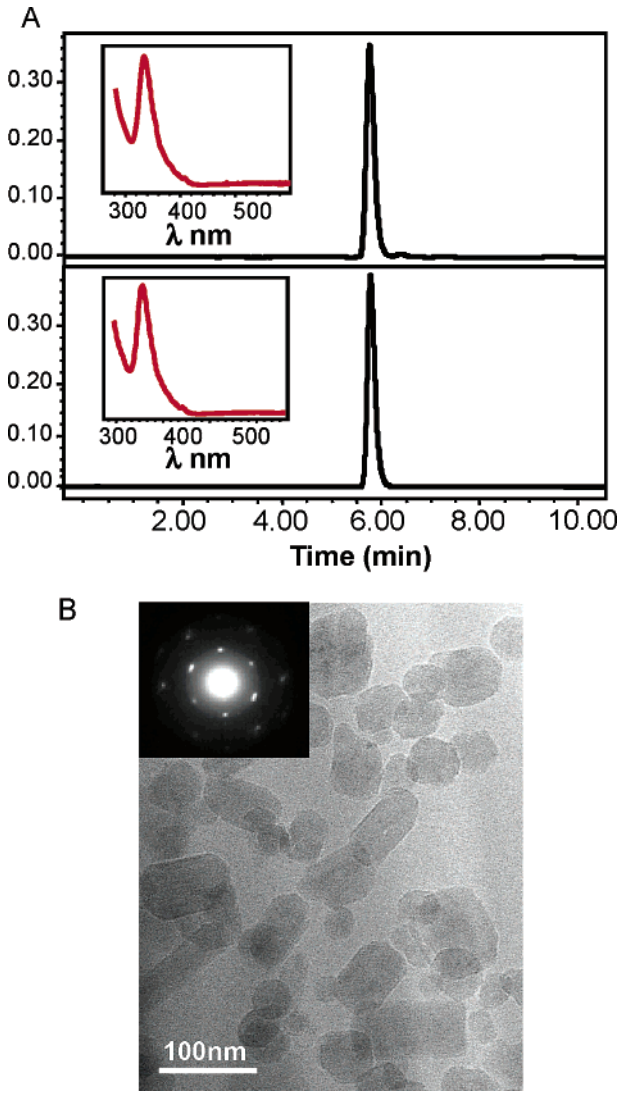

Figure 2. Spectroscopy, chromatography, and microscopy of the nano-C_{60} aggregated water-soluble fullerene species. (A) The chromatogram and UV/vis spectrum of the re-extracted C_{60} water suspension (top) as compared to C_{60} dissolved in toluene (bottom). (B) The cryo-TEM images and diffraction pattern of nano-C_{60}. Average size of nano-C_{60} crystalline nanoparticle is 100 nm.

In Vitro Cytotoxicity. To consistently evaluate the cytotoxicity of water-soluble fullerenes species, two cell lines, human dermal fibroblasts (HDF) and human liver carcinoma cells (HepG2) (ATCC), were cultured in Dulbecco's modification of Eagles media (DMEM).[58,59] Cells were grown to 70% confluency before exposure to each fullerene sample; each culture plate was incubated in the dark at 37 °C/5% CO_2 for 48 h. The concentrations of each fullerene species delivered were 0.24−2400 ppb. The LC_{50} value, the concentration at which 50% of cells die, was determined by evaluating cytotoxicity over the concentration range. Cytotoxicity was measured using a LIVE/DEAD Viability/Cytotoxicity Kit (Molecular Probes) for staining with calcein AM (live cells fluoresce green) and ethidium homodimer (dead cells fluoresce red) and validated using LDH (lactate

dehydrogenase) and MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assays.[60−64] Paraquat (methyl violgen) was used as a positive control, and LC_{50} values for this molecule using these cell lines (150 ppm) compared well to existing literature values (230 ppm).[18]

Cytotoxicity Observed in the nano-C_{60} Water-Soluble Fullerene Species In Vitro. We measured the cytotoxicity of four different water-soluble fullerene species on human dermal fibroblasts (HDF) and human liver carcinoma cells (HepG2). The differential cytotoxicity was first examined using a standard cytotoxicity screen. After each species was administered separately to HDF and HepG2 for 48 h, viability was determined by staining with calcein AM and ethidium homodimer (Molecular Probes); calcein AM is taken up by cells and becomes fluorescent when cleaved by esterase enzymes in viable cells, while viable cells are impermeable to the second fluorescent dye, ethidium homodimer. The use of two dyes reduces possible interference effects from toxicant interactions with the probes. To further verify cell death, the release of lactate dehydrogenase (LDH) was monitored as nano-C_{60} concentration increased.[62,63] The cell's DNA concentration was also determined for both normal and toxicated cells. Since the DNA concentration was the same for both sets of cells, and there was no change in MTT concentration, initial evidence suggests that the cell's internal organelles remain intact.

The least derivatized water soluble fullerene species is substantially more toxic to both cell lines than the highly derivatized water soluble fullerene species. Figure 2 shows the dose−response behavior of each fullerene type on human dermal fibroblasts; the lethal doses vary by 7 orders of magnitude depending on the derivatization of the cage. The nano-C_{60} aggregates are toxic to HDF cells at a LC_{50} value of 20 ppb. The larger crystalline and amorphous aggregates can be separated from possible molecular contaminants by centrifugation. We confirmed that the aggregates were the lethal species by comparing the cytotoxicity of both the supernatant and pellet of nano-C_{60} after centrifugation at 30 000 g for 30 min; the pellet had virtually identical effects to the original solution. By comparison, more highly soluble fullerenes had less pronounced biological effects. C_3 is cytotoxic to HDF cells at a LC_{50} value of 10 000 ppb, $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$ at a value of 40 000 ppb, and $C_{60}(OH)_{24}$ at a value of >5 000 000 ppb. Interestingly, the apparent cytotoxicity scales with the degree of derivatization of the fullerene cage.

Evidence of Leaky Cytoplasmic Membrane in the Presence of nano-C_{60}. We also evaluated the characteristics of cells exposed to fullerene species, in this case using the most toxic of the materials, nano-C_{60}. Based on several independent experiments, both HDF and HepG2 cells show evidence of a leaky plasma membrane after exposure to toxic concentrations of fullerenes. First, the concentration of lactate dehydrogenase, an enzyme that is commonly released after cell death, was monitored in the media at 490 nm in the presence of a tetrazolium dye (Sigma). For the cells dosed with 2.4−2400 ppb nano-C_{60}, an increasing amount of LDH was observed, thus suggesting the disruption of the plasma membrane. It is important to note that no evidence of DNA oxidation, protein oxidation, or release of mitochondrial dehydrogenase was observed at any nano-C_{60} dose. This suggests that the observed oxidative damage occurred exclusively at the cellular membrane and is likely associated with membrane injury rather than cell death.

To confirm the physical disruption of the membranes, a series of fluoresceinano-derivatized dextrans (Molecular Probes) of increasing molecular weights were added to cells dosed with 240 ppb nano-C_{60} after 30 h (Figure 3). The cells were incubated with the dextran solutions (2.5 mg/mL) for 30 min, rinsed thoroughly with phosphate-buffered saline (PBS), and fixed with 5% glutaraldehyde (Sigma). Dyes that diffuse into cells emit light, thus allowing for a visualization of the effectiveness of the cell membrane as a barrier. Because a normal cellular membrane is somewhat permeable, it is expected that smaller molecules will penetrate the membrane. However, in a cell with a leaky membrane, larger molecules will also move into the cell. While the smallest sized dye of 10 kDa proved to fluoresce in the control and toxicated cells, the medium and large sized dyes, 70 kDa and 500 kDa, proved to fluoresce only in the cells administered with the nano-C_{60} solution, thus exhibiting evidence of a leaky membrane (Figure 3). The 500 kDa dye was added to cells dosed with 240 ppb $C_{60}(OH)_{24}$, as well; however, no fluorescence was observed.

We correlated evidence for leaky membranes to the lipid peroxidation of the cells. In the presence of a radical species, the interior tail of the lipid bilayers undergoes a series of reactions; in particular, peroxy-radicals are formed on the alkene termini of the lipid bilayer. This peroxy-radical is hydrophilic and will thus associate extracellularly forming a hole in cytoplasmic membranes.[65] A fluorescent indicator for this radical, 1,1,3,3-tetraethoxypropane (MDTA), emits light only when associated with this species. Figure 3 shows the increasing emission of this probe as HDF cells are exposed to increasing levels of nano-C_{60}. This experiment was repeated for HepG2 cells, yielding the same results.

Nano-C_{60} Can Produce Oxygen Radical Species in Cell-Free Experiments. Experiments strongly suggest that the mechanism of cell death is oxygen radical induced peroxidation of the lipid bilayers of cells. We show in Figure 4 that in cell-free aqueous solutions nano-C_{60} can produce the superoxide anion and, in comparison, fully hydroxylated fullerenes cannot. We measured the appearance of the superoxide anion in two ways to rule out any fullerene interference with the photometric tests. In the first assay, iodophenol is used as an indicator for superoxide anion; this dye absorbs at 416 nm only when exposed to the superoxide anion. After addition to an aqueous nano-C_{60} solution (5 ppm) at a concentration of 200 μM, the visible absorption steadily increased (Figure 4); the same result was found regardless of the ambient light exposure. In addition, C_3, $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$, and $C_{60}(OH)_{24}$ aqueous solutions were examined. While C_3 exhibited a small change in emission, $C_{60}(OH)_{24}$ and $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)}$ aqueous solutions added to iodophenol in the same concentrations showed no change in emission.

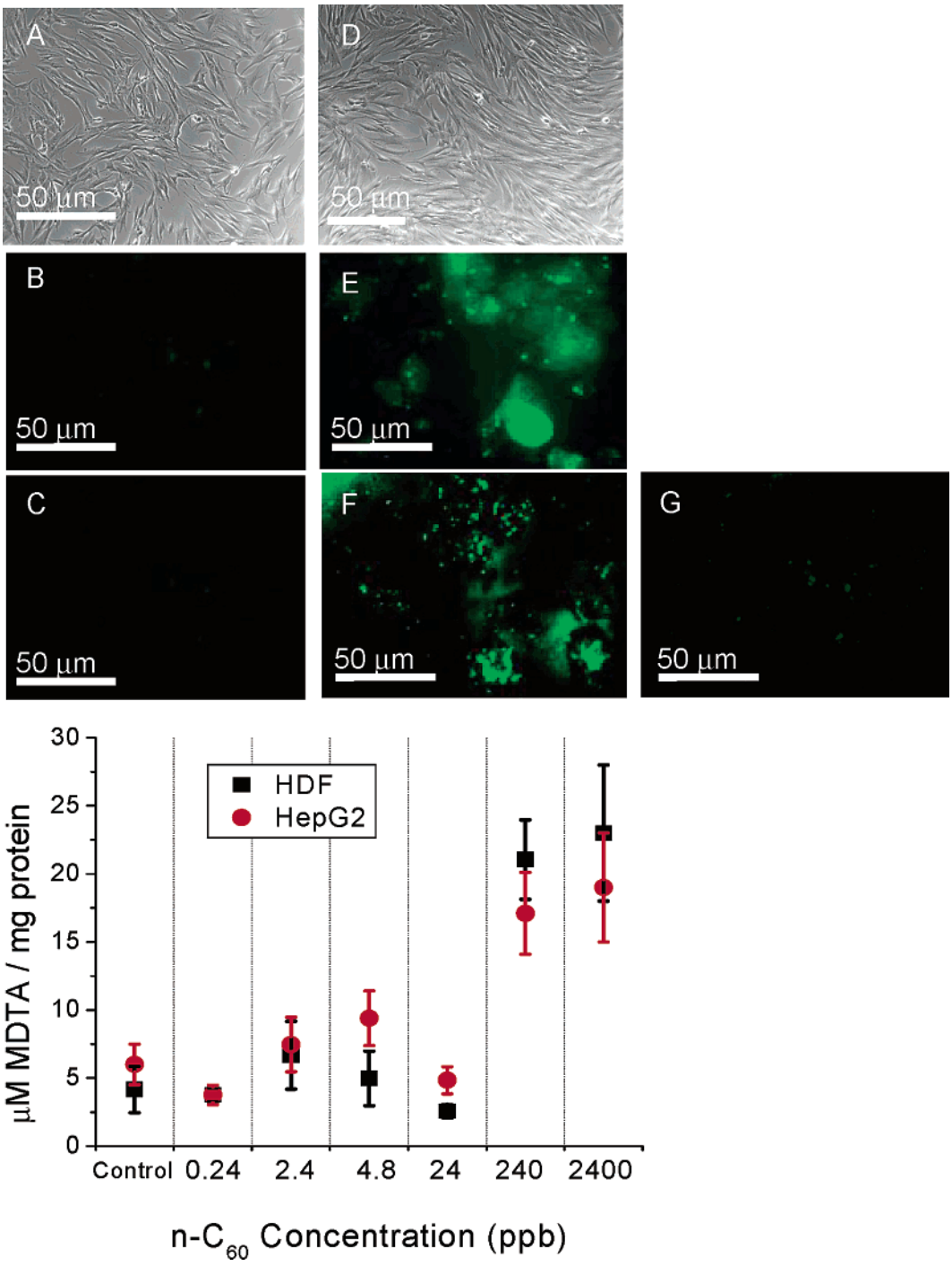

Figure 3. Fluorescence detection of disrupted plasma membranes. (A) Phase contrast image of normal healthy cells and (D) toxicated cells. Fluoresceinano-conjugated dextran of (B) 70 000 kDa, and (C) 500 000 kDa added to normal HDF cells. After cells had been dosed with 240 ppb nano-C_{60} and exposed for 24 h, fluorescence detection of the Dextran dye was observed for (E) 70 000 kDa and (F) 500 000 kDa. However, cells dosed with 240 ppb $C_{60}(OH)_{24}$ showed no signs of leaky membranes. (Bottom) Lipid peroxidation was observed in toxicated cells. As the nano-C_{60} concentration increases, the MDTA production increases.

We confirmed the ability of nano-C_{60} to provide oxygen radicals using the xanthine/xanthine oxidase reaction. Here, xanthine, a compound found in all cells, will fluoresce in the presence of iodophenol or luminol. Its fluorescence is reduced once it is oxidized to uric acid. In cells, xanthine oxidase converts dissolved O_2 to the superoxide anion, which in turn oxidizes xanthine and decreases its fluorescence. We observed a decrease in light emitted from the xanthine/xanthine oxidase solution only after the nano-C_{60} was added to it, a result consistent with the ability of the species to generate superoxide anions. The competing reaction of the oxidation of iodophenol loses, thus consistent with the ob served decreased light intensity. No decrease in light emission was observed when C_3, $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$, and $C_{60}(OH)_{24}$ were added to the xanthine solution, independently.

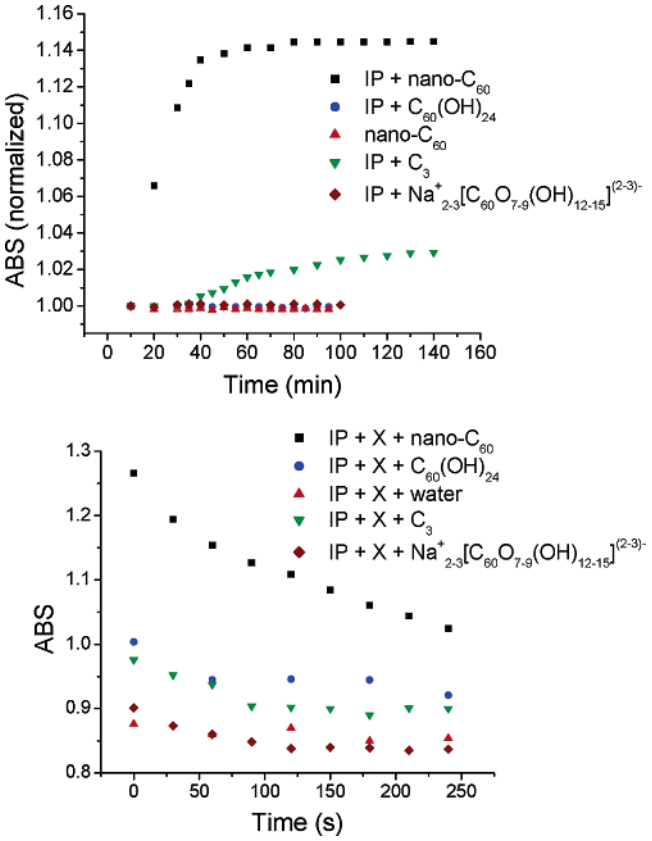

Figure 4. Presence of an oxygen radical species in the nano-C_{60} solution. At 416 nm, (A) iodophenol will fluoresce in the presence of nano-C_{60}, but not $C_{60}(OH)_{24}$. (B) A decrease in absorbance was observed only when nano-C_{60} was added to a xanthine. The same results were observed from the experiment in the presence and absence light. The absorbance profile of C_3 and $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$ is included as well.

A detailed understanding of the mechanism by which nano-C_{60} generates superoxide anions is beyond the scope of this paper; we note that other forms of water soluble fullerenes have been associated with superoxide anions in several cases.[43,44,66] Perhaps the ability for a fullerene to generate these species depends on the details of its chemical derivatization; ongoing efforts will further establish a connection between the superoxide anion generation of fullerenes and their cytotoxicity.

Summary and Conclusions. In conclusion, we have shown that nano-C_{60} is cytotoxic to HDF and HepG2 cells at the 20 ppb level. The C_3 and $Na^{+}_{2-3}[C_{60}O_{7-9}(OH)_{12-15}]^{(2-3)-}$ water-soluble fullerene species are less cytotoxic to HDF or HepG2 cells, while $C_{60}(OH)_{24}$ shows no cytotoxicity up to its limits of solubility. This provides striking evidence that water-soluble functional groups on the surface of a fullerene molecule dramatically decrease the toxicity of pristine C_{60}. After 30 hours of exposure to nano-C_{60}, cells begin to exhibit signs of leaky membranes and lipid oxidation. However, we have concluded that this toxicant does not oxidize the cell's proteins or disrupt the normal function of certain cellular organelles. In cell-free studies, fullerene materials can in some cases generate superoxide anions that could be the agent responsible for membrane oxidation and cytotoxicity. This work demonstrates that hydroxylation of the C_{60} cage could be used as a remediation for the possible unintentional biological effects of pristine fullerenes.

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