Stable Colloidal Dispersions of C60 Fullerenes in Water: Evidence for Genotoxicity

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Concerns regarding potential health risks and the environmental impact of engineered nanomaterials prompt a proactive approach to ensuring that the burgeoning nanotech industry is environmentally benign and sustainable [1-3]. Such approach should include studies on fundamental nanomaterials chemistry, likely significant sources and potential escape routes for nanomaterials, their transport and fate in the environment and living tissues, and nanomaterials toxicity. In view of the large diversity in chemistries involved, different types of nanomaterials ranging from semiconducting quantum dots to metal oxides to carbon-based nanoparticles need to be evaluated separately.

One salient example of a novel carbon-based nanoscale material of growing practical importance is C_{60} fullerene. C_{60} had been thought to exist in water only in molecular solutions of very low concentrations (less than 10^{-9} mg/L [4]) unless its surface was derivatized to render C_{60} more hydrophilic. However, it was shown that under certain conditions, pristine C_{60} can form suspensions of C_{60} clusters (nC_{60}) in water [5, 6]. While the complete information on suspension composition and surface chemistry of different types of nC_{60} is not yet available, nC_{60} particles in hydrosols have been shown to be hydrophilic [7] and charged (e.g. [8, 9]). It was also demonstrated that nC_{60} suspensions comprise particles in colloidal size range (e.g. [10]) and, possibly, hydrated molecular fullerene $C_{60}@\{H_2O\}_m$ [11, 12]. One implication of these findings is that fullerene hydrosols are stable and nC_{60} particles are likely to persist in the aqueous environment.

To evaluate the environmental impact of waterborne fullerenes, both their physicochemical properties and toxicity need to be assessed. Several important physicochemical characteristics of nC_{60} have been shown to strongly depend on nC_{60} preparation technique [7]. Techniques that have been developed to produce stable dispersions of fullerenes in water can be grouped into two categories: (i) methods based on solvent exchange wherein solution of fullerenes in an organic solvent is mixed with water and the organic solvent is then removed from the mixture and (ii) methods based on direct dispersion of powdered C_{60} in water followed by prolonged mixing of the dispersion. The solvent exchange method was used with such organic solvents as tetrahydrofuran (THF) [9, 10, 13, 14], NaOH-THF [11, 15], NaOH-DMSO and NaOH-DMF [15], benzene [12], toluene [6, 7], [16, 17]; or combinations of solvents: benzene-THF-acetone [5] and toluene-THF-acetone [7, 17]. A method based on C_{60} transfer from toluene to an aqueous micellar solution has also been reported [18]. Magnetic mixing, sonication [6-8, 11, 18], or heat, and further removal of organic solvent by rotary evaporation [7, 9, 10, 14] or by boiling [17] can be used to assist formation of aqueous fullerene species. The recently established method of nC_{60} preparation by extended mixing of powdered C_{60} in water produces more polydisperse suspensions of colloidal fullerenes [19] and is of especial interest as it

corresponds to intuitive environmental transport scenarios. In this paper, we will adapt the notation “initial solvent/colloidal species” [9] and thus will refer to suspensions prepared by transfer from ethanol and by mixing in water as EthOH/*n*C_{60} and aqu/*n*C_{60}, respectively.

Investigations of the biological activity and potential uses of fullerenes have been hampered by C_{60} poor water solubility [20]. With C_{60} joining the expanding range of solubilizable engineered nanomaterials, possibilities for beneficial uses of C_{60} increase; C_{60} has been shown to protect rat liver from damage by carbon tetrachloride [21] and protect against lipid peroxidation even better than vitamin E [22]. At the same time, potential toxicity of these nanomaterials needs to be evaluated as dermal pathways [23] and oral ingestion [24] become likely exposure routes. In an earlier study, *n*C_{60} prepared by solvent exchange using benzene-THF-acetone was found to have no effect on the proliferation rate of keratinocytes or fibroblasts [5]. Several other studies on the cytotoxicity of *n*C_{60} have been published recently. Fortner at al. [10] found that the growth of both *E.coli* and *Bacillus subtilis* were inhibited by THF/*n*C_{60} at a concentration of 0.4 mg/L. For THF/*n*C_{60}, Sayes at al. determined LC50 for human skin cells to be 20 □g/L [25].

There is evidence of the presence of residual intermediate solvent [7, 9, 10] most likely associated with *n*C_{60} particles either as adsorbed species or intercalated into the bulk of the *n*C_{60} cluster [26]. A recent paper by Lyon et al. [44] addressed the concern that the residual THF may interfere with toxicity measurements: the authors demonstrate that THF controls have no deleterious effect on the bacterial cultures being studied in contrast to the effect of THF/nC_{60} suspension. In our case, the residual solvent does not affect the genotoxicity results because the ethanol concentration used in the our work is well below the concentration shown to have no effect on tail moment in human lymphocytes [27].

Less information is available on the *geno*toxicity of fullerenes. Several early studies have shown that pristine C_{60} and some of its derivatives may cause DNA damage [28], [29]. To the best of our knowledge, no data has been published on whether *n*C_{60} also damages DNA. Based on the observations that *n*C_{60}: i) is capable of producing reactive oxygen species [14], ii) causes leaky cytoplasmic membrane [14], and iii) may include molecular fullerene C_{60}@$\{H_2O\}_m$ as a component of *n*C_{60} hydrosol [11, 12], it is hypothesized that *n*C_{60} will also result in DNA damage. This study tests the above hypothesis via use of the single cell gel electrophoresis assay, also known as Comet assay [30, 31]. Because the presence of organic solvent in *n*C_{60} suspensions may confound toxicity data, this study used preparation methods that were either free of organic solvent (extended mixing method) or employed solvent exchange method with ethanol used as the solvent, which is known to be non-genotoxic at the concentrations used [27].

In our studies, stable aqueous suspensions of colloidal C_{60} fullerenes free of toxic organic solvents were prepared by two methods: ethanol to water solvent exchange (EtOH/*n*C_{60} suspensions) and extended mixing in water (aqu/*n*C_{60} suspensions). The

extended mixing method resulted in the formation of larger ($\bar{d}_p$ ≈ 178 nm) and less negatively charged ($\bar{\zeta}$ ≈ - 13.5 mV) *n*C_{60} colloids than *n*C_{60} prepared by ethanol to water solvent exchange ($\bar{d}_p$ ≈ 122 nm, $\bar{\zeta}$ ≈ - 31.6 mV). Three different methods were evaluated for the measurement of *n*C_{60} concentration after filtered through a 0.45 μm filter. 1) The *n*C_{60} suspension was dried using rotary evaporation and the dry deposit was dissolved in toluene for the subsequent UV-vis absorption measurement using previously recorded calibration curve. 2) The *n*C_{60} suspension was filtered through an ultrafiltration (UF) membrane with a molecular weight cut-off of 2,000 Daltons in a dead-end filtration cell. The membrane was submerged into toluene and sonicated for 60 min while heated at ca. 45 ºC for 10 min to dissolve *n*C_{60} particles accumulated on membrane surface; after that, the absorption of the resulting solution was measured. 3) *n*C_{60} concentration was quantified by liquid chromatography / mass spectrometry (LC/MS) using a Quattro micro mass spectrometer (Waters, Milford, MA) interfaced to a Shimadzu LC-20AD ternary pump and a Shimadzu SIL-5000 auto sampler.

Genotoxicity of these suspensions was evaluated with respect to human lymphocytes using single cell gel electrophoresis assay (Comet assay). The assay demonstrated genotoxicity for both types of suspensions with a strong correlation between the genotoxic response and *n*C_{60} concentration, and with genotoxicity observed at concentrations as low as 2.2 □g/L for aqu/*n*C_{60} and 4.2 □g/L for EtOH/*n*C_{60}. Both samples caused statistically significant, concentration-depend DNA damage in human lymphocytes as measured by the Comet assay parameters, olive tail moment, % tail DNA, and tail length The Olive Tail Moments (OTM) for these two concentrations were 1.54 ± 0.24 and 1.34 ± 0.07 respectively, which in comparison to the negative control OTM of 0.98 ± 0.17 is statistically different with a *p* value of at least 0.05. Aqu/*n*C_{60} suspensions elicited higher genotoxic response than EthOH/*n*C_{60} for the same *n*C_{60} concentration. The results represent the first genotoxicity data for colloidal fullerenes produced by simple mixing in water.

We attribute this genotoxic response to the effect of *n*C_{60} colloids or molecular hydrated C_{60}@$\{H_2O\}_m$ or both. It should be emphasized that only indirect experimental observations indicated the presence of C_{60}@$\{H_2O\}_m$ [11, 12, 16] and confirmation of C_{60}@$\{H_2O\}_m$ presence by more direct methods (e.g., TEM) is needed. Therefore, we *hypothesize* that the response is due to one of or a combination of the following three reasons (Figure 4):

1) *n*C_{60} produces oxygen radicals and causes leaky cytoplasmic membranes. Both molecular C_{60} and *n*C_{60} produce oxygen radicals, which have been shown to cause lipid peroxidation in three different types of human cell lines [14]. There is evidence that *n*C_{60} can also cause "leaky" cytoplasmic membranes suggesting that oxygen radical, molecular C_{60}, and *n*C_{60} colloids may all have access to internal cellular organelles.

2) *n*C_{60} partitions to DNA. Using molecular models, Zhao et al [37] demonstrated that the binding energy of two C_{60} molecules in aqueous solution is -7.5 kcal/mol while the binding energy between a 20 nucleotide long oligonucleotide and C_{60} is between -27 to -42 kcal/mol. This binding energy is in the same range as the binding energy for signature oligonucleotides probes specifically designed to hybridize with their target sequences (-16 to -85 kcal/mol; [38]). Thus, a "partitioning" of C_{60} from aqueous solution into DNA matrix or other organic matrices (if present) is also possible.

3) Pristine and modified *C_{60}* cause DNA damage. Using an oligonucleotide attached to C_{60} carboxylic acid, Tokuyama et al. showed that upon photoactivation, C_{60} carboxylic acid cuts at guanine sites in a DNA sequence [28]. Later, Boutourine et al. confirmed that guanine sites in the vicinity of C_{60} are preferentially cut and presumed that this may be due to the effect of oxygen radical on DNA damage [29]. This characteristic of pristine C_{60} has also been implicated in virus inactivation [39] and in nonenzymatic cleavage of DNA [40], among others. Evidence is also available for an alternative mechanism (i.e., not implicating oxygen radical) of DNA damage due to electron transfer between C_{60} and oligonucleotides. Pristine C_{60} is capable of accepting up to 6 electrons [41].

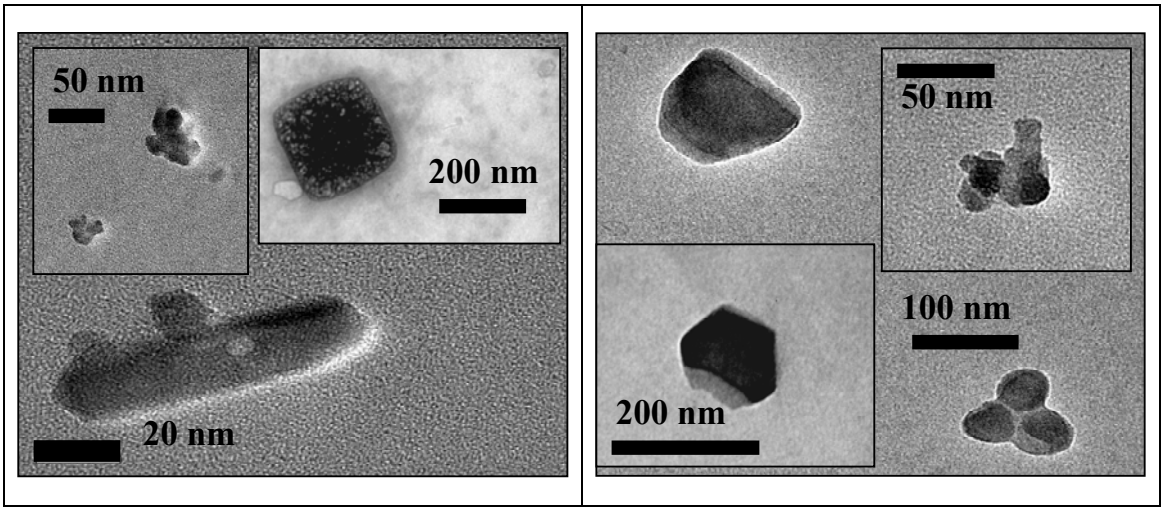

Figure 1 TEM micrographs of EthOH/*n*C_{60}

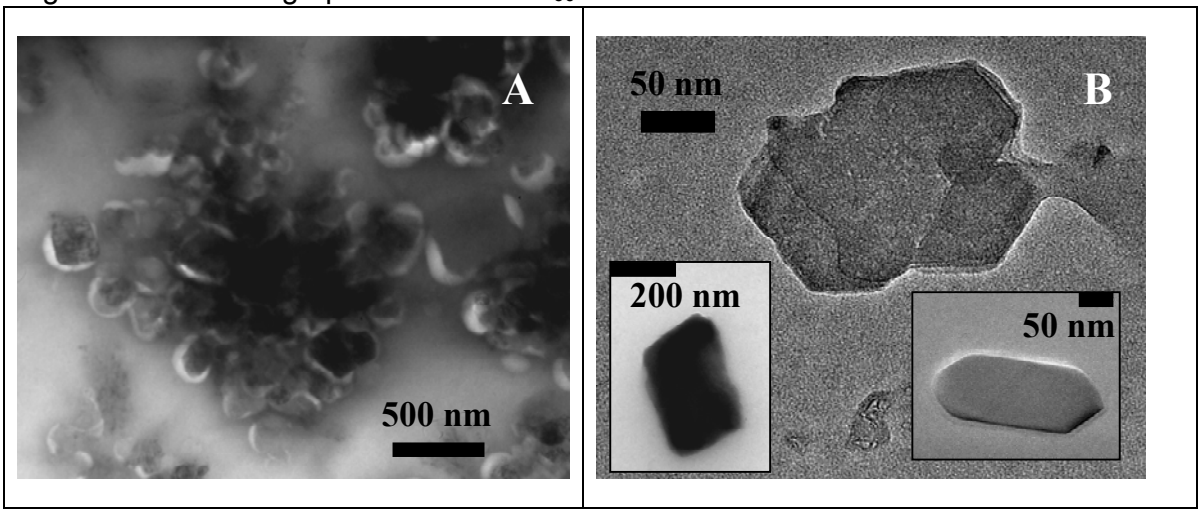

Figure 2 TEM micrographs of aqu/*n*C_{60} --2 weeks mixing

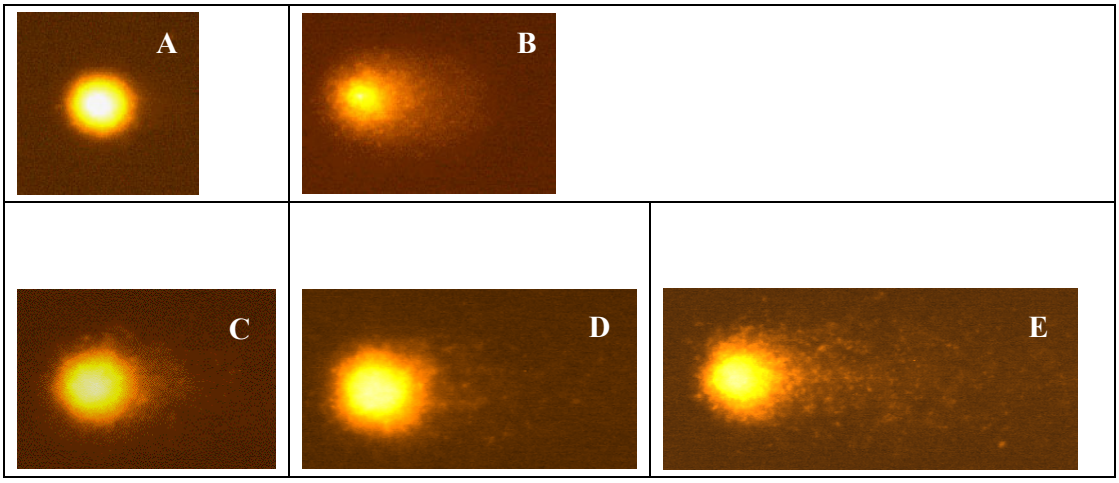

Figure 3 Human lymphocytes treated with fullerenes showing genotoxicity. Magnification: 400x

A: Nucleus from an untreated human lymphocyte (negative control)

B: Nucleus from an ethyl methanesulfonate (2 mM; positive control) treated human lymphocyte showing DNA damage

C, D, E: Nuclei from *n*C_{60} treated human lymphocytes showing DNA damage

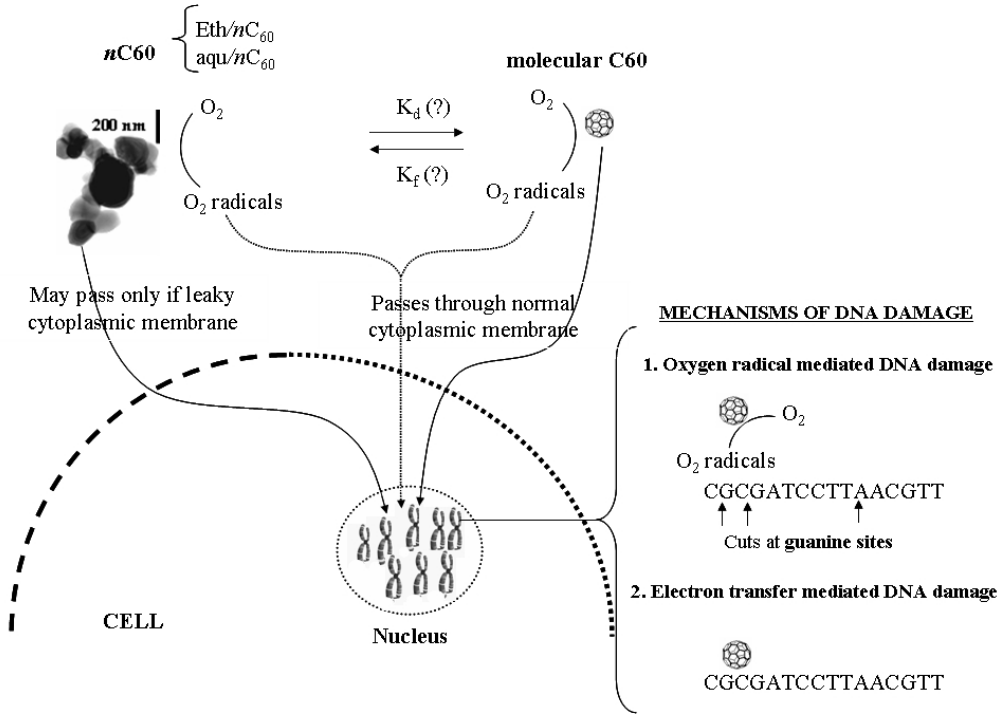

Figure 4 Possible mechanisms of genotoxicity

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