Mechanisms of Photochemistry and Reactive Oxygen Production by Fullerene Suspensions in Water

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Buckminsterfullerene (C\textsubscript{60}) is a known photosensitizer that produces reactive oxygen species (ROS) in the presence of light; however, its properties in aqueous environments are still not well understood or modeled. In this study, production of both singlet oxygen and superoxide by UV photosensitization of colloidal aggregates of C\textsubscript{60} in water was measured by two distinct methods: electron paramagnetic resonance (EPR) with a spin trapping compound, and spectrophotometric detection of the reduced form of the tetrazolium compound XTT. Both singlet oxygen and superoxide were generated by fullerol suspensions while neither was detected in the aqu/nC\textsubscript{60} suspensions. A mechanistic framework for photosensitization that takes into account differences in C\textsubscript{60} aggregate structure in water is proposed to explain these results. While theory developed for single molecules suggests that alterations to the C\textsubscript{60} cage should reduce the quantum yield for the triplet state and associated ROS production, the failure to detect ROS production by aqu/\textit{nC}_{60} is explained in part by a more dense aggregate structure compared with the hydroxylated C\textsubscript{60}.

1. Introduction

Commercial products containing fullerenes are already on the market. These include tennis racquets (1), epidermal growth factor (2), and facial antioxidant cream (3) to name a few. The availability and use of these products and a substantial increase in production forecasted for fullerene suspensions suggest that these materials may make their way into wastewater treatment influents and aquatic environments. The richness of organic chemistry allows for an uncountable number of fullerene variations through functionalization. Also, the behavior of these materials may be altered by the commercial and natural matrices in which they are immersed. However, evaluation of the surface and photochemistry of some relatively simple aqueous suspensions of fullerenes is an essential starting point for assessing the environmental impacts of fullerenes and basis for benchmarking more complex systems.

The properties of C\textsubscript{60} and other fullerenes have been described in numerous studies with respect to fullerene toxicity (4–8), antioxidant capacity (9–11), and characterization (12–15). The ability of C\textsubscript{60} to produce reactive oxygen species, or ROS, (e.g., \textit{O}_{2}^{-}) has received considerable attention, in part based on the implications for toxicity to cells (5) in both medical and environmental contexts as well as the potential applications implied for industrial or water treatment technologies (17, 18). However, unlike the conditions leading to ROS production via photosensitization by individual C\textsubscript{60} molecules in organic solvent, (19, 20), the conditions that lead to ROS production by C\textsubscript{60} in water and the reactive species formed are less well understood. We have previously reported on the ability of hydroxylated C\textsubscript{60} (fullerol) to produce ROS in water (17, 18), but have not considered the ROS speciation and underlying mechanisms.

Photosensitized molecules like fullerene are capable of transferring light energy to chemical oxidation potential in the form of ROS. When the photosensitized molecule transfers energy directly to an oxygen molecule, ROS formation may occur via a type II pathway primarily resulting in singlet oxygen (\textit{O}_{2}^{1} \textit{g}) (21). Type I ROS formation occurs when photosensitization increases interactions between a photosensitizer molecule and an electron donor, ultimately leading to the transfer of an electron to an oxygen and the production of radicals such as superoxide (\textit{O}_{2}^{-}). The efficiency of light energy transferred relative to the amount of ROS formed can be expressed as a quantum yield (21). Pristine fullerene suspended in a nonpolar solvent has a quantum yield near unity (19) indicating little to no loss of energy between initial photosensitization and formation of its triplet excited state (C\textsubscript{60}^{3}). However the introduction of fullerenes into a polar solvent, such as water, results in a very different chemical environment and physical configuration of C\textsubscript{60} compared with the organic solutions of C\textsubscript{60} that have been the subject of previous studies of ROS formation. In particular, stable suspensions of fullerenes in water tend to be present as colloidal aggregates. Indeed, the study of ROS generation by fullerenes in water is complicated by the low solubility of many of these materials in water. Nonetheless, stable suspensions of these materials may be produced either intentionally or naturally through encapsulation (22–25), functionalization (26–28), or aggregation (29–32).

The degree to which fullerenes are functionalized appears to affect the tendency of these molecules to form aggregates. Monofunctionalized (33) molecules tend to aggregate despite functionalization (34), bis-functionalized fullerene aggregate somewhat less due to steric hindrance, and poly-functionalized fullerenes exhibit greater stability with respect to limited aggregation. Nonetheless, aggregation of poly functionalized C\textsubscript{60} may readily occur and has been observed in some cases to increase with concentration (15), whereas in other cases, concentration does not seem to have an effect on aggregation (35). One of the more studied poly functionalized fullerenes is a hydroxylated form known as fullerol which can have a varying amount of hydroxyl groups on its surface depending on reaction conditions (26). Even with this surface modification, fullerol forms colloidal aggregates in suspensions that are stable to a maximum concentration of approximately 36.5 mM (18), depending on the number of hydroxyl groups added.

Stable colloidal suspensions of initially unfunctionalized C\textsubscript{60} can be made by extended stirring (25, 31) or sonication in water (aqu/nC\textsubscript{60}) (36) or through solvent exchange using organic solvents such as tetrahydrofuran (12, 29), or toluene (37). However, these latter colloidal suspensions of C\textsubscript{60} may...
contain residual quantities of the organic solvent (13). Differences in the aggregation state of C60 as well as functionalization of the C60 contained within these aggregates may affect the ability of suspensions of these aggregates to produce ROS (38). One premise examined in this paper is that a higher proximity of two C60 cages within a dense aggregate may decrease ROS production by increasing the likelihood of processes such as triplet–triplet annihilation and self-quenching. In addition, dense aggregates will expose less of the fullerene to the solution, potentially reducing the active surface area for ROS production. In this study, we compare ROS production by two colloidal suspensions of C60 having very different aggregate structures as quantified by X-ray diffraction, aqu/nC60 and fullerol. We further differentiate conditions favoring type 1 and type II photosensitization reactions by these two suspensions.

2. Materials and Methods

2.1. Chemicals. Fullerol (C60(OH)22-24), and C60 (99.9% pure) were purchased from MER (Tucson, AZ). Superoxide dismutase (bovine ethrocytes) (SOD), 5,5-dimethyl-1-pyrroline-1-oxide (DMPO), 2,2,6,6-tetramethyl-4-piperidinol (4-oxo-TEMP), Adenosine 5’-trihydrogen diphosphate (NADH), 2,2,6,6-tetramethyl-4-piperidine (TEMP), β-carotene, and XTT (2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide) were obtained from Sigma-Aldrich (St. Louis, MO). Deuterium Oxide (D2O) was purchased from Cambridge Isotope Laboratories (Andover, MA). Ultrapure water had resistivity greater than 10 MΩ cm and dissolved organic carbon concentration <3 µg/L.

2.2. Irradiation. Irradiation was performed with both low and medium pressure UV lamps. Low pressure (LP) UV irradiation was done in the presence of two 15 W fluorescent ultraviolet bulbs (Philips TLD 15W/08). These bulbs have an output spectrum peak at 365 nm and a total irradiance of 24.1 W/m² (17). For singlet oxygen measurements irradiation by a low pressure (LP) UV light source was necessary for proximity to the EPR equipment.

Medium pressure (MP) irradiation (Calgon Carbon Corporation Pittsburgh, PA) was provided by a bench scale collimated beam (39). These experiments were carried out in a Petri dish with known sample depth and surface area allowing for a calculation of UV fluence (mJ/cm²) (2). A UV radiometer and detector (International Light Inc., model 1700/SED 240/W) calibrated at 2 nm intervals in the range of 200–400 nm was used to measure UV irradiance at the top of the suspension. UV fluence (mJ/cm²) was calculated as the average irradiance multiplied by the exposure time. The average UV irradiance in the completely mixed sample was determined from the incident irradiance, UV absorbance, and sample depth using an integrated form of the Beer-Lambert law. Utilizing a shutter, samples of various concentrations were exposed to UV light for 5 minute time intervals under the broadband MP UV source, the fluence was calculated as the total UV output in the 200–300 nm region. UV fluence was used as a normalizing factor in determining the capability of each suspension to produce superoxide radicals. It should be noted that light under 300 nm in wavelength is more relevant to engineered systems rather than natural systems. Wavelengths larger than 300 nm are likely to be less effective in photosensitization. Our goal here, however, is to explore mechanisms of ROS formation by fullerenes rather than determine representative rates for either engineered or natural systems. Further study is needed to determine the rates of ROS that would occur in natural systems under typical solar illumination.

2.3. Instrumentation. EPR spectra were recorded at room temperature with a Varian E-6 spectrometer. The conditions for all measurements were: frequency, 9.27 GHz; power, 5 mW; modulation amplitude, 4 G; modulation frequency, 100 kHz. UV/vis spectrometry was performed using a Hitachi U-2000 spectrophotometer. X-ray diffraction (XRD) was performed using an X’Pert-Pro diffractometer from PANanalytical instruments equipped with a Co anode source, and an RTMS scanning detector was used. Dynamic light scattering was performed using a Zetasizer nano ZS (Malvern Instrument, Bedford, MA). This instrument employs a He–Ne laser (633 nm) and collects time variable scattering data at a fixed angle of 173°.

2.4. XRD Procedure. The structure of the C60 molecules in the fullerol and aqu/nC60 aggregates was characterized by X-ray scattering by samples prepared by depositing a few drops of aqu/nC60 or fullerol suspension on a silicon plate and allowing the water to evaporate. The 2 theta angle was continuously scanned from 10 to 90° with a 0.03° and 1 s per step, and the divergence slit was automated so that a 10 mm sample length was constantly irradiated.

2.5. Singlet Oxygen Concentration Determination with EPR Spin Trapping. The spin-trapping reagent: 4-oxo-TEMP was used to trap singlet oxygen. TEMP has a low detection limit and a relatively long adduct lifetime when compared with other spin traps. This makes it an ideal trap for suspensions containing low concentrations of singlet oxygen that must be irradiated for prolonged periods of time (40) for this reason it is widely utilized to detect singlet oxygen (41, 42). A mixture of the fullerol colloidal suspension and 80 mM TEMP was shaken in a 5 mL volumetric flask. This was poured into a sample tray for UV irradiation. Upon removal, the sample was taken up in a capillary tube, capped with clay, placed in quartz EPR tube, and positioned in the EPR sample holder. In each case the EPR parameters were held constant as was the TEMP concentration. Separate samples were irradiated under UV for up to 3 h. Signals were compared with the standard product of TEMP and singlet oxygen (TEMP-PO) order to determine singlet oxygen generation rate.

2.6. Superoxide Concentration Analysis by XTT. XTT reduction was employed to measure the production of superoxide. The reduction of XTT results in an increase in optical density at 470 nm that can be used to quantify the relative amount of superoxide present (43, 44). The concentration of superoxide was determined by comparing XTT reduction with and without a quencher for superoxide, superoxide dismutase (SOD), which allowed nonsuperoxide related XTT reactions to be accounted for. Samples were prepared by mixing 10 mL flasks with the appropriate suspension and 100 µM XTT. One set of experiments was done without SOD while in others, 10 U/mL SOD was added to the samples to quench any superoxide generated. In experiments irradiated by the MP UV lamp, flasks were poured into a Petri dish with surface area approximately 40 cm² and depth approximately 1 cm. SOD-containing samples served to eliminate the influence of background absorbance of suspensions at 470 nm. While the physical chemical properties of XTT and TEMP do not favor adsorption to fullerene surfaces, possible interference arising from XTT or TEMP adsorption to aqu/nC60 or fullerol aggregates was addressed by adding the compounds in excess as calculated from available fullerene surface area and KOW relationships (see Supporting Information).

2.7. Suspension Preparation. Aqu/nC60 was prepared from a supersaturated suspension of C60 in water (DDW). Here approximately 0.4 mg/mL of powdered C60 was added to DDW. The solution was then stirred and sonicated for approximately 6 h with a Branson sonifier ultrasonic cell disruptor (model S-250A). The final solution had a gold color, characteristic of C60 suspensions, and was filtered through a 0.45 µm methyl cellulose ester (MCE) filter (to remove unsuspended C60) and stored in the dark. The aqu/nC60 was also prepared by the same method in D2O. The final
suspension was found to be stable for periods up to at least 3 months as monitored by mean aggregate size using dynamic light scattering (DLS). In both D_2O and H_2O, the mean diameter of the aqu/nC_{60} was approximately 145 nm as determined by DLS number distribution. Fullerol suspensions were made by adding approximately 0.07 mg/mL powdered fullerol to DDW or D_2O. This suspension was made up in a volumetric flask and placed in the sonication bath for 2 h. During this time the suspension became gradually more gold colored as more fullerol was stabilized in the suspension. Once removed from sonication, the suspension was filtered through 0.45 µm MCE filters via vacuum to remove unsuspended particles. The final suspension contained aggregates with a mean diameter of 200 nm as determined by DLS and a total organic carbon concentration of 90 mg/L. The stock solution was stable with respect to size and concentration over a period of at least three months. UV/vis absorbance spectra for fullerol and aqu/nC_{60} suspensions have been previously reported (13, 45).

3. Results and Discussion

3.1. Singlet Oxygen Production by aqu/nC_{60}. EPR measurements were undertaken to compare the singlet oxygen generation capacities of the two types of C_{60} suspensions. Appearance of a distinct triplet signal in an induced magnetic field of the EPR indicates the presence of singlet oxygen (40). The aqu/nC_{60} did not produce a signal for the TEMP-singlet oxygen adduct after 60 min of LP UV irradiation, even when these suspensions were made in D_2O to increase detection sensitivity (Figure 1A). These results suggest that aqu/nC_{60} does not participate in detectable type II photosensitization reactions.

1B: Electron Paramagnetic Resonance signal for photosensitizers suspended in D_2O with 80 mM TEMP and irradiated under LP UV for 60 min. The absence of a triplet signal indicates the absence of singlet oxygen generation.

1C: Electron Paramagnetic Resonance signal for 40 µM fullerol suspended in D_2O with 80 mM TEMP. The suspension was irradiated under LP UV light for 15, 30, and 60 min. The field is measured between 3270 and 3320 G.

1D: Using the amplitudes of TEMP signals from Figure 1C and the standard signal from 0.05 µM TEMPO in Figure 1B the rate of singlet oxygen generation under LP UV is calculated to be 0.032 µM/min when 40 µM fullerol suspended in D_2O.

3.2. Singlet Oxygen Production by Fullerol. Deuterated water was also used to increase singlet oxygen sensitivity in experiments with the fullerol suspensions. In contrast with the aqu/nC_{60}, fullerol was observed to generate singlet oxygen via the type II photosensitization pathway. The response produced by the fullerol suspension was compared with a standard TEMPO adduct solution and a standard Rose Bengal sensitizing molecule (21). Figure 1B compares the signal corresponding to singlet oxygen generation produced by a solution of 40 µM Fullerol after 5 min LP UV irradiation with that produced by a separate suspension of 40 µM fullerol following 15 min LP UV irradiation. The Rose Bengal generated singlet oxygen at significantly higher rate than did fullerol. The amplitude of the 0.5 µM TEMPO adduct approximately matches the fullerol signal, meaning that 40 µM fullerol produces approximately 0.5 µM singlet oxygen after 15 min of LP UV irradiation.

Singlet oxygen generation with increasing UV fluence is illustrated in Figure 1C where a 40 µM suspension of fullerol
that aqu/nC60 experiments produced less superoxide than photosensitization. This was confirmed by the observation of fullerol suspended in water because singlet oxygen has but the signal is less distinct and 5 times lower in amplitude enough to be detected in suspensions of conventional H2O, (Figure 1D).

Singlet oxygen production by fullerol was significant enough to be detected in suspensions of conventional H2O, but the signal is less distinct and 5 times lower in amplitude for fullerol suspended in water because singlet oxygen has 1/10 the lifetime in nondeuterated water. Nonetheless, these measurements confirm significant singlet oxygen production by the fullerol in a water suspension.

3.3. Superoxide Production by Fullerol and aqu/nC60 Aggregates. XTT reduction was employed to measure the production of superoxide in the presence of NADH, a common reductant found in cells. As shown in Figure 2 fullerol suspensions generate superoxide in a concentration-dependent manner via type I reaction under medium pressure (MP) UV light. This indicates that the triplet state of fullerol is participating in a type I photosensitization reaction. This is not surprising considering that fullerol participates in type II singlet oxygen production (Figure 1B and C) and with the addition of a donor molecule NADH type I reactions occur by additional reaction steps (Figure 5).

On the other hand, it was deduced from the TEMP type II measurements of singlet oxygen (Figure 1A) that aqu/nC60 had a short-lived triplet state, possibly due to its aggregation state as we consider subsequently. Therefore, suspensions of aqu/nC60 would not be expected to participate in type I photosensitization. This was confirmed by the observation that aqu/nC60 experiments produced less superoxide than NADH alone indicating possible antioxidant properties (9, 46). It is important to note that since LP and MP UV provided UV fluences that likely differed by 1 order of magnitude, it is impossible to make a direct comparison of type I and type II production rates in this instance.

3.4. TEM and XRD Structural Analysis of Aggregates. Transmission electron microscopy of fullerol aggregates and aqu/nC60 show that while both suspensions have a similarly gold-colored appearance, the structure of the aggregates in the suspensions differ greatly (Figure 3). Fullerol suspensions consist of nearly spherical aggregates (n-scale) assembled into larger (m-scale) aggregates of the n-scale spheres. The diameter of a typical n-scale aggregate as observed by TEM (47) is slightly smaller than the diameter determined by DLS measurements and may indicate the presence of m-scale aggregates in the suspension. We have previously shown that the aqu/nC60 has a fractal m-scale structure (45) reflecting an apparently random assemblage of what appear to be more crystalline n-scale aggregates. However, it is the n-scale structure of the colloidal suspension that will likely determine the proximity of the majority of C60–C60 contacts, with implications for the rate of ROS generation due to both the potential for triplet–triplet annihilation, self-quenching, and greater accessibility to the reactive C60 surfaces.

Type I and type II photosensitizations by the fullerol and the lack of these reactions with regard to aqu/nC60 may indicate a longer lifetime for the triplet state of the hydroxylated C60 in the fullerol aggregates compared with that of the aqu/nC60 due to differences in structure. XRD was performed on the aggregates in order to obtain an indication of the distance between C60 cages, taken to be a quantitative indicator of the rates of triplet–triplet annihilation and self-quenching. In the fullerol diffractogram (Figure 4 inset), the absence of a diffraction peak implies a lack of order within the fullerol aggregate at the n-scale and a greater distance between C60 molecules.

In contrast, the aqu/nC60 diffractogram (Figure 4) shows a crystalline organization very similar to that of fullerene fine powder (fullerite). C60 molecules in aqu/nC60 that has been dried into a thin film appear to be assembled in a cubic face centered structure with the same lattice constants as that of C60 fullerite (a = b = c = 14.16 Å). This organization is apparently retained in spite of the subdivision of particles of the initial C60 powder as they form a stable suspension in water. It is reasonable to assume that such a lattice structure is inherited from the original crystal structure of C60 fullerite before sonication treatment in water. This tighter n-scale assembly favors triplet–triplet annihilation and reduces the surface of C60 effectively available for reaction with approaching oxygen molecules.

3.5. Proposed Framework. We propose a mechanistic framework (Figure 5) derived from literature and experimental evidence to explain the observed trends in ROS production and speciation in large part based on differences
in the structure of aggregates in the aqueous fullerol and nC60 suspensions. When fullerenes are illuminated under the appropriate wavelength, the electrons are excited from the ground state (cC60) to the singlet state (I). In the case of aqu/nC60 and fullerol, this process likely occurs at a higher rate in aqu/nC60 due to a stronger absorbance at UVA wavelengths tested in this study. The singlet state (cC60) can decay in three main manners: fluorescence (II); internal conversion (III); and intersystem crossing (ISC) (IV). The former two pathways return the fullerene to the ground-state while the latter leads to the relaxation of singlet C60 to the triplet state (3C60).

Unaltered C60 that is found inside aqu/nC60 has a high quantum yield for the triplet state (19) while altered fullerenes such as fullerol have a lower yield due to cage alteration that promotes fluorescence (II) and internal conversion (III) (48, 49). Interaction of the singlet state with oxygen can also result in the triplet state (VI, V). Pathway VI results in the production of singlet oxygen via type II photosensitization while pathway V only results in the formation of the triplet state because energy is released from oxygen in a nonsinglet form. The triplet state, 3C60, has a significantly longer lifetime than cC60 in solution allowing it to participate in type II formation of singlet oxygen to a greater extent than does the cC60 (IX). The triplet state is also susceptible to self-quenching (VII) via interaction with the ground state (cC60) and triplet–triplet annihilation (VIII) via interaction with another triplet (cC60). These processes are promoted by close interactions between fullerene cages, and as the XRD data in Figure 4 shows aqu/nC60 cages are keeping their tight crystalline form even after suspension, while the fullerol suspensions do not exhibit crystal structure (Figure 4 inset). TEMP measurements of singlet oxygen confirm that the triplet lifetime is significantly different in aqu/nC60 which has no trace of type II photosensitization when compared with standards and fullerol suspensions (Figure 1). Along the same lines, type I sensitization (X) occurs when the triplet state comes in contact with a donor molecule (NADH) that has a more negative reduction potential than cC60. Superoxide measurements in Figure 2 confirm that type I results parallel those found with type II. The resulting radical (cC60) can then pass the electron to ground-state oxygen to form superoxide (XI). Electrons from this radical could also donate to form other types of free radicals such as organic radicals.

The critical agent for producing ROS is the relatively long-lived triplet state (∼µs) compared with the short lifetime of the singlet state (∼ns) (19, 50, 51). Singlet oxygen generation by aqu/nC60 measured in Figure 1A validates the proposed mechanism in that aggregates more closely associated with each other (Figure 4) are shown to produce little to no singlet oxygen. Both the hydroxylated (fullerol) and underivatized varieties of C60 form stable colloidal suspensions in water. However, aggregation itself does not imply that these fullerenes are not able to produce reactive oxygen. The structure (and likely the size) of the aggregate appear to determine the potential for ROS production. The amorphous structure of the fullerol aggregates favors ROS formation while the tighter and more structured nature of aqu/nC60 may prevent ROS production. This occurs despite the higher absorption of UV light at the frequencies exposed and the predicted higher reactivity of the unaltered C60 cages (52). Accessibility of water to the fullerene surface may also be favored by the fullerol’s aggregate structure and hydroxylated functionality compared with the aqu/nC60. These facts, when taken in the context of the proposed mechanism and ROS measurements, indicate that fullerol has a long-lived triplet state in aqueous solution, while aqu/nC60 does not. Therefore, the aggregation characteristics affecting triplet lifetime have clear ramifications for the type I and type II photosensitized production of ROS in carbon based nanomaterials and thus should be considered more carefully in future analyses of these materials for toxicity and application in the aqueous environment.

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Supporting Information Available

Calculation and analysis of projected surface area and theoretical Koaw values of probes used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

Literature Cited


