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Active Oxygen Species Generated from Photoexcited Fullerene (C) as Potential Medicines: O versus O

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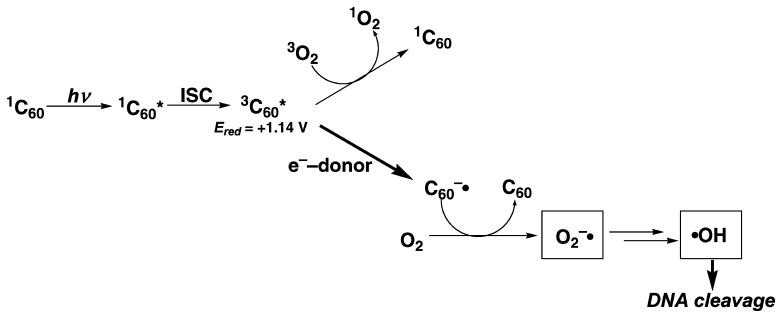

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Active Oxygen Species Generated from Photoexcited Fullerene (C_{60}) as Potential Medicines: $O_2^{-\bullet}$ versus $^1O_2$

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Abstract: To characterize fullerenes (C_{60} and C_{70}) as photosensitizers in biological systems, the generation of active oxygen species, through energy transfer (singlet oxygen $^1O_2$) and electron transfer (reduced active oxygen radicals such as superoxide anion radical $O_2^{-\bullet}$ and hydroxyl radical •OH), was studied by a combination of methods, including biochemical (DNA-cleavage assay in the presence of various scavengers of active oxygen species), physicochemical (EPR radical trapping and near-infrared spectrometry), and chemical methods (nitro blue tetrazolium (NBT) method). Whereas $^1O_2$ was generated effectively by photoexcited C_{60} in nonpolar solvents such as benzene and benzonitrile, we found that $O_2^{-\bullet}$ and •OH were produced instead of $^1O_2$ in polar solvents such as water, especially in the presence of a physiological concentration of reductants including NADH. The above results, together with those of a DNA cleavage assay in the presence of various scavengers of specific active oxygen species, indicate that the active oxygen species primarily responsible for photoinduced DNA cleavage by C_{60} under physiological conditions are reduced species such as $O_2^{-\bullet}$ and •OH.

Introduction

Photodynamic therapy (PDT) is an important approach to cancer treatment and is emerging as an antimicrobial chemotherapy with particular promise for the treatment of multidrug resistant pathogens. In a typical course of PDT, the combination of a photosensitizing agent and focused irradiation is used to elicit specific, controlled production of reactive species in a localized area, leading to cell destruction through several pathways, of which one of the most important is DNA strand cleavage. By using highly focused light irradiation, PDT has the potential to act specifically at the desired site of action with low levels of collateral damage to healthy cells.

Typical photosensitizers used for PDT are aromatic molecules, which can efficiently form a long-lived triplet excited state and which have the potential to generate highly reactive oxygen species. The ideal drug for PDT should have a high quantum yield to form reactive species, a long wavelength of absorption, and low toxicity in the nonilluminated state. While the majority of clinically employed photosensitizers for PDT are porphyrin-based, associated side effects and the search for more efficient therapeutic agents have led numerous groups, including our own, to investigate the potential of fullerene (C_{60} and C_{70}) derivatives as novel PDT drugs.[1] Such molecules are particularly attractive due to their long wavelength of absorption ((S−S absorption: 530, 920 nm (C_{60}); 570, ∼740, ∼900 nm (C_{70}). T−T absorption: 400, 740 nm (C_{60}); 525, 575, 960 nm (C_{70})),[2] high quantum yield (1.0 based on generation of singlet oxygen, $^1O_2$),[3−5] and lack of acute toxicity, except in rare cases,[6] in the absence of light. Despite these advantages, progress in the development of fullerene-based agents for PDT has been hampered by two major obstacles: (1) the low solubility of fullerenes in biologically relevant media[7] and (2) the uncertainty about the mechanism of action of photoexcited fullerenes in biological systems.

To realize the potential of these promising compounds, we have sought to overcome both of these limitations. In particular, our discovery of a novel approach to the effective solubilization of fullerenes in aqueous, physiologically relevant media[8] has enabled the study of the mechanism and biological activity of photoexcited fullerenes, leading to the discovery of DNA strand cleaving activity,[9] hemolytic activity,[10] antimicrobial photodynamic therapy[11] and cytotoxicity,[12,13] and, in the absence of irradiation, a chondrogenesis-promoting effect[14,15] and glutathione *S*-transferase-inhibitory activity.[16] The readily availability of aqueous fullerene solutions also led to the first mechanistic study of active oxygen generation by underivatized photoexcited fullerenes *in aqueous media*, leading to the

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(1) For reviews on photophysical properties of fullerenes, see: (a) Guldi, D. M.; Prato, M. *Acc. Chem. Res.* **2000**, *33*, 695−703. (b) Guldi, D. M. *Chem. Commun.* **2000**, 321−327. (c) Guldi, D. M.; Kamat, P. V. In *Fullerenes Chemistry, Physics, and Technology*; Kadish, K. M., Ruoff, R. S., Eds.; Wiley: New York, 2000; pp 225−281.

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discovery of superoxide ($O_2^{-\bullet}$) and hydroxyl radical (•OH), rather than the expected singlet oxygen ($^1O_2$), as the active species for DNA cleavage activity.[17]

We now present a full account of our studies documenting the specific generation of $O_2^{-\bullet}$ and •OH by photoexcited C_{60} solubilized by detergent in aqueous solutions, which are in marked contrast with previous explanations for the observed biological activity of photoexcited C_{60}. Prior studies on photoexcited C_{60}, which were performed in organic solvents or on water-soluble C_{60} derivatives, have suggested a critical role of $^1O_2$ generation and subsequent DNA cleavage.[18−20] Further experimentation on related derivatives, however, cast doubt on the role of $^1O_2$ in mediating the observed biological activity. In this regard, An et al. demonstrated that, while the DNA-cleaving activity of eosine derivatives (well-known $^1O_2$ generators) was suppressed by the addition of $^1O_2$ trapping reagents, the DNA-cleaving ability of photoexcited C_{60} was unaffected by such $^1O_2$ traps.[21]

The photochemical properties of fullerenes have been well studied. The first study of this type, reported by Foote and coworkers, described the appearance of an excited state of C_{60} ($^1C_{60}$* and $^3C_{60}$*) that subsequently afforded $^1O_2$ through an energy transfer mechanism (type II pathway, eq 1).[3] A closely

$$^1C_{60} \xrightarrow{h\nu} {}^1C_{60}^* \xrightarrow{\text{ISC}} {}^3C_{60}^* \xrightarrow{^3O_2 \rightarrow {}^1O_2} {}^1C_{60} \qquad (1)$$

related study on C_{70} was subsequently reported.[4] Almost simultaneously, Wasielewski et al. detected triplet-excited states of C_{60} and C_{70} ($^3C_{60}$* and $^3C_{70}$*) by an EPR method.[22] Other investigators extended these findings to the study of type II energy transfer by photoexcitation of fullerenes.[23]

Electron transfer (type I pathway, eq 2) reactions of photoexcited fullerene have been reported.[2] In 1991, Krusic et al. reported the generation of $C_{60}^{-\bullet}$ by photolysis in the presence of an electron donor.[2a] Shortly thereafter, Foote et al. described the generation of $C_{60}^{-\bullet}$ by photoirradiation of C_{60} in the presence of amines and reported the high first electron-reducing voltage of $^3C_{60}$* ($E_1 = +1.14$ V vs SCE in PhCN, $^1C_{60}$: $E_1 = -0.42$ V vs SCE in PhCN).[2b]

$$^1C_{60} \xrightarrow{h\nu} {}^1C_{60}^* \xrightarrow{\text{ISC}} {}^3C_{60}^* \xrightarrow[E_{red} = +1.14\ \text{V}]{\text{e}^-\text{–donor}} C_{60}^{-\bullet} \qquad (2)$$

In the present paper, we report the generation of active oxygen species from photoexcited fullerenes through both type I and II pathways, in solvents with a range of polarity values and we evaluate the contributions of these active species to photoinduced DNA cleavage under physiological conditions.

Results and Discussion

To clarify the active species principally responsible for the DNA cleavage upon photoirradiation, we employed biochemical (DNA-cleavage assay), chemical (NBT method), and physicochemical methods (EPR spin-trapping and near-infrared spectroscopy).

DNA Cleavage. First, we examined the DNA-cleavage activity of C_{60} and C_{70} to establish the effects of visible light, reductants, and initiator or quencher of $O_2^{-\bullet}$ and $^1O_2$. The widely used assay with pBR322 supercoiled DNA was employed. According to our previous report,[8] C_{60} and C_{70} were solubilized in water with poly(vinylpyrrolidone) (PVP). Figure 1 shows that

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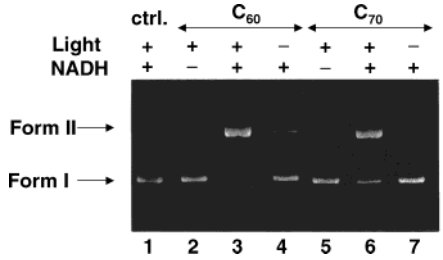


Figure 1. Photoinduced DNA cleavage by C_{60} and C_{70}. The pBR322 supercoiled plasmid was incubated with each chemical in TDC buffer for 4 h at 37 °C under irradiation with a 300-W photoreflector lamp. Lanes 1−3 and 5−6, incubation under visible light irradiation: lane 1, pBR322 DNA with 1.25% of PVP; lane 2, with 0.14 mM C_{60}; lane 3, with 0.14 mM C_{60} and 10 mM NADH; lane 5, with 0.07 mM C_{70}; lane 6, with 0.07 mM C_{70} and 10 mM NADH. Lanes 4 and 7, in the dark: lane 4, pBR322 DNA with 1.25% PVP 0.14 mM C_{60} and 10 mM NADH; lane 7 with 1.25% PVP 0.07 mM C_{70} and 10 mM NADH.

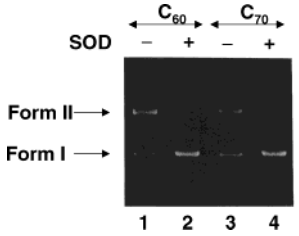
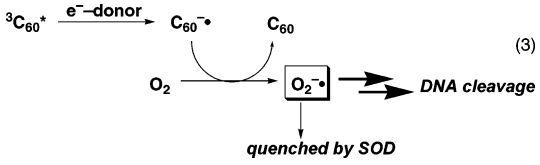


Figure 2. Effect of SOD on the photoinduced DNA cleavage by C_{60} and C_{70}. The pBR322 supercoiled plasmid was incubated with each reagent in TDC buffer for 2 h at 37 °C under irradiation with a 300-W photoreflector lamp. Lanes 1−4, PVP 1.25% incubation under visible light irradiation: lane 1, pBR322 DNA with 0.14 mM C_{60} and 10 mM NADH; lane 2, with 0.14 mM C_{60}, 10 mM NADH, and 0.04 units/mL of SOD; lane 3, with 0.07 mM C_{70} and 10 mM NADH; lane 4, with 0.07 mM C_{70}, 10 mM NADH, and 0.04 units/mL of SOD.

both photoexcited C_{60} and C_{70} cleave DNA (supercoiled form I) to give the nicked form II in the presence of NADH, a common reductant in vivo (lanes 3 and 6 for C_{60} and C_{70}, respectively). However, in the absence of NADH, neither C_{60} nor C_{70} had any effect on DNA even under photoirradiation (lanes 2 and 5 for C_{60} and C_{70}, respectively) indicating that electron transfer processes induced by the reductant are important for this activity in aqueous systems. The cleaving activity was clearly inhibited by the addition of superoxide dismutase (SOD), which quenches $O_2^{-\bullet}$ (Figure 2, lane 2 for C_{60} and lane 4 for C_{70}). This result suggested that $O_2^{-\bullet}$ is a key intermediate for DNA-cleaving activity, as shown in eq 3.



To confirm the role of $^1O_2$, the effects of $^1O_2$ stabilizers or quenchers were tested. Upon increasing the D_2O concentration in the solvent, which prolongs the lifetime of $^1O_2$, DNA cleavage was not affected, indicating the absence of a dominant role for $^1O_2$ in the DNA cleavage (data not shown). Likewise, the addition of $^1O_2$ quenchers such as sodium azide, L-histidine, and 2,5-dimethylfuran resulted in no significant decrease in the cleavage. These results support the notion that $^1O_2$, which was previously reported to be important for the photoinduced bioactivities, does not play a significant role in the expression of DNA-cleaving activity in these systems.

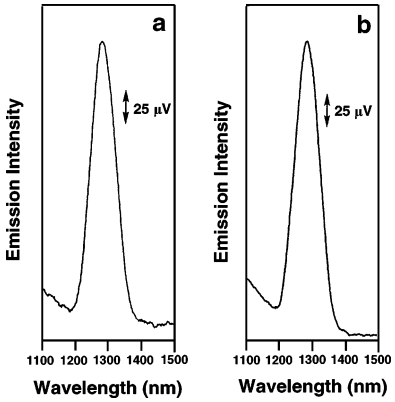


Figure 3. Near-infrared singlet oxygen luminescence emission spectra in C_{60} excited by Ar laser light at 514.5 nm with 200-mW output power. (a) 40 μM benzene solution, (b) 40 μM benzonitrile solution.

In vivo biological systems contain a similar concentration of NADH to that used in the in vitro tests employed in this paper. It is therefore possible that $O_2^{-\bullet}$ is generated in vivo and subsequently affords •OH, which is highly reactive and could directly attack DNA in the body. To compare the generation of oxyl radicals and $^1O_2$, we applied spectroscopic methods including near-infrared spectroscopy and EPR spin-trapping.

Detection of $^1O_2$. For the detection of $^1O_2$, a direct method (detection of near-infrared emission)[24] and an indirect method by EPR spin-trapping with 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP)[25] were employed.

A photoirradiated C_{60} solution in benzene and benzonitrile clearly showed the near-infrared $^1O_2$ luminescence emission at 1268 nm following excitation with 514.5-nm Ar laser light (Figure 3). However, we did not observe any $^1O_2$ luminescence emission in C_{60}/PVP aqueous solution. Therefore, we applied an EPR method with the spin-trapping agent 4-oxo-TEMP in order to confirm this result.

The EPR method with 4-oxo-TEMP, which reacts with $^1O_2$ to give the adduct 4-oxo-TEMPO (eq 4), was carried out with Rose Bengal, a typical water-soluble $^1O_2$ generator, as a positive control.

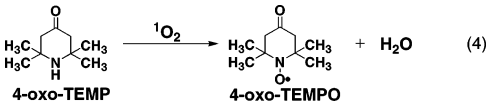


Figure 4a shows the specific signals of 4-oxo-TEMPO, which was produced by $^1O_2$ generated from photoexcited Rose Bengal, whereas no signal was detected in C_{60}/PVP aqueous solution under the same conditions (Figure 4b). This result shows that no $^1O_2$ was generated in an aqueous solution of C_{60}, even in the absence of a reductant.

Detection of $O_2^{-\bullet}$. The generation of $O_2^{-\bullet}$, which could potentially be the first-formed active oxygen species, was checked by EPR spin-trapping and the nitro blue tetrazolium method (NBT method).

5,5-Dimethyl-1-pyrroline-*N*-oxide (DMPO) was used as a spin-trapping agent for the detection of $O_2^{-\bullet}$ (eq 5). The reaction was carried out in the presence of DMSO in order to quench

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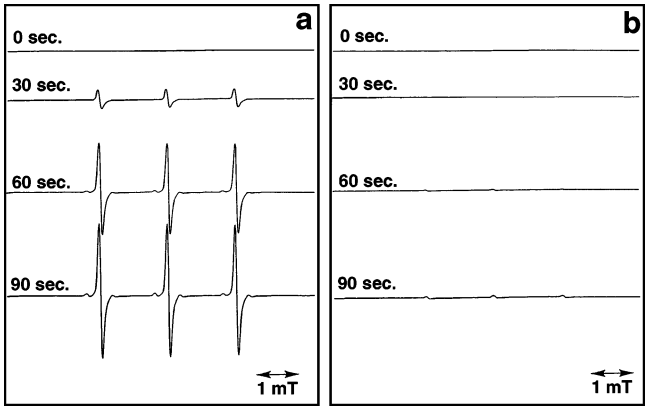


Figure 4. X-band EPR spectra of 4-oxo-TEMP adduct with $^1O_2$ generated in Rose Bengal (a) and C_{60} (b) aqueous solutions under irradiation with a 300-W photoreflector lamp. 4-Oxo-TEMP 80 mM in 50 mM phosphate buffer (pH 7). Rose Bengal or C_{60} 40 μM, PVP 0.4%. Irradiation time was 0, 30, 60, or 90 s. Experimental conditions: temperature 296 K, microwave frequency 9.394 GHz, microwave power 16 mW, field modulation 0.1 mT at 100 kHz, scan time 2 min.

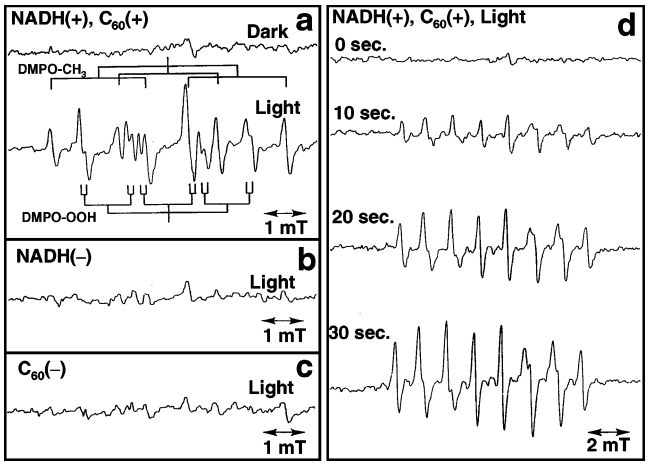
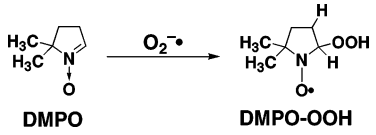


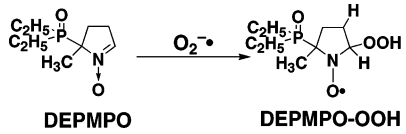
Figure 5. X-band EPR spectra of DMPO adduct with $O_2^{-\bullet}$ (a−c) and DEPMPO adduct with $O_2^{-\bullet}$ (d) generated in C_{60}/PVP aqueous solution under irradiation with a 300-W photoreflector lamp. (a−c) DMPO 0.722 M, DETAPAC 1 mM, DMSO 3.1 M, in 50 mM phosphate buffer (pH 7): (a) C_{60} 0.2 mM, PVP 2%, NADH 10 mM; (b) C_{60} 0.2 mM, PVP 2%; (c) PVP 2%, NADH 10 mM. Irradiation time: light 10 s, dark 0 s. Experimental conditions: temperature 296 K, microwave frequency 9.394 GHz, microwave power 16 mW, field modulation 0.1 mT at 100 kHz, scan time 2 min. (d): DEPMPO 50 mM, DETAPAC 1 mM, DMSO 3.1 mM in 50 mM phosphate buffer (pH 7), C_{60} 0.2 mM, PVP 2%, NADH 10 mM. Irradiation time was 0, 10, 20, or 30 s. Experimental conditions: temperature 296 K, microwave frequency 9.394 GHz, microwave power 16 mW, field modulation 0.1 mT at 100 kHz, scan time 2 min.

•OH generated under the reaction conditions used, since •OH could react rapidly with DMPO to give the adduct DMPO−OH (see eq 8), thus disturbing the spectrum of DMPO−OOH. Figure 5a shows the spectra obtained for DMPO−OOH, indicating the generation of $O_2^{-\bullet}$ from C_{60}/PVP aqueous solution in the presence of light and NADH.



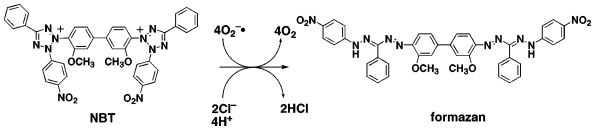
(5)

Since the lifetime of the DMPO−OOH adduct is short, 5-diethoxyphosphoryl-5-methyl-1-pyrroline-*N*-oxide (DEPMPO) was used as a spin-trapping reagent to form the more stable adduct DEPMPO−OOH (eq 6), allowing the effect of photoexposure time to be investigated. As shown in Figure 5d, the generation of $O_2^{-\bullet}$ increased in an irradiation time-dependent fashion.



(6)

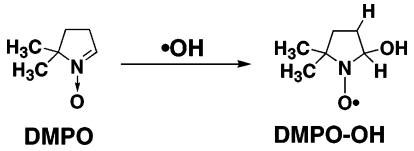
Since the quantitative detection of $O_2^{-\bullet}$ by means of spin-trapping is difficult, we employed the NBT method (eq 7) in order to quantify the amount of generated $O_2^{-\bullet}$.



(7)

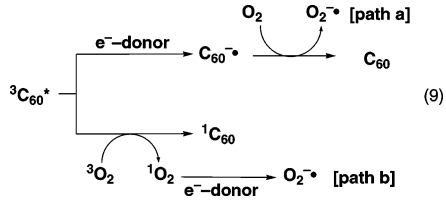
The effects of light irradiation duration, NADH concentration, and C_{60} concentration on the generation of $O_2^{-\bullet}$ were tested by the NBT method. Figure 6 shows that the generation of $O_2^{-\bullet}$ was affected by the duration of irradiation (a) and increased by the addition of NADH (b) or C_{60} (c) in a dose-dependent manner.

Detection of •OH. As reported by Cadet et al.,[26] $O_2^{-\bullet}$ itself does not cause DNA damage. However, the generation of •OH, a highly active radical which is known to cause DNA cleavage from $O_2^{-\bullet}$ via the Fenton reaction is possible under reducing conditions and the generation of •OH under the photoirradiation conditions was studied. As shown in Figure 7, the generation of •OH was detected as DMPO−OH (eq 8) in a photoexposure time-dependent manner in the presence of Fe(II).



(8)

Detection of C_{60} Radical Anion ($C_{60}^{-\bullet}$). From the results described above, it is clear that $O_2^{-\bullet}$ generated under photoirradiation is converted to •OH to cause DNA cleavage, but the role of fullerenes in generating $O_2^{-\bullet}$ is less clear. Upon photoirradiation, C_{60} is known to form a long-lived triplet state which can react with electron donors, providing two possible mechanisms for the generation of active oxygen species (eq 9).



(9)

In path a, $^3C_{60}$* is reduced to $C_{60}^{-\bullet}$ which subsequently reduces O_2 to $O_2^{-\bullet}$, and in path b, $^1O_2$, which is generated by energy transfer from $^3C_{60}$* to $^3O_2$, is reduced to $O_2^{-\bullet}$. To clarify which

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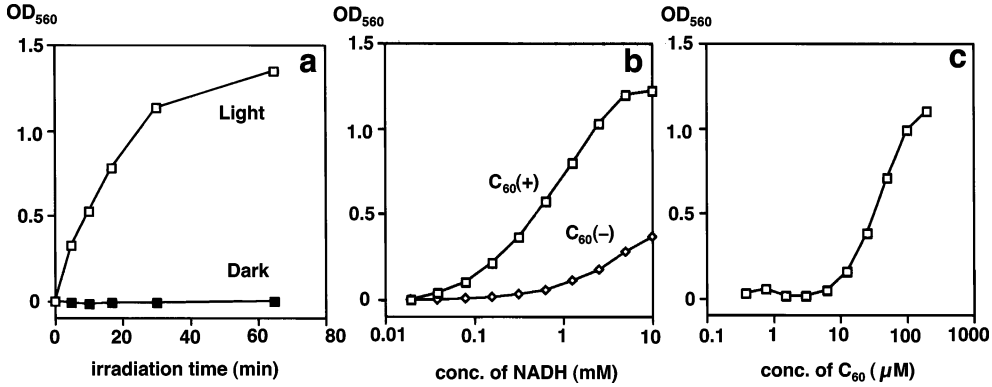


Figure 6. Photoinduced $O_2^{-\bullet}$ generation by C_{60} in PVP aqueous solution detected by the NBT method. Irradiation: 300-W photoreflector lamp at 0−5 °C. (a) Time course of $O_2^{-\bullet}$ generation with 0.2 mM C_{60}/2.5% PVP and 10 mM NADH. Light (□): under photoirradiation. Dark (■): without photoirradiation. (b) Dose-dependent effect of NADH on the photoinduced $O_2^{-\bullet}$ generation by C_{60}. Irradiation time was 30 min with 0.2 mM C_{60}/2.5% PVP (C_{60}(+)) (□) or 2.5% PVP (C_{60}(−)) (◇). (c) Dose-dependent effect of C_{60} on the $O_2^{-\bullet}$ generation by photoexcited C_{60}. Irradiation time was 30 min with 10 mM NADH.

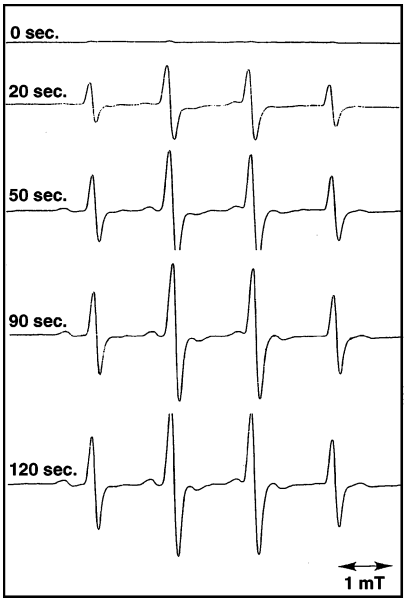
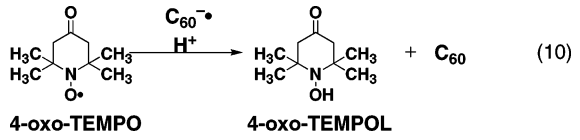


Figure 7. X-band EPR spectra of the DMPO adduct with •OH generated in C_{60}/PVP aqueous solution under irradiation with a 300-W photoreflector lamp. DMPO 0.72 M, Fe(II)-DETAPAC 0.2 mM, in 50 mM phosphate buffer, C_{60} 0.2 mM, PVP 2%, NADH 10 mM. Irradiation time: 0, 20, 50, 90, and 120 s. Experimental conditions: temperature 296 K, microwave frequency 9.394 GHz, microwave power 16 mW, field modulation 0.1 mT at 100 kHz, scan time 2 min.

pathway is most likely, we tested the detection of $C_{60}^{-\bullet}$ and the effect $^1O_2$ scavengers on the generation of $O_2^{-\bullet}$.

Since $C_{60}^{-\bullet}$ is not sufficiently stable to be detected at room temperature, we applied a convenient trapping method with 2,2,6,6-tetramethyl-4-piperidone *N*-oxide (4-oxo-TEMPO), which can react with $C_{60}^{-\bullet}$ (eq 10).



(10)

In the presence of C_{60} and NADH, a time-dependent decrease of 4-oxo-TEMPO peaks was observed under aerobic conditions (Figure 8a), whereas no reduction of the peaks was observed in the absence of NADH or C_{60} (Figure 8b and c). Importantly, no decrease of the peaks was observed in the presence of

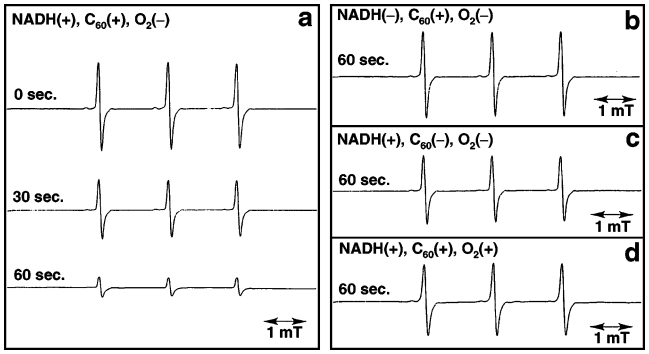


Figure 8. X-band EPR spectra of 4-oxo-TEMPO. Experimental conditions: temperature 296 K, microwave frequency 9.394 GHz, microwave power 16 mW, field modulation 0.1 mT at 100 kHz, scan time 2 min. (a) In degassed C_{60}/PVP aqueous solution under irradiation (0, 30, or 60 s) with a 300-W photoreflector lamp. 4-Oxo-TEMPO 4 μM, C_{60} 0.2 mM, PVP 2%, NADH 10 mM in 50 mM phosphate buffer (pH 7). (b) In degassed C_{60}/PVP aqueous solution without NADH under irradiation (60 s) with a 300-W photoreflector lamp. 4-Oxo-TEMPO 4 μM, C_{60} 0.2 mM, PVP 2% in 50 mM phosphate buffer (pH 7). (c) In degassed PVP aqueous solution without C_{60} under irradiation (60 s) with a 300-W photoreflector lamp. The 4-oxo-TEMPO 4 μM, PVP 2%, NADH 10 mM in 50 mM phosphate buffer (pH 7). (d) In aerobic C_{60}/PVP aqueous solution under irradiation with a 300-W photoreflector lamp. Oxo-TEMPO 4 μM, C_{60} 0.2 mM, PVP 2%, NADH 10 mM in 50 mM phosphate buffer (pH 7).

Table 1. Effect of $^1O_2$ Scavengers on Photoinduced $O_2^{-\bullet}$ Generation from Photoexcited C_{60}[a]

$^1O_2$ scavengers	OD_{560} in light	OD_{560} in dark	relative generation of $O_2^{-\bullet}$ (%)
	1.38	0.09	100
DABCO[b]	1.32	0.09	95.4
L-histidine	1.33	0.09	96.2
2,5-dimethylfuran	1.36	0.09	98.6

[a] Irradiation: 300-W reflector lamp, 30 min, 0−5 °C, with 0.2 mM C_{60}/2.5% of PVP and 10 mM NADH. [b] DABCO: 1,4-diazabicyclo[2,2,2]octane.

oxygen, indicating that $C_{60}^{-\bullet}$ transfers an electron to oxygen, rather than 4-oxo-TEMPO, to give $O_2^{-\bullet}$ (Figure 8d). This finding suggests that $O_2^{-\bullet}$ is formed via path a.

To investigate the potential that path b is operative, the effect of $^1O_2$ scavengers on $O_2^{-\bullet}$ generation was also studied by NBT the method. As shown in Table 1, there was no significant effect of $^1O_2$ scavengers on the generation of $O_2^{-\bullet}$, further suggesting that the $O_2^{-\bullet}$ generation proceeds via path a in eq 9.

Conclusions

Using spectroscopic methods (EPR spin-trapping and the NBT method), it was shown that oxyl radicals ($O_2^{-\bullet}$ and •OH) are generated from photoexcited C_{60} under physiological conditions, especially in the presence of biological reducing agents. Taking into account the results of DNA cleavage studies, we believe it is likely that reduced oxygen species ($O_2^{-\bullet}$ and •OH) are the key active species for the observed biological activities. This is in contrast to previous studies of photoexcited fullerenes, which were performed under conditions where singlet oxygen is indeed generated. However, the identification and study of the active species under physiological conditions are essential for the further development of photodynamic therapies based on fullerene and its derivatives as photosensitizers. Much work has been done on biological activities, such as enzyme inhibition or radical scavenging activity, of water-soluble fullerene derivatives. [27−34] However, the photobiological properties of C_{60} described in this paper offer a unique and a potentially promising platform for the design and development of novel therapeutic agents.

Experimental Section

EPR experiments were carried out with a JEOL JES-FE 2XG ESR spectrometer system (JEOL Ltd., Tokyo) and recorded under the following conditions: temperature 296 K, microwave frequency 9.394 GHz, microwave power 16 mW, field modulation 0.1 mT at 100 kHz, scan time 2 min. The C_{60} and C_{70} aqueous solutions were prepared with poly(vinylpyrrolidone) K30 (PVP, Wako Ltd., Tokyo), as reported previously.[8]

DNA Cleavage Assay. A super coiled plasmid DNA (pBR322, 660 μg mL^{-1}, Sigma Co. Ltd.) was diluted with 1 M Tris-HCl buffer (pH8.0) to 12.5 μg mL^{-1}. The DNA solution, fullerene solutions, and other reagent solutions (reductant or quencher) were mixed well in TDC buffer (Tris-HCl buffer with KCl and $MgCl_2$) and subjected to photoirradiation with a 300-W photoreflector lamp at a distance of 20 cm at 37 or 0−5 °C. After irradiation, an aliquot (20 μL) of the irradiated solution was mixed with bromophenol blue-glycerol solution (5 μL) and subjected to agarose gel electrophoresis (10% agarose in Timiza base EDTA (TBE) buffer, 70 V, 2 h). The gel was stained with ethidium bromide and photographed on the transilluminator for analysis of the ratio of Form I (supercoiled) and Form II (nicked).

Singlet Oxygen Detection (Near-Infrared Spectroscopy). According to the method of Arakane et al.,[35] singlet oxygen was detected by using a near-infrared spectrometer (near-infrared Ge detecter, model 403HS, Applied Detector Co., USA), cooled with liquid nitrogen, to obtain luminescence emission spectra from a test solution circulated in a quartz flow cell excited with argon laser light (Ar laser equipment, Innova 70-4, Coherenet Inc., USA) at 514.5 nm with 200-mW output power. The argon laser beam was chopped at 800 Hz by an acousto-optic modulator (A-160, Hoya Co., Japan) with a driver (110-DS, HOYA Co., Japan). The output signal from the Ge detector was led through a preamplifier (model 116, E. G. & G. Princeton Applied Research, USA) to a lock-in amplifier (model 124A, E. G. & G. Princeton Applied Research, USA), synchronized with an internal standard signal, and recorded on an XY recorder.

Singlet Oxygen Detection (EPR Spin-Trapping Method). Singlet oxygen was also detected by an EPR method using 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP) as a spin-trapping reagent. As a standard, $^1O_2$-generating compound Rose Bengal was employed. To a C_{60}/PVP or a Rose Bengal/PVP aqueous solution (100 μL), 250 mM phosphate buffer (50 μL), distilled water (80 μL), and 1 M 4-oxo-TEMP (20 μL) were added and mixed well under an aerobic condition. The mixed solution was introduced into a flat cell, irradiated with a 300-W photoreflector lamp at a distance of 15 cm, and immediately subjected to EPR measurement. The generation of singlet oxygen was detected as an EPR signal due to 4-oxo-TEMPO formed by the reaction of $^1O_2$ with 4-oxo-TEMP.

Detection of Superoxide Radical Anion (EPR Spin-Trapping Method). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) or 5-diethoxyphosphoryl-5-methyl-1-pyrroline *N*-oxide (DEPMPO) was used as a spin-trapping agent. To a C_{60}/PVP or a PVP aqueous solution (100 μL), 5 mM DETAPAC in 250 mM phosphate buffer (50 μL), DMSO (55 μL), 100 mM NADH or distilled water (25 μL), and DMPO or 625 mM DEPMPO (20 μL) were added and mixed well under an aerobic condition. The mixed solution was collected in a flat cell, irradiated with a 300-W photoreflector lamp at a distance of 15 cm, and subjected immediately to EPR measurement. The generation of $O_2^{-\bullet}$ was detected as signals due to DMPO−OOH or DEPMPO−OOH formed by the reaction of $O_2^{-\bullet}$ with DMPO or DEPMPO, respectively.

Detection of Superoxide Radical Anion (NBT Method). The generation of $O_2^{-\bullet}$ was also detected by the NBT (nitro blue tetrazolium) method as reported by Nagano et al.[36] To a solution of C_{60}/PVP or PVP (100 μL), 2.4 mM NBT (20 μL), 1 mM EDTA (20 μL), 250 mM phosphate buffer (4 μL), and a 10-fold concentrated solution of reductant (20 μL) were added and mixed well under an aerobic condition. The reaction mixture was irradiated with a 300-W photoreflector lamp at 1−5 °C and then diluted with 50 mM phosphate buffer (0.8 mL) for the measurement of OD_{560}.

Detection of Hydroxyl Radical (EPR Spin-Trapping Method). 5,5-Dimethyl-1-pyrroline *N*-oxide (DMPO) was used as a spin-trapping agent. To a C_{60}/PVP or a PVP aqueous solution (100 μL), 0.2 mM Fe(II)-DETAPAC in 250 mM phosphate buffer (50 μL), distilled water (55 μL), 100 mM NADH or distilled water (25 μL), and DMPO (20 μL) were added and mixed well under an aerobic condition. The mixed solution was collected in a flat cell, irradiated with a 300-W photoreflector lamp at the distance of 15 cm, and immediately subjected to EPR measurement. The generation of •OH was detected as signals due to DMPO−OH formed by the reaction of •OH and DMPO.

Detection of C_{60} Radical Anion (EPR Spin-Trapping Method). According to the method reported by Hadjur et al.,[37] the generation of $C_{60}^{-\bullet}$ was detected as a decrease of the signal of 4-oxo-2,2,6,6-tetamethylpyperidine *N*-oxide (4-oxo-TEMPO) due to the reaction of $C_{60}^{-\bullet}$ and 4-oxo-TEMPO to give 4-oxo-TEMPOL, which does not exhibit any radical signal. To a C_{60}/PVP or a PVP aqueous solution (100 μL), 250 mM phosphate buffer (50 μL), distilled water (5 μL),

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100 mM NADH or distilled water (25 μL), and 20 μM 4-oxo-TEMPO (50 μL) were added and mixed well under an anaerobic or aerobic condition. The mixed solution was introduced into a flat cell, irradiated with a 300-W photoreflector lamp with a distance of 15 cm, and immediately subjected to the EPR measurement.

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