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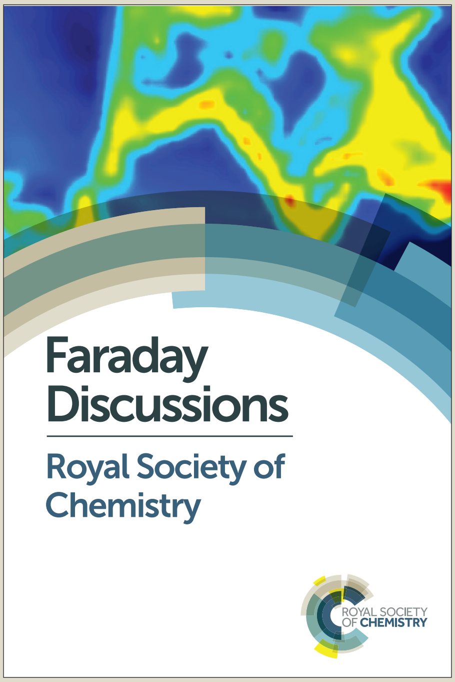

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ARTICLE

Water-soluble fullerene materials for bioapplications: photoinduced reactive oxygen species generation

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The photoinduced reactive oxygen species (ROSs) generation from several water-soluble fullerenes was examined. The macromolecular or small molecular water-soluble fullerene complexes/derivatives were prepared and their $^1O_2$ and $O_2{\bullet}^-$ generation were evaluates by EPR spin-trapping methods. As a result, efficient $^1O_2$ generation was detected from photoexcited C_{60} not only in organic solvents but also in aqueous media and especially from small molecule C_{60} carboxylic derivative, whereas efficient $O_2{\bullet}^-$ generation was observed in the aqueous solution of C_{60}/γ-CD complex under photoirradiation.

1 Introduction

Due to the unique physical and chemical properties of fullerenes – such as photosensitivity and metal encapsulation – biological application of fullerenes for photodynamic therapy (PDT) and MRI contrast enhancement are considered as a promising area. The photosensitivity of C_{60} and C_{70}, which was initially reported by Foote and co-workers in the early 1990's,[1, 2] is unique and useful due to (1) high quantum yield in $^1O_2$ generation and (2) long excitation wavelength. The quantum yield of $^1O_2$ generation in benzene was estimated to be 1.00. The lifetime of S_1 and T_1 of C_{60} are 1.2 ns and 250 μs respectively. Since both of these properties are ideal as PDT agents, many research groups became interested in using fullerenes as photobiomaterials. The main obstacle in such applications of fullerenes was their extremely low solubility, especially in water or water-miscible solvents due to their hydrophobic surfaces. Therefore, derivatization or complexation of fullerenes with water-soluble moieties (ideally biocompatible ones) is essential. The initial studies on bioactive C_{60} derivatives, reported independently in 1993 by the Nakamura-group[3] and the Wudl-group,[4] showed the preparation of water-soluble C_{60} materials by covalent attachments of water-soluble moieties that enabled *in vitro* biological assays of C_{60}. In their study, the Nakamura-group reported the $^1O_2$ generation from their C_{60} derivatives under photoirradiation and DNA damage *in vitro*.

According to the reports by the Foote and co-workers, photoexcited C_{60} can cause both of an energy transfer reaction (*type II* in Fig. 1) to generate singlet oxygen ($^1O_2$)[1] and an electron transfer reaction (*type I* in Fig. 1) to generate C_{60} radical anion ($C_{60}{\bullet}^-$).[1, 5] The *type I* reaction mechanism is possible due to the redox potential of triplet excited state of C_{60} ($^3C_{60}$*) ($^3C_{60}$*: $E_1 = +1.14$ V vs SCE in PhCN, $^1C_{60}$: $E_1 = -0.42$ V vs SCE in PhCN) and electron transfer reaction can occur easily in the presence of an electron donor to provide $C_{60}{\bullet}^-$. (The quantum yield of electron transfer of C_{60} was 1.00 in benzonitrile and 0.52 in benzonitrile-benzene (1:1)). In our previous study, we reported the observation of superoxide radical anion ($O_2{\bullet}^-$) *via* $C_{60}{\bullet}^-$ generated in the presence of physiological concentrations of biological electron donor (NADH) and efficient DNA cleavage.[6, 7] However, the mechanism and conditions, that control which reaction pathway (*type I* or *II*) is favoured, are still unclear. It is important to clarify the mechanisms of ROS generation from photoexcited C_{60} in bio-relevant condition for the future clinical applications and for the evaluation of safety/toxicity of these nanomaterials.

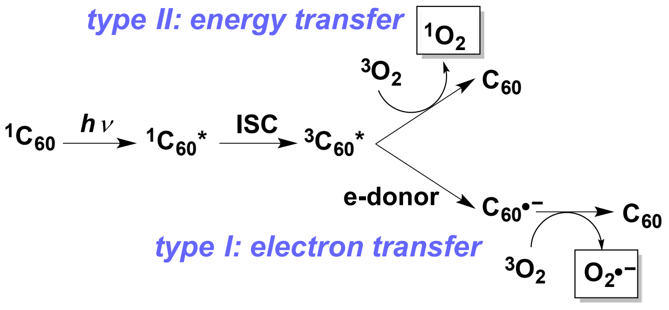


Fig. 1. Two pathways of generating reactive oxygen species (ROSs) from photoexcited C_{60}. The *type II* is *via* an energy transfer reaction from triplet excited state of C_{60} ($^3C_{60}$*) to generate singlet oxygen ($^1O_2$) and the *type I* is *via* an electron transfer reaction from C_{60} radical anion ($C_{60}{\bullet}^-$) to generate superoxide radical anion ($O_2{\bullet}^-$) which will be further converted to more reactive hydroxyl radical (•OH) to cleave DNA molecules.

In this study, we have prepared four kinds of water-soluble C_{60} materials by (1) complexation with poly(vinylpyrrolidone) (PVP) (C_{60}/PVP complex, **1** in Fig. 2), (2) copolymerization with PVP (C_{60}-PVP copolymer, **2** in Fig. 2), (3) complexation with γ-cyclodextrin (γ-CD) (C_{60}/γ-CD, **3** in Fig. 2), and (4) chemical functionalization of C_{60} (C_{60} carboxylic acid, **4** in Fig. 1). We have tested their ability to generate ROSs and evaluated their properties as PDT agents.

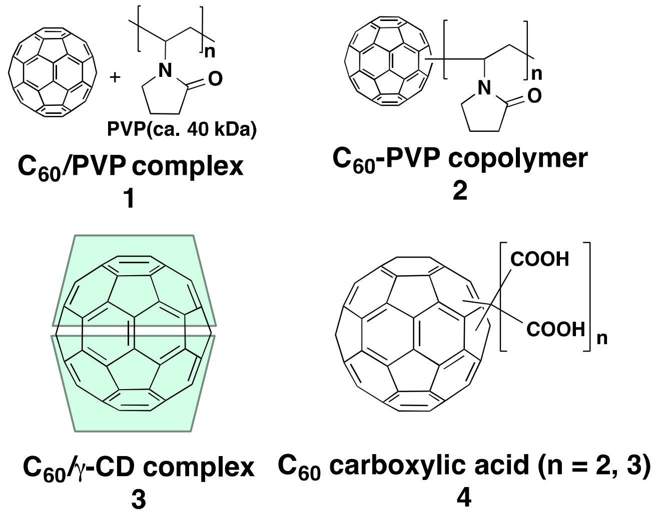


Fig. 2. Water-soluble C_{60} materials used in this study. Two macromolecular materials C_{60}/PVP complex **1** and C_{60}-PVP copolymer **2** and two small molecular materials C_{60}/γ-CD complex **3** and C_{60}-carboxylic acid **4**.

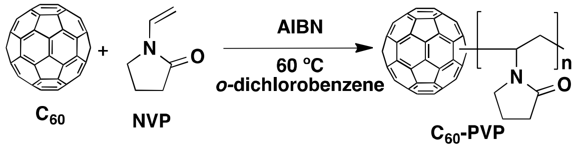
2 Experimental

2.1 Preparation of water-soluble C_{60} complexes or derivatives:

General. C_{60} (99.5%) was purchased from MTR Ltd. (Cleveland, OH, USA). All the other reagents were purchased from corresponding suppliers and purified as described when needed. All the solvents were purchased from Acros Organic (Thermo Fischer Scientific, Inc., Geel, Belgium).

C_{60}/PVP complex. C_{60}/PVP complex was prepared from C_{60} and poly(vinylpyrrolidone) K-30 (Alfa Aesar, Warf Hill, MA, USA) based on the reported procedure.[8] A toluene solution of C_{60} and a chloroform solution of PVP were mixed well and slowly concentrated *in vacuo* to give a thin film of complex. After drying the residue by high vacuum, milli-Q water was added to provide a transparent brownish solution of C_{60}/PVP complex.

C_{60}-PVP copolymer. C_{60}-PVP (1:200) copolymer[9] was prepared by the copolymerization of C_{60} and 200 equiv of *N*-vinylpyrrolidone (NVP, Acros, distilled before use) in the presence of azobisisobutyronitrile (AIBN, Aldrich, 98%, 0.2 equiv to NVP) as a radical initiator. The reaction was carried out in dichlorobenzene at 60 ºC (Scheme 1).[9] A solution of C_{60} (72.0 mg, 0.10 mmol), AIBN (656 mg, 4.0 mmol), and *N*-vinylpyrrolidone (distilled, 2.22 g, 20.0 mmol) in *o*-dichlorobenzene (2.5 mL) was degassed four times and stirred at 60 ºC under N_2 atmosphere until the substance became very viscous (2 days). $CHCl_3$ (2 mL) was added to the reaction mixture to provide uniform dark brown solution, which was subsequently added to Et_2O (200 mL) to precipitate the C_{60} polymer. The resulting precipitate was separated by centrifugation and the residue was again dissolved with $CHCl_3$ and precipitated in Et_2O (2 times). The final powder was collected and dried under vacuum to obtain C_{60}-PVP copolymer (1:200) (2.2 g, isolated yield 96 %).



Scheme 1. Preparation of C_{60}-PVP by radical polymerization of NVP in the presence of C_{60} as an end-cap reagent.

C_{60}/γ-cyclodextrin complex. The solutions of C_{60} (40 mg) in toluene (20 mL) and γ-cyclodextrin (γ-CD, TCI, ≥98%, 134 mg) in water (20 mL) were mixed in a flask and refluxed for 30 hours with vigorous stirring. The reaction mixture was cooled to room temperature and aqueous layer was separated and centrifuged for 30 min (4000 rpm). After removing the supernatant, cold water was added to the precipitate and centrifuged again (4000 rpm, 40 min at 10 ºC). The obtained precipitate was dried under high vacuum for 15 hours.

Bis- and tris-Bingel C_{60} carboxylic acid derivatives. The bis- and tris-Bingel adducts of C_{60} ($C_{60}(C(COOH)_2)_n$, n = 2 or 3) were synthesized based on a reported procedure.[10] Briefly, to a solution of C_{60} (493 mg, 0.68 mmol) in toluene (500 mL), NaH (60%, Aldrich, 1.46 g, 34 mmol as NaH) and subsequently 3 equiv of diethyl bromomalonate (350 μL, 2.05 mmol, prepared by bromination of diethylmalonate) were added and stirred at room temperature under N_2 for 12 hours. Additional NaH (60%, 1.16 g) was added and reaction mixture was stirred for another 12 hours. The reaction mixture was filtered through celite, concentrated *in vacuo*, and purified by SiO_2 column chromatography (SiO_2 150 g, solvent: hexane-toluene) to separate mono adduct (eluted by hexane-toluene (3:7)), bis-adducts (eluted by hexane-toluene (1:4)) and tris-adducts (eluted by hexane-toluene (1:1)). The obtained bis- and tris-adducts were subjected to the deprotection (by NaH and MeOH) without further isolation of each regioisomer (regioisomeric mixture).

2.2 Detection of reactive oxygen species by ESR and chemical reaction

General. ESR spectra were recorded on Bruker spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany) equipped with a microwave bridge X-band ER 082C. Light irradiation and ESR measurements were carried out on the sample in a disposable capillary with 50 μL volume (wiretrol, Drummond, Broomall, PA, USA), which was placed inside an ESR tube with 4 mm thin wall precision quartz 250 mm (Wilmad, Vineland, NJ, USA).

$^1O_2$ generation by ESR spin-trapping method. $^1O_2$ was detected by an EPR method using 2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP, Acros, 98%) as a spin-trapping reagent. To a 0.5 mM fullerene (20 μL) in distilled water, 1 M 4-oxo-TEMP (10 μL), 1 M phosphate buffer (5 μL), and distilled water (65 μL) were added and mixed well under an aerobic condition. The mixed solution was introduced into a capillary, placed inside an EPR tube, and irradiated by a 200-W photoreflector lamp at a distance of 10 cm for 5 min, and then immediately subjected to EPR measurement. The generation of singlet oxygen was detected by an ESR signal corresponding to 4-oxo-TEMPO formed by the reaction of $^1O_2$ with 4-oxo-TEMP. Measurement conditions: temperature 296 K, microwave frequency 9.375 GHz, microwave power 10 mW, receiver gain 2.0 x 10^3, modulation amplitude 4.00 G, modulation frequency 100 kHz, sweep time 20.48 sec.

$^1O_2$ generation by ADPA method. A mixture of C_{60} material (50 μM) and anthracene dipropionic acid (ADPA, Sigma-Aldrich, 50 μM) in PBS(–) (1 mL) was irradiated in a quartz cell by 200-W photoreflector lamp at a distance of 15 cm for 1 min. The decrease of the OD_{400} was measured as relative amount of $^1O_2$ generation, which converted ADPA (with absorbance at 400 nm) to a cycloadduct (no absorbance at 400 nm).

$O_2{\bullet}^-$ generation by ESR spin-trapping method. $O_2{\bullet}^-$ was detected by an ESR method using 5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-*N*-oxide (DEPMPO, Enzo Life Sciences, ≥99%) as a spin-trapping reagent. To a 0.5 mM fullerene aqueous solution (20 μL), 565 mM DEPMPO in water (8 μL), NADH (10 μL), 5 mM DETAPAC (20 μL), DMSO (26 μL) 1 M phosphate buffer (5 μL), and water (11 μL) were added and mixed well under an aerobic condition. The mixed solution was collected in a capillary (50 μL) inside of an ESR tube and irradiated with a 200-W photoreflector lamp at a distance of 10 cm for 1 min. After light irradiation, the sample was immediately subjected to ESR measurement. The generation of $O_2{\bullet}^-$ was detected as signals corresponding to DEPMPO-OOH formed by the reaction of $O_2{\bullet}^-$ with DEPMPO. Measurement conditions: temperature 296 K, microwave frequency 9.375 GHz, microwave power 10 mW, receiver gain 2.0 x 10^3, modulation amplitude 4.00 G, modulation frequency 100 kHz, sweep time 20.48 sec.

Results and discussion

Four kinds of water-soluble C_{60} materials (Fig. 2) were prepared to compare their ROS generation properties. C_{60}/PVP complex and C_{60}-PVP copolymers are both polymeric materials made by a combination with water-soluble non-ionic polymer PVP. The advantage of the use of PVP is lack of toxicity and this has been used as a dispersion reagent in medicine and cosmetics for a long time. In addition, such polymeric materials have advantage in their enhanced permeation and retention (EPR) effect in tumors and other inflammatory diseases. Compared to the C_{60}/PVP with water-solubility of 0.56 mM, C_{60}-PVP copolymer with covalently bound PVP provided better solubility (10-15 mM). Both the PVP complexes and copolymers can be alco obtained using C_{70} as a fullerene core instead of C_{60}. C_{60}/γ-CD was prepared by complexation in two-solvent system (toluene and water) and provided sufficient water-solubility. The tris-Bingel adduct of C_{60} carboxylic acid was prepared by the Hirsch-Bingel reaction of C_{60} and a bromo malonate derivative and isolated as a mixture of regioisomers of tris-adducts (separated from mono- and bis-adducts). Since the tris-adduct was thoroughly soluble in water after deprotection of ethyl esters compared to the mono- and bis-adducts (the deprotected bis-adduct was also water-soluble but solubility of tris-adduct was better), the tris-adduct (as a mixture of regioisomers) was used for the ROS generation study.

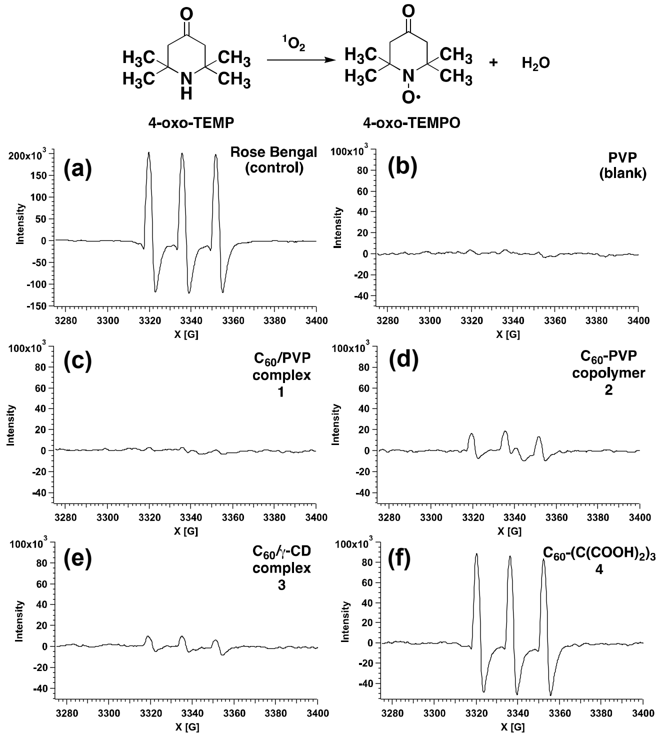


Fig. 3. X-band ESR spectra of 4-oxo-TEMP adduct with $^1O_2$ generated in Rose Bengal (a), PVP (b), C_{60}/PVP complex (c), C_{60}-PVP copolymer (d), C_{60}/γ-CD complex (e), and C_{60} carboxylic acid tris-Bingel derivative (f) in aqueous solution under irradiation of 200-W photoreflector lamp (1 min). 4-oxo-TEMP 80 mM in 50 mM phosphate buffer (pH 7). Rose Bengal or C_{60} 0.04 mM, PVP 0.4%. Experimental condition: temperature 296 K, microwave frequency: 9.375 GHz, microwave power: 10 mW, modulation amplitude 4.00 G, modulation frequency: 100 kHz, scan time 20.5 sec.

First, the $^1O_2$ generation through *type II* energy transfer pathway from each water-soluble C_{60} materials was measured under photoirradiation by ESR method in the presence of

2,2,6,6-tetramethyl-4-piperidone (4-oxo-TEMP) as a spin-trapping reagent, which reacts with $^1O_2$ to provide 4-oxo-TEMPO (see the scheme in Fig. 3). Rose Bengal was used as a standard compound for photoinduced $^1O_2$ generator in aqueous solution. As shown in Fig. 3a, strong peaks corresponding to the 4-oxo-TEMPO were observed in Rose Bengal solution. Interestingly, although there is no generation of $^1O_2$ in C_{60}/PVP complex **1** solution (Fig. 3c), significant generation of $^1O_2$ was observed for C_{60}-PVP copolymer **2** solution in good agreement to our previous report (Fig. 3d). This $^1O_2$ generation was observed even in higher efficiency with the C_{70}-PVP copolymer (data not shown). In our previous paper, we thought that photoexcited C_{60} provides $^1O_2$ in less polar organic solvents such as benzene or benzonitrile, but not in aqueous media.[7] However based on this current result, photoexcited C_{60} indeed generates $^1O_2$ in polar solvent. The difference in $^1O_2$ generation between C_{60}/PVP complex and C_{60}-PVP copolymer could be explained by the rate of aggregation. It is known that the pristine C_{60} aggregates do not generate ROS due to the self-quenching of $^3C_{60}$*.[11] Since the solubility of C_{60}-PVP copolymer is better than the one of C_{60}/PVP complex, C_{60}-PVP solution is less aggregated than C_{60}/PVP solution to provide better $^1O_2$ generation.

Interestingly, two small molecule C_{60} materials (C_{60}/γ-CD **3** and C_{60} carboxylic acid **4**) showed completely different properties in $^1O_2$ generation. Whereas C_{60}/γ-CD complex showed only little generation of $^1O_2$ (Fig. 3e), C_{60} carboxylic acid showed very efficient $^1O_2$ generation (Fig. 3f). The $^1O_2$ generation of the C_{60} carboxylic acid tris-Bingel adduct **4** was higher than the one with bis-Bingel adduct with less substitution (data not shown) presumably due to the solubility difference (generally $^1O_2$ generation from C_{60} derivative decreases with higher substitution[12]). This $^1O_2$ generation from **4** was pretty high and in the same range as the one from Rose Bengal.

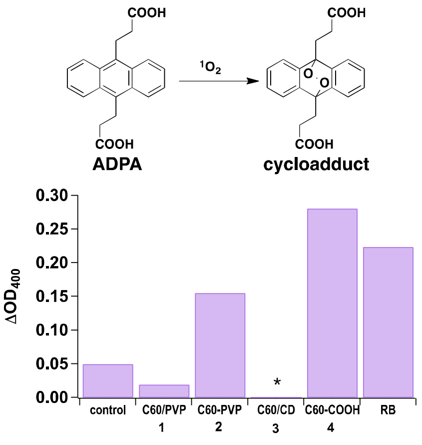


Fig. 4. Detection of $^1O_2$ generation from photoexcited C_{60} materials by ADPA method. C_{60} material: 50 μM, anthracene dipropionic acid (ADPA): 50 μM, in PBS(–) (1 mL). Irradiation: 200-W photoreflector lamp at a distance of 15 cm for 1 min. The generation of $^1O_2$ was estimated by the decrease in OD_{400}. *In the measurement of C_{60}/γ-CD complex, insoluble precipitates were observed during the light irradiation and did not provide a reliable data.

$^1O_2$ generation was also measured by an alternative chemical method using anthracene dipropionic acid (ADPA) as a diene for cycloaddition of $^1O_2$. The relative amount of $^1O_2$ generation measured by the decrease of OD_{400} corresponding to the decay of ADPA to cycloadduct was in good agreement to the results detected by the ESR method (Fig. 4).

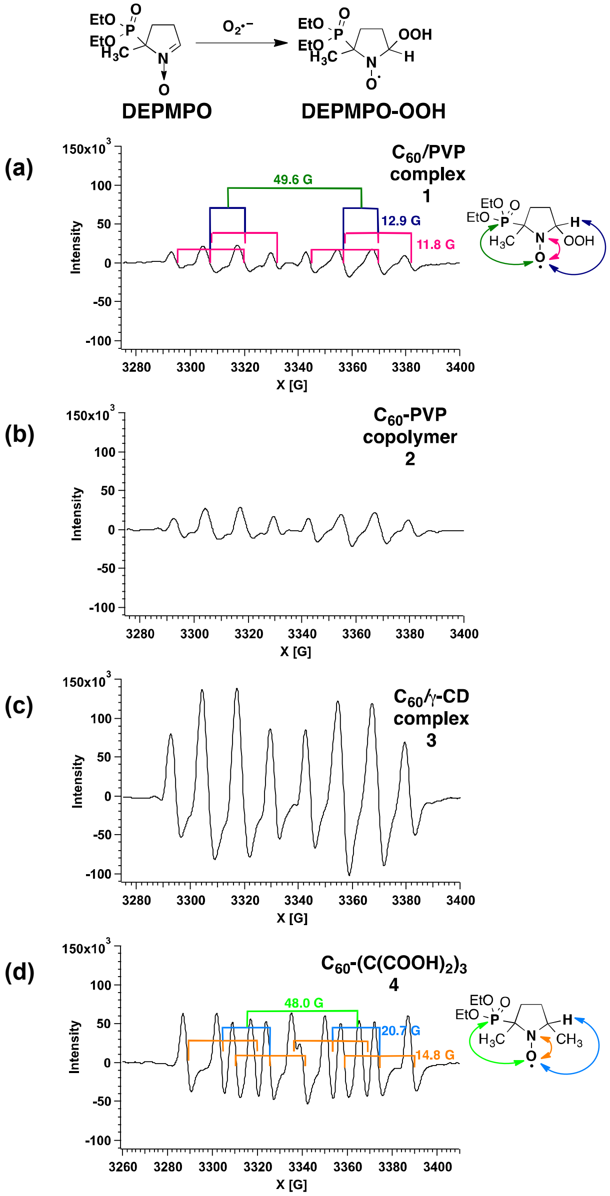
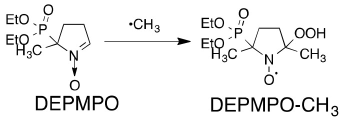


Fig. 5. X-band ESR spectra of DEPMPO adduct with O_2•$^-$ generated in C_{60}/PVP complex (a), C_{60}-PVP copolymer (b), C_{60}/γ-CD complex (c), and C_{60} carboxylic acid tris-Bingel derivative (d) in aqueous solution under irradiation of 200-W photoreflector lamp (1 min). DEPMPO 50 mM, DETAPAC 1 mM, DMSO 3.1 mM in 50 mM phosphate buffer (pH 7). C_{60} 0.2 mM, PVP 2%, NADH 10 mM. Experimental condition: temperature 296 K, microwave frequency: 9.375 GHz, microwave power: 10 mW, modulation amplitude 4.00 G, modulation frequency: 100 kHz, scan time 20.5 sec.



Next, the generation of $O_2^{\bullet-}$ through alternative *type I* electron transfer pathway was measured by ESR method using DEPMPO (5-(diethoxyphosphoryl)-5-methyl-1-pyrroline-N-oxide) as a spin-trapping reagent.[13] Compared to DMPO (5,5-dimethyl-1-pyroline *N*-oxide) which is commonly used for the detection of $O_2\bullet^-$, the use of DEPMPO has advantage in the quantitative detection of $O_2\bullet^-$, since $O_2\bullet^-$ adduct of DEPMPO (DEPMPO-OOH) has sufficiently longer lifetime. Photoirradiation was carried out in the presence of physiological concentration of NADH as a biological electron donor. The peaks of the DEPMPO adducts observed are shown in Fig. 5.

Consistent with our previous results,[7] $O_2\bullet^-$ generation from C_{60}/PVP complex under photoirradiation was clearly observed (Fig. 5a). Similarly, C_{60}-PVP copolymer also generated $O_2\bullet^-$ in the same range of efficiency but with a slight increase (Fig. 5b). Very interestingly, the generation of $O_2\bullet^-$ from C_{60}/γ-CD complex in aqueous solution was very efficient (Fig. 5c). This may be explained by the no self-aggregation of C_{60} in C_{60}/γ-CD system due to the complete bi-cap encapsulation of C_{60} by γ–CD (no chance that C_{60} interact with the other C_{60}) and does not cause any self-quenching of $^3C_{60}$*.

$O_2\bullet^-$ generation was not observed in solutions of C_{60} carboxylic acid (Fig. 5d). Instead, peaks corresponding to $\bullet CH_3$ adduct of DEPMPO (DEPMPO-CH_3, scheme in the foot note in Fig. 5) were clearly observed. In general, the generation of $\bullet CH_3$ in such $O_2\bullet^-$ generation detection system with spin-trapping reagents (DMPO or DEPMPO) is observed as a result of the reaction of •OH and DMSO, which is added to quench •OH with simultaneously generating $\bullet CH_3$. (The spin-trapping reagents can react not only $O_2\bullet^-$ but also •OH in much faster reaction rate and providing more stable adduct, which disturbs the detection of signal of $O_2\bullet^-$ adduct.) Therefore, it seemed that •OH generated much more efficiently in a solution of C_{60} carboxylic acid. However in the separate experiment of •OH detection using DMPO as a spin-trapping reagent, there is no significant difference in the generation of •OH between C_{60}-PVP system and C_{60} carboxylic acid system (data not shown). Therefore, the mechanism of this $\bullet CH_3$ generation is still not clear.

Table 1. Summary of ROS generation from water-soluble C_{60} materials

	C_{60} materials	types of the materials		solubility [mM]	$^1O_2$	$O_2\bullet^-$
1	C_{60}/PVP	macromolecule	complex	0.56[8]	–	++
2	C_{60}-PVP	macromolecule	derivative	10-15[9]	++	++
3	C_{60}/γ-CD	small molecule	complex	0.1[14]	+	+++
4	C_{60}-(C(COOH)$_2$)	small molecule	derivative	75[15]	+++	($\bullet CH_3$)

The results of the ROS generation from four water-soluble C_{60} materials are summarized in Table 1. Two small molecule C_{60} materials, C_{60}/γ-CD complex **3** and C_{60} carboxylic acid derivative **4** showed completely opposite tendency in the generation of $^1O_2$ and $O_2\bullet^-$. Whereas **4** generated $^1O_2$ (no $O_2\bullet^-$), **3** generated $O_2\bullet^-$ (little $^1O_2$). It has been reported that the charge separation of $^3C_{60}$-electron donor is dependent on the polarity of the environment.[16] This phenomenon in the current results may be explained by the polarity around C_{60}. In C_{60}/γ-CD, the environment around C_{60} is rather polar (note that CD is a suger derivative without aromatic moieties) to provide longer lifetime of charge-separation. Further experiments such as laser-flash-photolysis will be required to prove this speculation. The reason of the generation of $\bullet CH_3$ in the solution of C_{60} carboxylic acid is not clear. Further ESR spin-trapping experiments using various scavengers (such as NaN_3, L-His, and 2,5-dimethylfuran for $^1O_2$ and superoxide dismutase for $O_2\bullet^-$) in combination with photo DNA cleavage tests to clarify the mechanism.

Conclusions

Four different types of water-soluble C_{60} materials were prepared and tested for their photoinduced ROS generation ability using ESR spin trapping methods. All of the C_{60} materials clearly showed the generation of either $^1O_2$, produced *via* an energy transfer mechanism, or $O_2\bullet^-$, *via* an electron transfer mechanism. Although all reactions were carried out in aqueous media, the tendency of the generation of $^1O_2$ or $O_2\bullet^-$ were clearly different. We speculated that this is due to the aggregation state and polarity of the environment around C_{60} itself. Further experiments in combination with various scavengers for $^1O_2$ and $O_2\bullet^-$ and *in vitro* photo DNA cleaving tests are required for further evaluation on the mechanism in ROS generation from C_{60} in aqueous media.

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Notes and references

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