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Enhancement of photoinduced reactive oxygen species generation in open-cage fullerenes†

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Photodynamic therapy is an important tool in modern medicine due to its effectiveness, safety, and the ability to provide targeted treatment for a range of diseases. Photodynamic therapy utilizes photosensitizers to generate reactive oxygen species (ROS). Fullerenes can be used as photosensitizers to produce ROS in high quantum yields. Open-cage fullerenes are a subclass of fullerenes characterized by a partially open structure, with one or more openings or apertures. The promising electrochemical properties of open-cage fullerenes motivated us to investigate their use for DNA-cleavage and ROS generation under visible light irradiation through type I electron transfer and type II energy transfer reactions. Our results show that open-cage C₆₀ fullerenes are more efficient for photoinduced cleavage of DNA and ROS generation *via* both the type I electron transfer and type II energy transfer pathways than pristine C₆₀ or a C₆₀ pyrrolidine derivative without open-cage. The greater efficiency of ROS generation by open-cage C₆₀ fullerene in type I and type II reactions can be attributed to the increased rate of the initial intersystem crossing process, resulting from larger total reorganization energies, as indicated by computationally calculated relative rates using the Marcus equation, and the lower reduction potential of the open-cage derivative **3**, as determined by CV, which facilitates a more efficient generation of the corresponding radical anion (C₆₀^{•−}).

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Introduction

Photodynamic therapy (PDT),^{1–3} discovered over a century ago, has evolved into a well-established treatment for cancer and infections.^{4–6} PDT involves the use of photosensitizers (PSs),^{7,8} which are non-toxic dyes activated by light absorption. This activation first forms an excited singlet state of the PS, which then transitions to a long-lived excited triplet state (Scheme 1). In the presence of oxygen, the triplet state undergoes

photochemical reactions, producing reactive oxygen species (ROS) that can destroy cancer cells, pathogenic microbes, and unwanted tissue.

Fullerene C₆₀ is particularly well-suited PS for PDT due to its ability to generate ROS in high quantum yields.^{9–11} However, its hydrophobic nature and poor solubility in biological media have hindered the biomedical applications of fullerenes in its pristine form. Therefore, significant efforts have been made over the years to synthesize PSs based on water-soluble fullerene derivatives.¹² As one of the highly suited derivatives, polyhydroxy fullerenes (fullerols)^{13,14} were reported with enhanced aqueous solubility, however, their precise structure often remains unknown. To synthesize water-soluble C₆₀ derivatives with well-defined structures, two synthetic methods were mainly employed; Bingel–Hirsch cycloaddition reaction^{15,16} and 1,3-dipolar cycloaddition (Prato reaction).¹⁷ Using the former reaction, fullerene carboxylic acid derivatives^{18,19} and the latter, fullerene quaternary ammonium derivatives^{20,21} were reported as water-soluble derivatives resulting in recent studies with further advanced properties^{22–27} in combination with biomolecules, including sugars^{24,25} and peptides,²⁸ polymers,^{29,30} and nanoparticles.^{31,32} Despite these intensive reports on C₆₀-based PDT-PS, to date, there have been no studies on open-cage fullerene derivatives reported in this context.

Fullerene-based PSs offer considerable advantages over conventional PSs based on the suitable properties of C₆₀ with (1)

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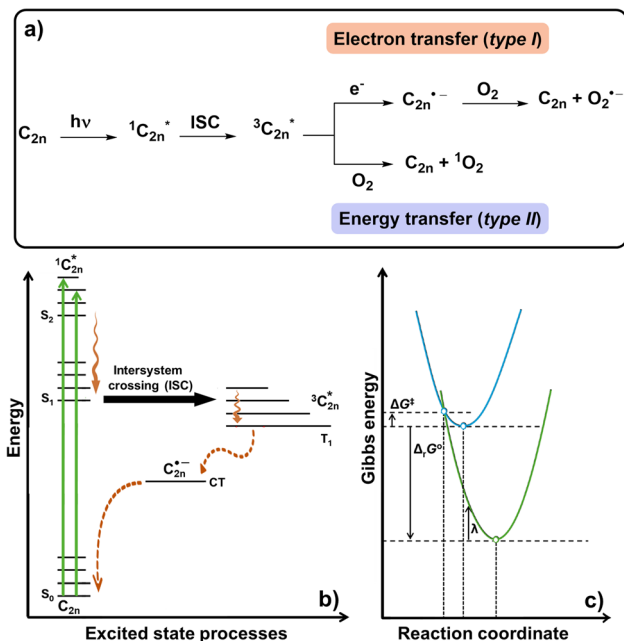
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† Electronic supplementary information (ESI) available: Details of the synthesis of **1–3** and preparation of **P1–3** complexes with corresponding spectral data, experimental methods for DNA cleavage tests, and ESR spin-trapping methods. Computational details, energies of lowest-lying S₁ and T₁ excited states estimated in Franck–Condon and relaxed geometries, and Gibbs energy ΔG⁰, and total reorganization energy λ_T, for ISC type II, and type I reactions. See DOI: <https://doi.org/10.1039/d4sc05428h>

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Scheme 1 (a) Photoinduced reactive oxygen species generation from fullerenes (C_{60} and C_{70}) via electron transfer (type I) and energy transfer (type II) pathways. (b) Schematic representation of the excited state processes that take place from C_{2n} to ${}^3C_{2n}^*$ (types I and II) and from here to $C_{2n}^{\bullet-}$ (type I). (c) Marcus parabolic model describing the charge transfer from a D–A (donor–acceptor, blue curve) to D^+-A^- (green curve).

high photostability and reduced photobleaching and (2) quantitative singlet oxygen (1O_2) generation having a quantum yield of close to 1.⁹ Notably, C_{60} follows two photophysical mechanisms for ROS generation – type I electron transfer and type II energy transfer (Scheme 1a), whereas conventional PDT dyes mainly rely on a single mechanism with type II. In type I electron transfer,^{10,33} the fullerene in its triplet excited state (${}^3C_{60}^*$), which has a relatively high redox potential (${}^3C_{60}^*$: $E_1 = +1.14$ V vs. SCE in PhCN), interacts with an electron donor such as NADH to form the radical anion $C_{60}^{\bullet-}$, which further produces $O_2^{\bullet-}$ (Scheme 1b), the ROS which can be subsequently converted to the most toxic ROS, $\cdot OH$, by the Fenton reaction. Alternatively, in the type II energy transfer pathway, ${}^3C_{60}^*$ reacts with O_2 to generate 1O_2 , another ROS.^{9,34} Rates of these two processes can be estimated using the Marcus parabolic model (Scheme 1c).^{35,36}

Previously, we have observed the generation of $O_2^{\bullet-}$ and $\cdot OH$ in aqueous solution of C_{60} complexed with poly(vinylpyrrolidone) (PVP) by means of electron spin resonance (ESR) spin-trapping methods under visible light irradiation.^{37,38} More recently, we have reported on the photoinduced oxidative DNA damage and related ROS generations by water-soluble C_{60} -PEG and two isomeric C_{70} -PEG derivatives³⁹ with full-eropyrrolidine skeletons.⁴⁰ It was found that the photoinduced cleavage of DNA by C_{60} -PEG via type I electron transfer is much higher than that by C_{70} -PEG, whereas the reverse is true for type II energy transfer.³⁰ Computationally estimated relative rates of

each step of the type I and type II reactions were able to explain the differences in photoinduced ROS generation of C_{60} -PEG and C_{70} -PEG in water.

Open-cage fullerenes are known as interesting molecules due to their unique shapes and properties. They are obtained by selective cleavage of one or more C–C bonds of the fullerene cage. Once opened, these fullerenes can act as host molecules encapsulating guests within their cavity. In the molecular surgery procedure,⁴¹ the fullerene is first opened, then a guest molecule is introduced in the cavity, and finally the structure is closed to generate an endohedral fullerene. Some open-cage derivatives of C_{60} were found useful as electron accepting materials in organic solar cells.⁴²

In 2018, we prepared bis-fulleroid derivatives by a Rh-catalyzed [2+2+2] cycloaddition of diynes and C_{60} to yield a cyclohexadiene-fused intermediate, which transformed into a bis-fulleroid through a [4+4]/retro-[2+2+2] rearrangement.^{43,44} The obtained bis-fulleroid can overcome ring expansion through a photooxygenation process to deliver an open-cage derivative with a 12-membered hole in the C_{60} cage structure. The same procedure was applied to open an orifice in C_{70} .⁴⁵ The obtained open-cage C_{60} derivatives above were incorporated in perovskite solar cells as an electron transport layer (ETL), resulting in solar cells with higher performances than those of previously reported solar cells containing standard $PC_{61}BM$ material as ETL.⁴² The improved performance of this open cage C_{60} derivative was attributed, at least in part, to the increased energy levels of the HOMO and LUMO orbitals due to the open cage structure.

The promising electrochemical properties and remarkable stability of these open-cage C_{60} above prompted us to study these compounds for their photoinduced ROS generation via type I electron transfer and type II energy transfer. To this end, we considered to test on C_{60} and three C_{60} derivatives 1–3 shown in Fig. 1. To have more bio-relevant conditions, we prepared water-soluble complexes of C_{60} and compounds 1–3 with PVP (C_{60}/PVP , **P1**, **P2**, and **P3**) and tested by photoinduced oxidative DNA damages. The generation of responsible ROS (1O_2 and $O_2^{\bullet-}$) by C_{60}/PVP and complexes **P1–3** were measured by ESR in the presence of spin-trapping agents. Finally, computational calculations were performed to investigate the relative reaction rate of each step to understand the mechanisms in ROS generation from C_{60} and 1–3 derivatives.

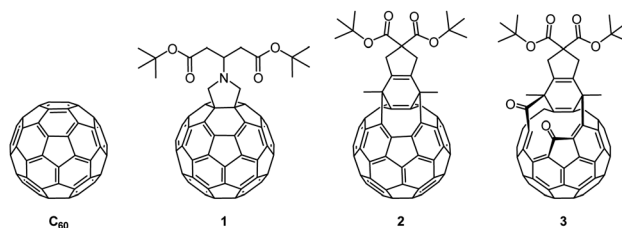


Fig. 1 Molecular structure of the C_{60} and C_{60} derivatives 1–3. The water-soluble complex of each compound with poly(vinylpyrrolidone) (PVP) (C_{60}/PVP , **P1**, **P2**, or **P3**) was prepared for DNA cleavage tests and ROS generation measurements.



Results and discussion

Preparation of water-soluble complexes of C₆₀ and C₆₀ derivatives 1–3 with PVP

A fulleropyrrolidine derivative **1** (ref. 46) and two open cage C₆₀ derivatives **2** and **3** (ref. 42 and 44) were synthesized based on the reported procedures. The UV-vis absorption spectra for the three compounds were registered showing the intense absorption in the UV region and lower intensity broad bands in the visible region, with the expected characteristic bands (430 nm for **1**; 259 and 325 nm for **2**; and 254 and 324 nm for **3**, see Fig. S1–S3†). Cyclic voltammetry was also recorded for the three compounds showing first reduction potentials of -1.18 V for **1**, -1.26 V for **2** and -1.06 V for **3** (see Fig. S4–S9†). These data altogether also allowed us to experimentally determine the HOMO and LUMO energies for the three compounds (see Table S1†).

Since these compounds were not soluble in water, aqueous solutions of C₆₀ and compounds **1–3** were prepared by forming complexes with a neutral polymeric detergent, PVP, as described in the literature.⁴⁷ Each PVP complex, C₆₀/PVP, **P1**, **P2**, or **P3**, was water-soluble and revealed sufficient absorption intensity in visible wavelength region (Fig. S10†), showing good potentials as PDT-PSs. The characteristic bands of the absorption spectra were not altered by the presence of the PVP especially in the visible region, suggesting that the photophysical processes remain unaffected by the presence of PVP (see Fig. S11–S13†).

Photoinduced ROS (¹O₂ and O₂^{•-}) generation

Generation of ROS from the photoexcited C₆₀/PVP and **P1–3** complexes were investigated by ESR spin trapping methods. ¹O₂ generation *via* type II energy transfer reaction (see Scheme 1) by each compound was measured using a spin-trapping reagent, 4-oxo-TEMP (scheme in Fig. 2a bottom). Peaks corresponding to the ¹O₂ adduct of 4-oxo-TEMP (4-oxo-TEMPO) were observed in all solutions after irradiation by green LED light (528 nm max) for 10 min (Fig. 2a). Fig. 2b (upper) shows double integration analyses of the ESR signal to estimate the relative amount of ¹O₂ generation. The results revealed that the generation of ¹O₂ by bis-fulleroid **P2** and open-cage **P3** were significantly higher than that of unsubstituted C₆₀, which was in parallel with the absorption intensity at 528 nm varies in the order **P1** ≈ **P2** > **P3** > C₆₀/PVP (Fig. S10†). Despite **P1** having relatively higher absorbance at this wavelength, it revealed only slightly higher ¹O₂ generation than C₆₀. Generation of ¹O₂ was observed in an irradiation-time-dependent manner in all complexes (Fig. 2b bottom, Fig. S14–S17†), clearly indicating that ¹O₂ generations were due to the photoexcitation of C₆₀/PVP, **P1**, **P2**, and **P3**. The fact that **P2** and **P3** show almost identical behaviour can be explained by the facile transformation of **2** into **3** in the presence of light and oxygen.

The generation of the other ROS, O₂^{•-}, which can be produced *via* type I electron transfer pathway (Scheme 1) by photoexcited C₆₀/PVP and complexes **P1–3**, was investigated in the presence of electron donor NADH using DEPMPO as a spin

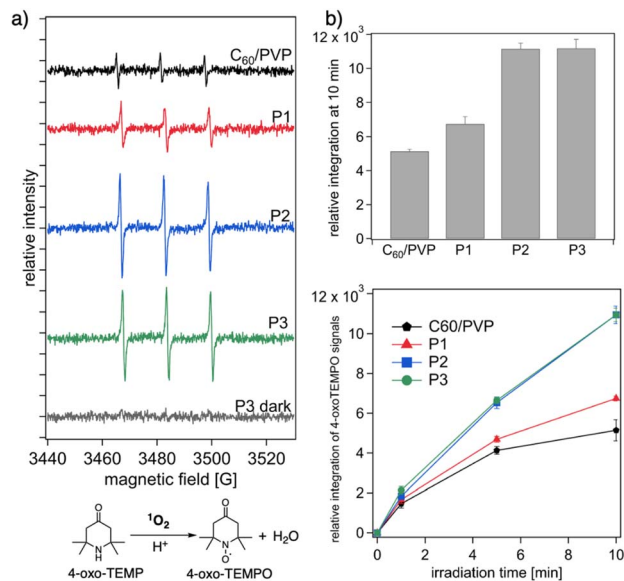


Fig. 2 Photoinduced generations of ¹O₂ by C₆₀/PVP and **P1–P3** in aqueous solutions. (a) X-band ESR spectra of ¹O₂ adduct of 4-oxo-TEMP generated in aqueous solution of C₆₀/PVP, **P1**, **P2**, and **P3**, under photoirradiation for 10 min and scheme for the reaction of ¹O₂ with a spin trapping reagent, 4-oxo-TEMP. (b) Relative amount of ¹O₂ generation estimated by double integration of the peaks corresponding to 4-oxo-TEMPO after 10 min irradiation (upper) and from time-dependent measurements (lower). C₆₀ or C₆₀ derivatives **1–3**: 40 μM; 4-oxo-TEMP: 80 mM; in 60 mM phosphate buffer (pH 7.0). Photoirradiation conditions: green LED (528 nm, 93 lm W⁻²), 4 cm distance, room temperature, 0–10 min. Measurement conditions: temperature 298 K; microwave frequency 9.78 GHz; microwave power 10 mW; receiver gain 5.02 × 10⁴; modulation amplitude 1.00 G; modulation frequency 100 kHz; sweep time 83.89 s. Measurements were triplicated.

trapping reagent (scheme in Fig. 3a bottom). Upon irradiation with green LED, all complexes showed O₂^{•-} generation in aqueous media observed as signals of DEPMPO-OOH (Fig. 3a). When the ESR spectra were quantified by double integration, the relative amount of O₂^{•-} produced by each complex was clearly estimated (Fig. 3b). The amount of O₂^{•-} generation was in a similar trend to that of ¹O₂ generation; **P2** and **P3** had much higher generation than C₆₀/PVP system and **P1** (Fig. 3b). The irradiation-time-dependency was also observed as shown in Fig. 3c and S18–S21.† It is known that O₂^{•-} can be produced not only *via* the type I pathway but also *via* ¹O₂, which was once generated through the type II energy transfer pathway, in the presence of electron donors. To detect the O₂^{•-} generated exclusively *via* the type I electron transfer pathway, ESR experiments in the presence of L-histidine (His), a ¹O₂ quencher, were performed. As shown in Fig. 3b, the reduced amount of O₂^{•-} generation was observed in all complexes in the presence of His, while **P2** showed only a limited amount of decrease (Fig. 3b). This was presumably due to the rapid reaction of **P2** with ¹O₂ to form **P3**, effectively quenching this species. However, the possibility of a faster rate for the type I pathway reaction relative to the type II pathway cannot be ruled out.



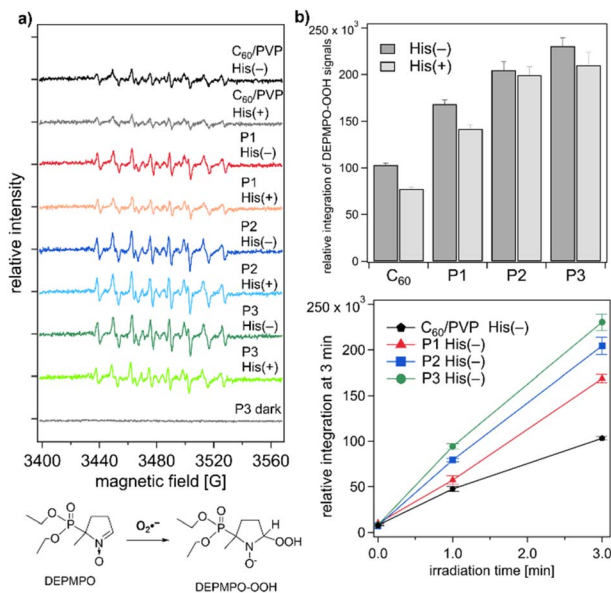


Fig. 3 Photoinduced generations of $O_2^{\bullet-}$ by C_{60}/PVP or $P1-3$ in aqueous solutions. (a) X-band ESR spectra of $O_2^{\bullet-}$ adduct of DEPMPPO in aqueous solution of C_{60}/PVP , $P1$, $P2$, and $P3$; and scheme for the reaction of $O_2^{\bullet-}$ with a spin trapping reagent, DEPMPPO. (b) Relative amount of $O_2^{\bullet-}$ generation estimated by double integration of the peaks corresponding to DEPMPPO-OOH. C_{60} or C_{60} derivatives 1-3; 40 μM ; DETAPAC: 1 mM; NADH: 10 mM; L-histidine: 10 mM; DEPMPPO: 113 mM; in phosphate buffer (60 mM, pH 7.0). Photoirradiation conditions: green LED (528 nm, 93 $lm W^{-1}$), 4 cm distance, room temperature, 0-3 min. Measurement conditions: temperature 298 K; microwave frequency 9.78 GHz; microwave power 10 mW; receiver gain 5.02×10^4 ; modulation amplitude 1.00 G; modulation frequency 100 kHz; sweep time 83.89 s. Measurements were triplicated.

Photoinduced DNA cleavage

In order to evaluate their potential as PDT-PSs, a photo DNA cleavage assay was performed using C_{60}/PVP and $P1-3$. Supercoiled pBR322 DNA was used as a DNA substrate to observe the DNA cleavage ability of the compounds by quantifying the amount of damaged DNA (form II nicked DNA) in relative to the intact ones (form I). Under visible light irradiation (green LED 527 nm), cleavages of the DNA by all four complexes were observed (Fig. 4 and S23-S26[†]). $P2$ and $P3$ showed stronger DNA cleavage upon photo irradiation than that of C_{60}/PVP and $P1$. These results were in line with the ROS generation measured by ESR spin trapping methods, where $P2$ and $P3$ showed higher generation of both 1O_2 and $O_2^{\bullet-}$ than C_{60}/PVP and $P1$. We also observed that DNA cleavage in absence of NADH was significantly lower in all compounds, suggesting that type I pathway plays a crucial role in photoinduced DNA damage, assuming that 1O_2 and $O_2^{\bullet-}$ have an analogous damage power. Noteworthy, generation of form II nicked DNA in the absence of NADH is significantly higher for $P3$ than the other compounds including C_{60}/PVP , which indicates increased involvement of ROS generation by type II pathway from this complex in relation to others.

Quantum chemical modelling

To further investigate the mechanism of ROS generation from considered open cage fullerenes through the type I and type II

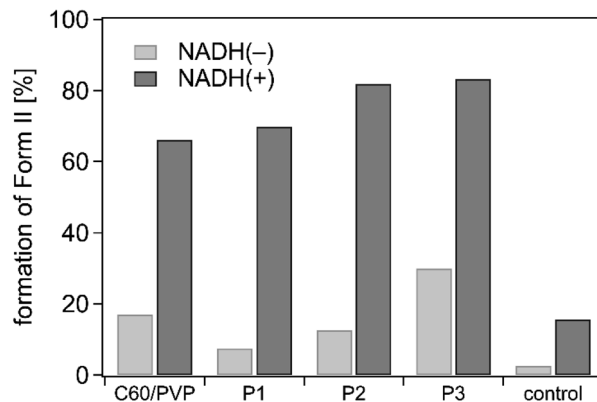


Fig. 4 Photoinduced DNA cleavage by C_{60}/PVP and $P1-3$ in the absence or presence of NADH as an electron donor. Data is obtained from gels shown in Fig. S11 and S12 in the ESI.† Reaction conditions: pBR322: 25 $ng \mu L^{-1}$; C_{60}/PVP or $P1-3$: 0.125 mM; NADH: 10 mM; in Tris-HCl buffer (pH 8.0, 1 mM EDTA); green LED (527 nm, 45 $lm W^{-1}$), at room temperature, for 25 min. Electrophoresis: 100 V, 80 min, 1% agarose in $0.5 \times$ TBE buffer (pH 8.3).

reactions, computational calculations were performed. Taking into account the moderate size of the studied systems, C_{60} fullerene as well as derivatives 1-3 were considered without any simplification. The NADH structure was replaced with a model compound represented by one nucleoside containing only nicotinamide as the base (see Fig. S15[†]). The applicability of such a replacement for the sake of simulation of photoinduced reactive oxygen generation has been previously demonstrated by us.³⁰ The rate of excited state processes, including intersystem crossing (ISC), type II (energy transfer), and type I (electron transfer), both part 1 and part 2 (schemes in Tables 1 and 2), were estimated using the classical Marcus equation:⁴⁸

$$k_{ET} = \frac{2\pi}{\hbar} |H_{AB}|^2 \frac{1}{\sqrt{4\pi\lambda_i k_b T}} \exp\left(\frac{-(\lambda_i + \Delta G^0)^2}{4\lambda_i k_b T}\right) \quad (1)$$

where $|H_{AB}|$ is the electronic matrix element describing the electronic coupling between the electronic states of the reactants and the products, ΔG^0 is the Gibbs energy, and λ_t is the total reorganization energy. λ_t is the sum of two components,

Table 1 Sum of Gibbs energies and total reorganization energies ($\Delta G^0 + \lambda_t$) in eV and relative rates (F = fast, M = medium, S = slow) for ISC and type II reaction estimated with respect to reference C_{60} fullerene

Compounds	ISC		Type II	
	($\Delta G^0 + \lambda_t$)	Rel. rate	($\Delta G^0 + \lambda_t$)	Rel. rate
1	-0.345	M	-0.097	F
2	-0.109	F	-0.037	F
3	-0.240	F	0.095	F
C_{60}	-0.411	S	-0.282	S

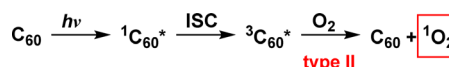
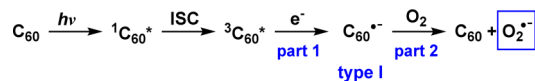


Table 2 Sum of Gibbs energies and total reorganization energies ($\Delta G^0 + \lambda_t$) in eV and relative rates (F = fast, M = medium, S = slow) for ISC and type I reaction (part 1 and part 2) estimated with respect to reference C_{60} fullerene

	ISC		Type I			
	$(\Delta G^0 + \lambda_t)$	Rel. rate	Part 1		Part 2	
	$(\Delta G^0 + \lambda_t)$		$(\Delta G^0 + \lambda_t)$	Rel. rate	$(\Delta G^0 + \lambda_t)$	Rel. rate
1	-0.345	M	0.418	M	0.295	F
2	-0.109	F	0.584	S	0.230	F
3	-0.240	F	0.412	M	0.485	M
C_{60}	-0.411	S	0.273	F	0.445	M



the internal reorganization energy (λ_i) and the external reorganization energy (λ_s). In accordance with Marcus theory, the dependence between the electron/energy transfer (ET/EnT) rate and driving force (ΔG^0) is described by a Gaussian function. The rate increases with the driving force until the reaction's reorganization energy matches the driving force ($\Delta G^0 = -\lambda_t$). Further increase in the driving force leads to a decrease in the ET rate in the so-called Marcus inverted region. A detailed description of reorganization energy estimation is provided in the computational details section in the ESI.† It is well known that $|H_{AB}|$ values are very sensitive to the mutual orientation of the species involved in ET/EnT processes. Their precise estimation is especially problematic for intermolecular processes where the relative position of donor and acceptor sites can vary in a wide range. The structural similarity of the systems under consideration allowed us to also assume the similarity of effective $|H_{AB}|$ values. This assumption, in turn, allowed us to estimate relative rates with respect to reference C_{60} fullerene (eqn (2)).

$$\frac{k_{ET}^{P_x}}{k_{ET}^{C_{60}}} \cong \exp\left(\frac{(\lambda_t^{C_{60}} + \Delta G^{0,C_{60}})^2}{\lambda_t^{C_{60}}} - \frac{(\lambda_t^{P_x} + \Delta G^{0,P_x})^2}{\lambda_t^{P_x}}\right) \quad (2)$$

Taking into account that the sizes of **1–3** conjugates are relatively small, the effect of geometrical relaxation in the corresponding excited state can be significant and must be properly accounted for. Indeed, comparison of excited state energies for **1–3** fullerenes in vertical ground state geometries and equilibrium first excited state geometries revealed that the difference was 0.48–0.63 eV and 0.45–0.59 eV for singlet and triplet states, respectively (Table S1, ESI†). Thus, for all studied processes, geometry relaxation in the corresponding excited state was accounted for accordingly.

In accordance with Scheme 1, the photoinduced reactive oxygen species generation is a sequential process consisting of photoexcitation and ISC stages followed by ET or EnT processes. As seen in Fig. S1,† the absorbance of **1–3** derivatives is 1.37 to 1.84 times higher than that of the parental C_{60} fullerene. Thus,

the initial stage of reactive oxygen generation for the systems of interest is expected to be more efficient compared to C_{60} .

Analyses of $(\Delta G^0 + \lambda_t)$ as well as relative rates for ISC and type II energy transfer are summarized in Table 1. The Gibbs energy for triplet-to-singlet oxygen conversion (${}^3O_2({}^3\Sigma_g^-) \rightarrow {}^1O_2({}^1\Delta_g)$) process was taken from experiment.⁴⁹

For all studied systems, the ISC reaction occurs in the inverted Marcus region ($|\Delta G^0| > \lambda_t$). ISC rates for **2** and **3** are predicted to be the fastest among the systems. At the same time, $(\Delta G + \lambda_t)$ values for C_{60} and the **1** conjugate are significantly higher, suggesting their slower ISC rates. Table S2 in ESI† contains the individual ΔG^0 and λ_t values for the ISC and type II reaction, for the systems of interest. The type II energy transfer for **1–3** conjugates is characterized by very small $(\Delta G + \lambda_t)$ values, suggesting a nearly barrierless process with a fast reaction rate. In contrast, the type II reaction in the reference C_{60} occurs in inverted region and is expected to be slower as compared to **1–3**. Taking into account that ISC and 1O_2 generation are two linked and consecutive reactions, where the product of the first reaction serves as the substrate for the second one, it can be argued that the rate of the entire process is determined by the rate of the slowest reaction. The experimentally observed more efficient 1O_2 generation by **P2** and **P3** complexes, followed by **P1** and C_{60} (**P2** > **P3** > **P1** > C_{60} /**PVP**), together with the fact that the absorption intensity at 528 nm varies in the order **P1** \approx **P2** > **P3** > C_{60} (and thus does not correctly describe the observed order) allows us to assume that the ISC stage is the rate-determining step. Interestingly, **2** and **3** have relatively large total reorganization energies as compared to **1** and C_{60} for the ISC step, which translates into relatively low $(\Delta G^0 + \lambda_t)$ values.

The photoinduced ROS generation that proceeds through electron transfer reaction (type I reaction) can be divided into two steps. The first step (part 1) is the reduction of fullerene by the electron transfer from NADH. The second step (part 2) is the reduction of molecular oxygen and generation of the superoxide radical ion $O_2^{\cdot -}$ (see Table 2 footnote). Analyses of $(\Delta G^0 + \lambda_t)$ and relative rates for the initial ISC process and the two subsequent steps of the type I reaction are summarized in Table 2. Individual ΔG^0 and λ_t values for the ISC and both stages of the type I reaction are provided in Table S2, ESI.†

In contrast to the type II reaction, both part 1 and part 2 stages of the type I reaction take place in the normal Marcus region ($|\Delta G^0| < \lambda_t$). Similar to the generation of 1O_2 , the type I reaction and the preceding ISC are linked reactions, and the rate-determining stage will be the slowest one. Following the experimental results of $O_2^{\cdot -}$ generation in the presence of the electron donor NADH, the bis-fulleroid **P2** and open-cage fullerene **P3** demonstrated a superior capability for superoxide generation compared to the closed-cage **P1** and the parental C_{60} . Additionally, the **P1** conjugate outperformed C_{60} . The observed efficiency of the studied systems towards $O_2^{\cdot -}$ radical anion generation suggested that in the case of the type I reaction, again ISC was the slowest, and thus the rate-determining stage. Experimental support for the relative rates determined for type I part 1 can be found in the determined reduction potentials that vary in the order **3** < **1** < **2**.



Conclusions

Four types of water-soluble complexes of C₆₀ and C₆₀ derivatives (1–3) with PVP (C₆₀/PVP and P1–3) were prepared and tested for oxidative damage of DNA and responsible ROS generation under visible light irradiation. While compound 1 is a standard fulleropyrrolidine derivative, compounds 2 and 3 are bis-fulleroid and open-cage fullerene, respectively. Our results show that species 2 and 3 are more efficient than 1 and C₆₀ for both the generation of O₂^{•−} via type I electron transfer pathway and the generation of ¹O₂ and via type II energy transfer reaction in line with the results of photo DNA cleavage by these compounds. The highly efficient generation of ROS by 2 and 3 in type I and type II reactions was explained by (1) the higher rate for the initial ISC process due to larger total reorganization energies of 2 and 3, as shown by computational results based on Marcus equation, and (2) the reduced reduction potential of open-cage derivative 3, as determined by CV. These altogether allow a more efficient generation of the corresponding anion radical (C₆₀^{•−}). Since 2 can be easily converted into 3 upon light and oxygen exposure, 3 is the best candidate for PDT identified in this study, highlighting the potential of open-cage derivatives for the development of more efficient photosensitizers in photodynamic therapy.

Experimental

General

Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. All ¹H and ¹³C NMR spectra were recorded on a Bruker ASCEND 400 spectrometer equipped with a 5 mm BBFO probe or and Bruker 600 spectrometer equipped with a CryoProbe using CDCl₃ as a deuterated solvent. Mass spectrometry analyses were recorded on a Bruker micrOTOF-Q II mass spectrometer (high resolution), equipped with electrospray ion source and a Bruker Daltonics solariX spectrometer.

Synthetic methods

Synthesis of 1, 2, and 3. Compound 1 was prepared by reported methods.⁴⁶ Spectroscopic data for 1: ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 1.57 (s, 18H), 2.80 (dd, *J* = 15.0, 6.6 Hz, 2H), 2.98 (dd, *J* = 15.0, 6.6 Hz, 2H), 4.21 (quint, *J* = 6.6 Hz, 1H), 4.55 (s, 4H); ¹³C NMR (CDCl₃, 150 MHz) δ (ppm): 28.4, 38.5, 54.8, 63.3, 69.9, 81.4, 136.6, 140.3, 142.0, 142.2, 142.3, 142.8, 143.2, 144.7, 145.4, 145.6, 145.9, 146.1, 146.2, 146.4, 147.5, 154.9, 171.1; ESI-HRMS (*m/z*) calculated for [M + H]⁺ = 1006.2013; found 1006.2002. Compounds 2 and 3 were prepared following reported methods for similar derivatives.^{42,44} Spectroscopic data for 2: ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 1.49 (s, 9H), 1.54 (m, 9H), 2.88 (s, 6H), 3.21–3.27 (m, 2H), 3.34–3.40 (m, 2H); ¹³C NMR (CDCl₃, 101 MHz) δ (ppm): 27.92, 27.95, 28.1, 39.9, 44.4, 59.8, 81.7, 81.8, 126.7, 135.2, 135.7, 136.5, 136.8, 137.1, 137.6, 139.0, 139.1, 140.0, 140.1, 140.5, 140.9, 143.3, 143.4, 143.6, 143.7, 143.8, 143.9, 144.0, 144.4, 144.5, 144.6, 144.7, 145.2, 145.3, 145.7, 145.8, 149.8, 151.8, 170.6, 170.9; ESI-HRMS (*m/z*)

calculated for [M + Na]⁺ = 1063.1880; found 1063.1877. Spectroscopic data for 3: ¹H NMR (CDCl₃, 400 MHz) δ (ppm): 1.60 (s, 9H), 1.68 (s, 9H), 2.29 (s, 3H), 2.63 (s, 3H), 3.44 (d, *J* = 16.0 Hz, 1H), 3.72 (d, *J* = 16.8, 1H), 4.05 (d, *J* = 16.0 Hz, 1H), 4.34 (d, *J* = 16.8, 1H); ¹³C NMR (CDCl₃, 101 MHz) δ (ppm): 28.2, 28.3, 32.0, 32.3, 40.3, 41.6, 43.6, 52.4, 60.3, 81.5, 129.3, 131.6, 131.8, 132.0, 132.3, 133.2, 133.3, 135.4, 135.8, 135.9, 136.2, 136.3, 136.7, 137.12, 137.13, 137.6, 137.8, 138.8, 139.7, 140.0, 140.3, 140.4, 140.5, 140.6, 140.9, 141.0, 141.3, 141.8, 142.0, 142.5, 142.8, 142.9, 143.0, 144.2, 144.4, 144.5, 144.73, 144.75, 145.24, 145.25, 145.5, 145.78, 145.80, 145.86, 145.91, 145.94, 146.0, 146.08, 146.13, 146.2, 146.4, 146.5, 147.1, 147.5, 147.8, 147.89, 147.93, 148.8, 150.0, 151.1, 155.9, 170.7, 172.3, 191.4, 202.6; ESI-HRMS (*m/z*) calculated for [M + Na]⁺ = 1095.1778; found 1095.1768.

Preparation of PVP complexes C₆₀/PVP and P1–3. Water soluble complexes were prepared by complexation of C₆₀ and compounds 1–3 with PVP (C₆₀/PVP, P1, P2, and P3) following the reported method.⁴⁷ C₆₀ or C₆₀ derivatives 1–3 (0.0011 mmol) were dissolved in toluene (1.0 ml) and 2.0 ml of CHCl₃ containing 100 mg of PVP (K-30) was added. The resulting solution was sonicated for one hour and the solvents were then slowly evaporated under vacuum to afford PVP complex of C₆₀ (C₆₀/PVP) and P1–3.

Detection of ROS generation by ESR with spin-trapping method. ESR spectra were recorded on a Bruker EMX, Continuous Wave X-Band EPR spectrometer (Bruker BioSpin GmbH, Rheinstetten, Germany). Each sample was taken into 50 μL Blaubrand® intraMark capillaries (Brand GMBH, Wertheim, Germany) and placed inside the Suprasil® ESR tube with a diameter of 4 mm, length of 250 mm, and a wall thickness of 0.8 mm (SP Wilmad-Lab Glass, New Jersey, US) for measurement. 4-Oxo-TEMP was purchased from ABCR (Karlsruhe, Germany) and purified by sublimation prior to use. DEPMPO was purchased from Enzo Life Sciences AG (Farmingdale, NY, USA) and used as it is. L-Histidine, DETAPAC, DMSO, and NADH were purchased from Sigma-Aldrich (St. Louis, Missouri, USA). Light irradiation was performed by green LED light (Lumitronix, PowerBar V3, true green, 528 nm, 93 lm W^{−1}) with a diameter of 8.5 cm. ESR measurement conditions: microwave frequency 9.78 GHz, microwave power 10 mW, receiver gain 5.02 × 10⁴, modulation amplitude 1.00 G, modulation frequency 100 kHz, 10 scan average, sweep time 83.89 s. All measurements were repeated 3 times.

For ¹O₂ measurement, a mixture (50 μL) containing PVP complex of 1–3 (P1–3) or C₆₀ (40 μM) and 4-oxo-TEMP (80 mM) in phosphate buffer (60 mM, pH 7.0) was prepared and O₂ was bubbled for 30 s right before placing into the capillary. Each sample solution was then irradiated and the X-band ESR spectrum was recorded immediately.

For O₂^{•−} measurement, a mixture (50 μL) containing PVP complex P1–3 or C₆₀/PVP (40 μM), DETAPAC (1 mM), NADH (10 mM), L-histidine (10 mM), and DEPMPO (113 mM) in phosphate buffer (60 mM, pH 7.0) containing 20% (v/v) DMSO was prepared and O₂ was bubbled for 30 s right before placing into the capillary. Each sample solution was then irradiated and the X-band ESR spectrum was recorded immediately.



DNA cleavage assay. Supercoiled plasmid pBR322 DNA (New England Biolabs) was used as a DNA substrate. A mixture (20 μL solution) of pBR322 (25 $\mu\text{g mL}^{-1}$), PVP complex of 1–3 (**P1–3**) or C_{60} (0.125 mM), and NADH (10 mM) was prepared in Tris–HCl buffer (10 mM, pH 8.0) containing 1 mM EDTA. Each solution was taken into round bottom 96-well microliter plate and irradiated green LED light (with 527 nm max) for 15 min by Lumidox® II 96-well LED Array (Analytical Sales and Services, Inc., New Jersey, USA). Subsequently, 4 μL of Gel Loading Dye, Purple (6 \times) (New England Biolabs) was added to each solution before loading to an agarose gel. Gel electrophoreses were performed in 0.5 \times TBE buffer at 100 V for 80 min. The gels were stained with GelRed® (Millipore) and visualized by gel imaging system (ChemiDoc, BioRad). The analysis of the images was performed using ImageJ for quantification.

Computational details

Geometry optimizations was performed employing the range-separated functional from Handy and coworkers' CAM-B3LYP⁵⁰ and Ahlrichs' Def2-SVP basis set.^{51,52} The empirical dispersion D3 correction with Becke–Johnson damping was employed.^{53–55} Vertical excitation energies were calculated with the same functional and basis set using Tamm–Dancoff approximation (TDA) formalism.⁵⁶ This method was proven to give accurate results for charge transfer process involving fullerenes.⁵⁷ Solvent effects were estimated using COSMO-like polarizable continuum model^{58,59} in monopole approximation considering water as solvent. The chosen model demonstrated excellent performance towards the solvation energy prediction for both, neutral and charged fullerene-based systems.^{60–62} Calculations were performed with the Gaussian 16 (rev. C01)⁶³ and Orca 5.0.3 programs.⁶⁴ Full computational details including how **P1–3** and NADH were modelled can be found in the ESI.†

Data availability

The data supporting this article have been included as part of the ESI.†

Author contributions

The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Conflicts of interest

There are no conflicts to declare.

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