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High Efficiencies for Singlet Oxygen Generation of Fullerenes and Their Phototoxicity

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Although fullerenes have been known as strong photosensitizers (PS), the quantitative evaluations of their activities have been insufficient, especially in water. We measured the near-infrared (NIR) emission from the singlet oxygen ($^1O_2$) produced by C_{60} and C_{70} dissolved in water using dispersants of γ-cyclodextrin (γ-CD) or poly(vinylpyrrolidone) (PVP), and estimated the $^1O_2$ generation efficiencies. The obtained results show that C_{60} and C_{70} can generate $^1O_2$ with 2–3 times higher efficiencies than the well-known PS, rose bengal (RB), and the $^1O_2$ generation efficiencies depends on the dispersants. Phototoxicity of C_{60} and C_{70} were also examined by using rat fibroblast cell line (5RP7). The cell viabilities after irradiations were consistent with the corresponding $^1O_2$ generation efficiencies. Our results that fullerenes have high $^1O_2$ generation efficiencies assure the potential applications of fullerenes as PS for photodynamic therapy (PDT).

Compared to current medical treatment including surgery, radiation therapy, and chemotherapy, photodynamic therapy (PDT) is known as an effective and selective method for destroying diseased tissues without damaging the surrounding healthy tissues.[1] The key components of PDT are photosensitizers (PS), light, and tissue oxygen. Usually, the PS is excited from a ground singlet state to an excited singlet state by light. It then undergoes intersystem crossing to a longer-lived excited triplet state. When the PS encounters an oxygen molecule in a tissue, an energy transfer can take place that allows the PS to relax to its ground singlet state, and create an excited $^1O_2$. The $^1O_2$ molecule is an aggressive chemical species and will very rapidly react with any nearby biomolecules. Ultimately, these destructive reactions will result in cell killing (phototoxicity).

Fullerenes have been expected to be very strong PS because the triplet energies of C_{60} and C_{70} are 1.5–1.6 eV which is approximately 0.6–0.7 eV higher than the $^1O_2$ energy,[2,3] and the quantum yields of triplet formation for C_{60} and C_{70} are almost unity.[4–6] Actually, Foote and co-workers found that fullerenes produce $^1O_2$ in large quantities as measured by $^1O_2$ luminescence at $\approx$1270 nm.[4–6] The quantum yield of $^1O_2$ generation is nearly unity for C_{60} and slightly lower for C_{70} in benzene.[4–6] From the high quantum yield of $^1O_2$ generation, the fullerenes are regarded as a potential PS for PDT and other uses,[7] however for biological uses, the lack of solubility of fullerenes in water has been a problem. To confirm the biological activities of photoexcited fullerenes, water-soluble fullerene derivatives were prepared.[8–11] For instance, Nakamura and coworkers reported photodriven DNA cleavage and inhibition of HIV protease and other enzymes by using functionalized C_{60}.[8,9] In their reports, hydrophilic groups such as carboxylic acid groups solubilized fullerenes.[8,9] Another research group led by Kenyon, Wudl, et al. also reported inhibition of certain HIV enzymes by water-soluble fullerene derivatives.[10,11]

However, according to a systematic study by Prat et al. on the relationship between the chemical functionalization of fullerenes and $^1O_2$ generation efficiencies, the $^1O_2$ quantum yield decreases as the area of the conjugated fullerene core decreases.[12] This means that chemical perturbation of the π system of the fullerene changes the photophysical properties substantially. Therefore, the solubilization of fullerene without chemical structure changes is a better approach for PDT applications. Yamakoshi et al. reported biological activities of unmodified fullerenes dissolved in poly(vinylpyrrolidone) (PVP) aqueous solutions.[13–15] More recently, Ikeda et al. reported the introduction of cell death and DNA cleavage by PDT with water-soluble liposomes incorporating unmodified fullerenes.[16,17]

Here we report quantitative evaluation of the $^1O_2$ generation efficiencies of unmodified fullerenes in water, which has not yet been well studied. NIR emission from $^1O_2$ produced by photoexcitation of water-soluble fullerenes (C_{60} and C_{70}) was measured for fullerenes dispersed in water using γ-cyclodextrin (γ-CD) and PVP (Figure 1). The observed emission intensities are directly compared with that of RB under the same experimental conditions. Furthermore, we examine the photo-induced cytotoxicity of the water-soluble fullerenes by WST-1 (2-(4-iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2*H*-tetrazolium monosodium salt) cell proliferation assay using rat fibroblast cell line (5RP7). The results indicate that water-soluble fullerenes have 2–3 times higher $^1O_2$ yields than RB, resulting in the substantial phototoxicities.

Results and Discussion

We first investigated the C_{60} and C_{70} aqueous solutions with γ-CD (Figures 2a and 2b).[18] The optical absorption spectra are

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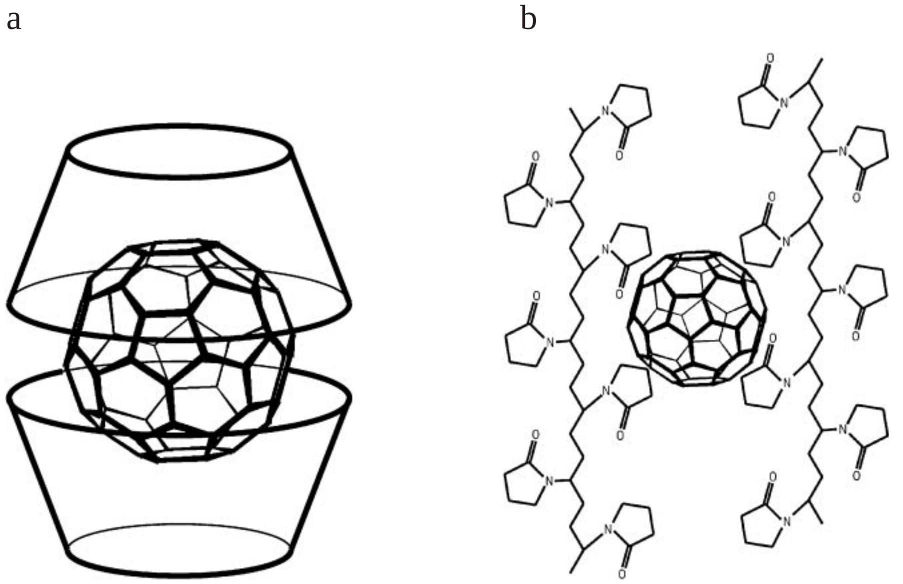

Figure 1. Schematic illustrations of (a) C_{60}–γ-CD and (b) C_{60}–PVP.

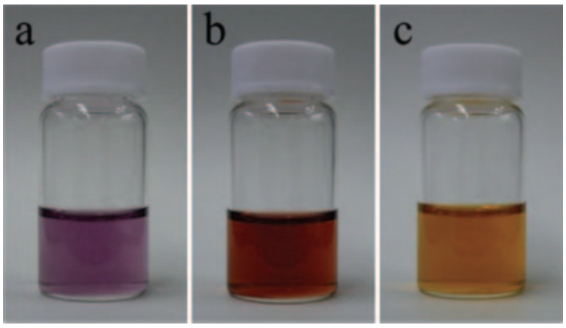

Figure 2. Photographs of (a) C_{60}–γ-CD, (b) C_{70}–γ-CD, and (c) C_{60}–PVP aqueous solutions.

very close to those of the C_{60} and C_{70} in an organic solvent (see Supporting Information), which suggests that C_{60} and C_{70} are dissolved in water as monomers by forming stable complexes with γ-CD.[18,19]

Figure 3a shows a typical NIR emission spectrum corresponding to the $O_2(^1\Delta_g) \rightarrow O_2(^3\Sigma_g^-)$ transition at ≈1275 nm after the photoexcitation of fullerene aqueous solutions.[20,21] To estimate the efficiencies of $^1O_2$ generation, the emission intensities (I) were corrected for the concentration (C) and the molar extinction coefficient (ε), that is the absorbance ($A = \varepsilon C$), of the PS. For instance, Figure 3b shows the obtained I from C_{60}–γ-CD aqueous solution as a function of the excitation wavelength. An excellent agreement between the excitation wavelength dependence of I and the optical absorption spectrum strongly suggests the validity of this correction. The obtained I/A for C_{60}–γ-CD and C_{70}–γ-CD aqueous solutions are listed in Table 1. Each value is an average of more than ten I/A at different excitation wavelengths and normalized to that of RB aqueous solution.

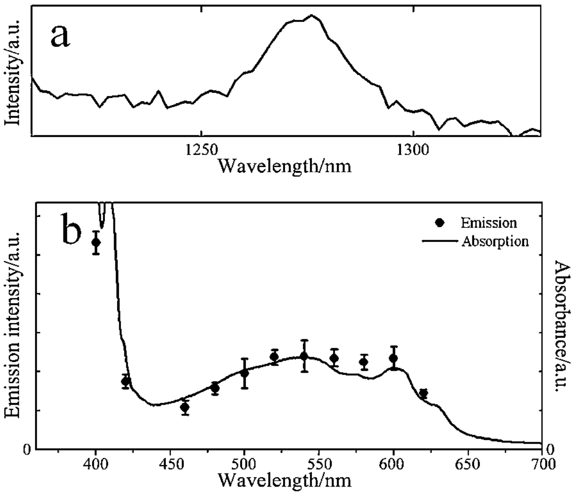

Figure 3. (a) Typical $^1O_2$ emission spectrum by photoexcitation of C_{60}–γ-CD–H_2O. (b) The observed $^1O_2$ emission intensities (I) as a function of the excitation wavelength, together with an optical absorption spectrum of C_{60}–γ-CD–H_2O.

Table 1. The $^1O_2$ Generation Efficiencies (I/A), the Amount of $^1O_2$ Generated under the 633 nm Irradiations (I^{633}) and the Cell Viabilities of Various Photosensitizers

	PS			
	C_{60}–γ-CD	C_{70}–γ-CD	C_{60}–PVP	RB
I/A	2.4 ± 0.3	2.8 ± 0.3	0.7 ± 0.2	1.0 ± 0.1
$I^{633\ \mathrm{a)}}$	1	5.7	0.37	—
Viability/%	66 ± 12	13 ± 10	98 ± 16	—

a) Normalized to C_{60}–γ-CD.

Larger I/A should suggest the higher $^1O_2$ generation efficiency. The I/A values of ≈2.4 and ≈2.8 for C_{60}–γ-CD and C_{70}–γ-CD, respectively, indicate that C_{60} and C_{70} can generate $^1O_2$ with 2–3 times higher efficiencies than RB. Similar I/A values between C_{60} and C_{70} are consistent with the fact that the quantum yields of triplet formation for both C_{60} and C_{70} are almost unity.[4,5] Note that the relative values of 2.4 for C_{60} to RB are significantly smaller than that in the organic solvents (≈4.8)[22] whereas the $^1O_2$ generation efficiency of RB is insensitive to the solvent.[23] This means that the quantum yield of the $^1O_2$ formation from C_{60} in water is lower than that in organic solvent. The decrease of the $^1O_2$ generation efficiencies is presumably attributed to the γ-CD surrounding the fullerene molecules, which may disturb the efficient energy transfer between fullerene and O_2 molecules. A similar trend was observed in a water-soluble fullerodendrimer system.[24] The surrounding dendrimer decreases the encounter rate of $^1O_2$, resulting in the decrease of the $^1O_2$ generation efficiencies.

Sometimes fullerenes are dissolved in water by using water-soluble polymers for biological applications. Especially, PVP has frequently been used for the dispersant.[13–15] Hence we also investigated the $^1O_2$ formation ability of C_{60} in PVP aqueous solution (Figure 2c). In comparison to C_{60}–γ-CD, the $^1O_2$ generation efficiency (I/A) of C_{60}–PVP shows a much smaller value (≈0.7) (Table 1). The lower efficiency can be attributed to the aggregation and clustering of C_{60}. Indeed it is known that the formation of clusters decrease the triplet lifetime of C_{60} by 2–3 orders of magnitude.[25] Evidence for clustering can be obtained by optical absorption spectroscopy.[18,25] The typical fullerene bands at 260 and 330 nm become broader than those of C_{60}–γ-CD (see Supporting Information). The yellow color of the C_{60}–PVP solution is due to this absorption change and strongly suggests that substantial amounts of C_{60} aggregate in the PVP solution (Figure 2c).[25]

We then examined the phototoxicity of C_{60}–γ-CD, C_{70}–γ-CD, and C_{60}–PVP by using rat fibroblast cell line (5RP7). The concentrations of fullerenes were set to be 10 μM for all cases. After 24 h incubation with these solutions, the cells were rinsed with phosphate-buffered saline (PBS) several times and replaced by fresh medium. Then light irradiation at 633 nm (3 mW cm^{-2}) for 1 h was carried out for each cell.

Figure 4 shows laser scanning confocal microscope images after these treatments. Calcein-AM (3′,6′-di(*O*-acetyl)-4′,5′-bis[*N*,*N*-bis(carboxymethyl)aminomethyl]fluorescein tetraacetoxymethyl ester) and PI (3,8-diamino-5-[3-(diethylmethylammonio)propyl]-6-phenylphenanthridinium diiodide) were used to visualize the living (green) and dead (red) cells, respectively. In the C_{60}–γ-CD case (Figure 4a), both living and dead cells were observable after the irradiation. The numbers of cells stained by Calcein-AM and PI seem to be comparable. On the other hand, almost all cells were dead in the C_{70}–γ-CD case (Figure 4b). A big difference between C_{60} and C_{70} is due to the amount of the generated $^1O_2$. In spite of the almost same I/A values (Table 1), C_{70} can more effectively generate $^1O_2$ under these experimental conditions because the molar extinction coefficient at 633 nm (ε^{633}) of C_{70} is ≈4.9 times larger than that of C_{60}. On the contrary, almost all cells were living in the C_{60}–PVP (Figure 4c). This result is consistent with the lower value of I/A for C_{60}–PVP (Table 1).

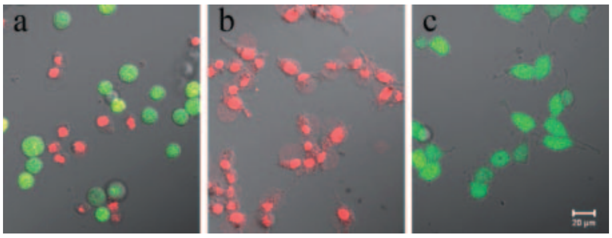

Figure 4. Laser scanning confocal microscope images of 5RP7 cells treated with a) C_{60}–γ-CD, b) C_{70}–γ-CD, and c) C_{60}–PVP aqueous solutions, and irradiated at 633 nm for 1 h. Calcein-AM and PI were added to stain the living (green) and dead (red) cells, respectively.

To evaluate the phototoxicity more quantitatively, we conducted WST-1 assays on these fullerene solutions. Figure 5 shows the cell viabilities after irradiation at 633 nm for 1 h. Compared to the dark (no irradiation) condition, the cell viabilities significantly decrease in the C_{60}–γ-CD and the C_{70}–γ-CD systems after irradiation. The cell viabilities of ≈66 and ≈12% were obtained for C_{60}–γ-CD and C_{70}–γ-CD, respectively, indicating that the PDT activity of C_{70}–γ-CD is ≈2.6 times larger than that of C_{60}–γ-CD in these conditions. On the contrary, there is no essential difference in the cell viability between dark and light conditions in the C_{60}–PVP case.

To investigate the correlation between the observed phototoxicity and the $^1O_2$ generation efficiency, we compare the cell lethality (100-viability (%)) with the amount of $^1O_2$ generated under the 633 nm irradiations (I^{633}) for each water-soluble fullerene (Figure 6). The I^{633} values were estimated by $I^{633} = \varepsilon^{633}(I/A)$ for each solution (Table 1). It is clearly seen that there is a positive correlation between the cell lethality and I^{633}, which indicates that the observed phototoxicity is mainly due to the generated $^1O_2$.

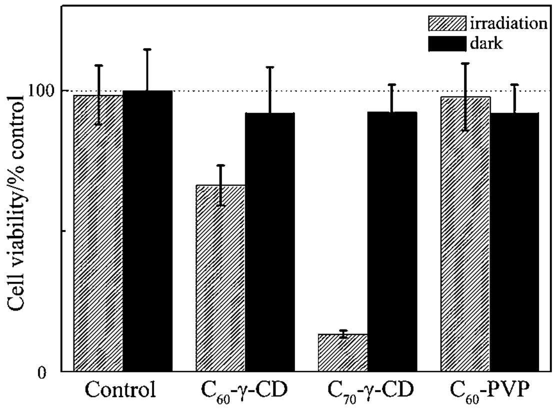

Figure 5. WST-1 assay of 5RP7 cells with C_{60}–γ-CD, C_{70}–γ-CD, and C_{60}–PVP aqueous solutions after 1 h irradiations (633 nm).

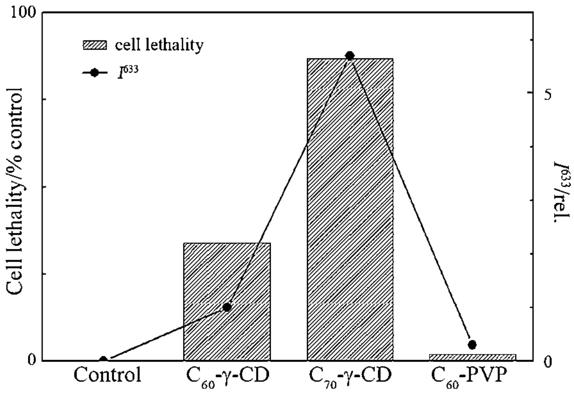

Figure 6. Cell lethality for C_{60}–γ-CD, C_{70}–γ-CD, and C_{60}–PVP, respectively, and the corresponding $^1O_2$ generation (I^{633}).

As application of nanotechnology expands, assessment of the potential risks of nano-materials to human health and the environment becomes more important. The present results show that water-soluble C_{60} and C_{70} have lower toxicities under dark conditions and their phototoxicities strongly depend on the dispersed forms. The current information should be useful for further toxicity studies of nano-materials.

Conclusion

We investigated the $^1O_2$ generation efficiencies of C_{60} and C_{70} in water and directly compared them with that of RB. The obtained results show that C_{60} and C_{70} can generate $^1O_2$ with 2–3 times higher efficiency than RB, but it depends on the water dispersant. Phototoxicity of C_{60} and C_{70} were also examined by WST-1 assay using rat fibroblast cell line (5RP7). The cell viabilities after irradiations are consistent with the amount of generated $^1O_2$ under the experimental condition. The present finding reveals that fullerenes have great potential as PS for PDT because of high $^1O_2$ generation efficiencies and almost no dark toxicity.

Experimental

We here used γ-CD and PVP as water dispersants for fullerenes. C_{60}–γ-CD was prepared by ball milling a mixture containing 10 mg of C_{60} and 120 mg of γ-CD for 90 min at 30 Hz, followed by addition of 5 mL of water.[6] The aqueous solutions containing C_{60}–γ-CD were filtered with a membrane filter (pore size: 0.2 µm). After letting the aqueous solution stand for 3 days, purple crystalline plates separated. C_{60}–γ-CD solution was prepared by dissolving 10 mg of the crystals in 10 mL of water containing 50 mg of γ-CD.

C_{70}–γ-CD was prepared by the same procedure. A mixture containing 5 mg of C_{70} and 50 mg of γ-CD was milled and dissolved in 3 mL of water. C_{70}–γ-CD solution was filtered with a membrane filter (pore size: 0.2 µm).

C_{60}–PVP aqueous solution was prepared as follows. C_{60} (0.72 mg) dissolved in toluene (1.0 mL) was added to 10 mL of $CHCl_3$ containing 100 mg of PVP (K-25). The solution was mixed and the solvent thoroughly evaporated under vacuum. The residue was dissolved in 10 mL of water.

The NIR emission spectra from $^1O_2$ in fullerene aqueous solution after photoexcitation were measured with a Horiba SPEX Fluorolog 3-2 Triax spectrometer equipped with a near-infrared photomultiplier (Hamamatsu H9170-75). A Xe lamp was used for photoexcitations. Typical lamp power was $\approx 0.1\,\mathrm{mW\,mm^{-2}}$. The raw data were corrected for wavelength-dependent instrumental factors and excitation lamp intensities. The emission intensity (I) was obtained by the curve fitting. Optical absorption spectra were recorded with a Shimadzu UV-3150 spectrometer.

RB (Wako Pure Chemical Industries) was used as a reference sample in NIR emission measurements. We also tried methylene blue (MB) (Tokyo Kasei Kogyo) as a reference sample. However, it was difficult to detect the emission spectrum of $^1O_2$ from the MB aqueous solution with a good signal-to-noise ratio because strong fluorescence from MB overlapped with it.

5RP7 cells were obtained from the Health Science Research Resources Bank (HSRRB, Tokyo, Japan) and were cultured in Dulbecco's minimal essential medium (D'MEM) supplemented with 10% fetal bovine serum (FBS) containing 100 units $\mathrm{mL^{-1}}$ penicillin-streptomycin, at 37 °C, 5% of CO_2. Initially, the cells in the log phase were seeded in a 96-well plate at a concentration of 2.0×10^4 cells/well (100-µL cell suspension in culture medium/well). After seeding, the 96-well plates were incubated at 37 °C, 5% CO_2 for 24 h before the fullerenes were treated.

After adding C_{60}–γ-CD, C_{70}–γ-CD, or C_{60}–PVP to the culture medium, the cells were incubated again for 24 h, washed with PBS three times, and replaced by fresh culture medium (100 µL). The concentration of C_{60}–γ-CD, C_{70}–γ-CD, or C_{60}–PVP was set to be 10 µM. The treated cells were irradiated with light ($3\,\mathrm{mW\,cm^{-2}}$) at a wavelength of 633 nm for 60 min.

For laser scanning confocal microscopy, irradiated cells were washed three times with PBS, and replaced by the double staining solution, PBS containing Calcein-AM (0.2 µM) and PI (0.6 µM). After 10 min incubation at 37 °C, 5% CO_2, cells were observed by laser scanning confocal microscope (LSM5 PASCAL, Zeiss) (excitation at 488 and 543 nm; Filter 505–530 nm, 560 nm).

Phototoxicity was measured by WST-1 assay. 10 µL of WST-1 solution was added to each well and incubated at 37 °C for 1 h after irradiation. Then, absorbance values of the samples ($\lambda =$ 450 nm) were measured with Microplate Reader (model 680, Bio-RAD). The relative cell viability was then calculated from the absorbance values (as a percent of the negative control).

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Supporting Information

UV–vis–NIR absorption spectra of C_{60}–γ-CD, C_{70}–γ-CD, and C_{60}–PVP aqueous solutions. This material is available free of charge on the Web at: http://www.csj.jp/journals/bcsj/.

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