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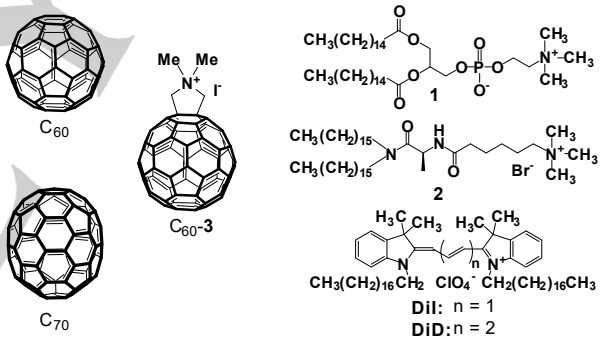
COMMUNICATION

Improvement of Photodynamic Activity of Lipid-Membrane-Incorporated Fullerene Derivative by Combination with a Photo-Antenna Molecule

Daiki Antoku,[a] Shuhei Satake,[a] Tomoya Mae,[a] Kouta Sugikawa,[a] Hisakage Funabashi,[b] Akio Kuroda,[b] and Atsushi Ikeda*[a]

Abstract: The weak absorbance of pristine C_{60}, C_{70}, and fullerene derivatives at wavelengths over 600 nm hampers the use of these molecules as photosensitizers (PSs) for photodynamic therapy (PDT). The coexistence of light-harvesting antenna molecules with a fullerene derivative in lipid membrane bilayers solved this issue. By controlling the location of the C_{60} derivative in the lipid membrane, the liposomal dyad system for PDT improved the photodynamic activity via an efficient photoenergy transfer from antenna molecules to the fullerene derivative. The photodynamic activity was found to be much higher than those of dyad systems using pristine C_{60} and C_{70}.

Photodynamic therapy (PDT) is a next-generation non-invasive treatment for various types of tumors. Cancer cell death is generally induced by the activity of reactive oxygen species (ROS) such as singlet oxygen ($^1O_2$), which are produced by photochemical reactions between photoexcited photosensitizers (PS) and dissolved molecular oxygen.[1] Fullerenes and their derivatives have attracted significant attention as PS of PDT,[2–4] because of the formation of a long-lived triplet excited state and the photoproduction ability of ROS with high quantum yields. Although a lipid-membrane-incorporating C_{60} ($LMIC_{60}$) showed high photodynamic activity toward HeLa cells under photoirradiation between 350–500 nm,[5] the light absorption of C_{60} between 600–700 nm is too low to show photodynamic activity at wavelengths above 600 nm, which are the most suitable wavelengths for PDT. We have reported previously that a solution to this problem is the coexistence of light-harvesting antenna molecules and pristine C_{60} in lipid bilayers by a biomimetic approach of photosynthesis.[6] The photodynamic activity of the dyad systems can be improved by suitable choice of fullerenes and/or antenna molecules. In lipid-membrane-incorporating C_{60} and 1,1'-dioctadecyl-3,3,3',3'-tetramethylindodicarbocyanine (DiD: dialkylated carbocyanine lipid membrane probes) molecules ($LMIC_{60}$-DiD), the quantum yield of energy transfer between DiD and C_{60} was less than 50% because fluorescence quenching of DiD by C_{60} transfer was about 50% in the lipid membranes. There are two explanations for the low quantum yield of the energy transfer: (i) the ability of C_{60} to accept the energy from DiD is low, and (ii) the distance between C_{60} and DiD is too far for the energy transfer to occur and molecular oxygen has to migrate into the membrane to contact the excited C_{60}, because C_{60} is located in the hydrophobic core of the lipid bilayer. In this paper, we overcome these issues by employing C_{70}[7] and C_{60} derivatives[8,9] as fullerenes and combine these derivatives with DiD in lipid membranes.



DiD was used as a light-harvesting antenna molecule because dialkylated carbocyanine lipid membrane probes have no appreciable cytotoxicity[10] and have an absorption maximum (λ_{max}) of 648 nm in liposomes, which matches the optimal wavelength range for PDT. In contrast, 1,1'-dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine (DiI), which is structurally similar to DiD, was employed as a reference antenna molecule because DiI has a λ_{max} at 551 nm and barely absorbs light above 600 nm (Figure S1). LMIfullerene-antenna molecule dyads were prepared via the fullerene exchange method from the γ-cyclodextrin (γ-CDx) cavity to antenna molecule contained liposomes (liposome-antenna molecule), as described previously.[4,8,9] A cationic lipid **2** (Figure 1) was added to improve intracellular uptake of LMIfullerene-antenna molecule dyads.[4b] LMIfullerene-antenna molecule dyads composed of antenna molecules, 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (**1**), lipid **2** and fullerene, were produced in a molar ratio of 1:36:4:2. The ^{1}H NMR peaks assigned to the *γ*-CDx•C_{60}-**3** complex disappeared completely after the C_{60} derivative-exchange reaction (Figures. 1 and S2).[9b] The result indicated that all of the C_{60}-**3** molecules had been released from the *γ*-CDx cavities in the presence of the liposomes. Furthermore, peaks belonging to the guest molecules and the

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lipids are known to disappear completely in these systems as a consequence of peak broadening after the formation of the liposomes. The disappearance of the peaks belonging to the C_{60}-**3** molecules in the current study indicates that all of these molecules were completely incorporated into the liposomes (Figure 1, blue circles). The coexistence of C_{60}-**3** with DiD in the lipid membrane was confirmed by following the quenching of the fluorescence in the spectrum of LMIC_{60}-**3**-DiI. The size distributions of the liposomes were studied using dynamic light scattering (DLS), which measures the average hydrodynamic diameters (D_{hy}) of the LMIfullerene-DiD. Unfortunately the D_{hy} values of LMIfullerene-DiD could not be determined accurately because the absorption of DiD interferes with the laser equipped in the DLS instrument. Therefore, LMIfullerene-DiI was used for DLS measurements (Figure S1). Table S1 shows that the hydrodynamic diameters (D_{hy}) changed from 70–90 nm before the exchange reactions to 71, 62 and 76 nm for LMIC_{60}-DiI, C_{70}-DiI and C_{60}-**3**-DiI, respectively, indicating that the incorporation of C_{60}, C_{70}, and C_{60}-**3** had minimal impact on the size of the liposomes.

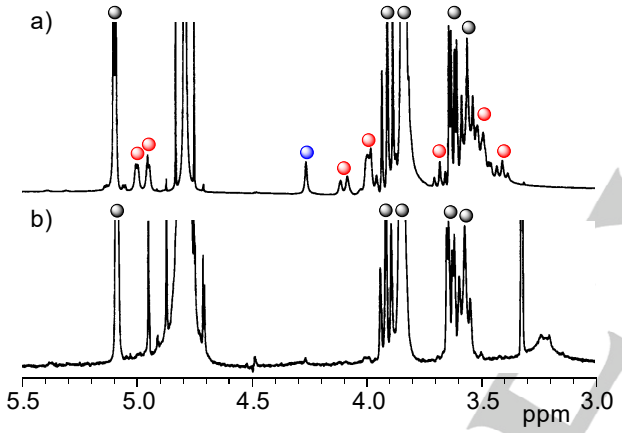


Figure 1. Partial ^{1}H NMR spectra of the C_{60}-**3**•γ-CDx complex a) before and b) after addition of liposome-DiD in D_2O (●: free γ-CDx; ●: γ-CDx in the C_{60}-**3**•γ-CDx complex; ●: C_{60}-**3** in the C_{60}-**3**•γ-CDx complex) [**1**] = 1.0 mM, [C_{60}-**3**]/[**1**] = 5.0 mol%, [DiD]/[**1**] = 2.5 mol%.

The UV-vis absorption spectra of liposome-DiD and LMIC_{60}-**3**-DiD were compared (Figure 2, blue and red lines). Although the absorption maximum of DiD did not shift in the presence of C_{60}-**3**, peaks of C_{60}-**3** in LMIC_{60}-**3**-DiD sharpened when compared with those in LMIC_{60}-**3** (Figure 2, red and green lines). The result indicates that C_{60}-**3** units are isolated and self-aggregation is prevented by DiD in the lipid membrane, but the C_{60}-**3**-DiD interaction scarcely exists in the ground state. The control of the self-aggregation of C_{60}-**3** units leads to a high quantum yield for the energy transfer between C_{60}-**3** and DiD because of the suppression of self-quenching of excited C_{60}-**3**.[4d] The fullerene-dependent fluorescence quenching of DiD was analyzed to confirm that the photon energy absorbed by DiD antenna molecules was transferred to fullerene in LMIfullerene-DiD (Figure 3). The fluorescence quenching values by C_{60}, C_{70}, and C_{60}-**3** were estimated to be 55, 59, and 87%, respectively, indicating that the light energy absorbed by DiD is more efficiently transferred to C_{60}-**3** when compared with that of C_{60} and C_{70}. Furthermore, the result strongly supports the coexistence of C_{60}-**3** with DiD in the lipid membrane. We suggest two possible explanations for the different fluorescence quenching values for C_{60}, C_{70}, and C_{60}-**3**: (i) the energy transfer efficiency between C_{60}-**3** and DiD is higher than those between C_{60} or C_{70} and DiD and (ii) the distance between C_{60}-**3** and DiD is shorter than those between C_{60} or C_{70} and DiD (Scheme 1). To test explanation (i), we compared the fluorescence quenching values of DiD in aqueous solutions by the γ-CDx•C_{60}, C_{70}, and C_{60}-**3** complexes in the absence of liposomes (Figure 3b). The value of C_{60} (78%) was higher than that of C_{60}-**3** (61%), indicating that the primary quenching effect of C_{60} is higher than that of C_{60}-**3**. This result showed that explanation (i) is incorrect. Thus, these results indicate that explanation (ii) is the main reason because C_{60} is buried in the central area of the lipid bilayer membrane (Scheme 1a),[11] whereas C_{60}-**3** is located at the surface of the lipid membrane (Scheme 1c).[9b]

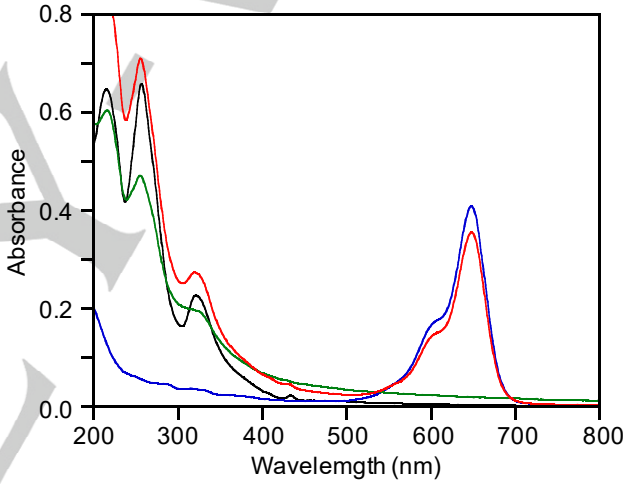
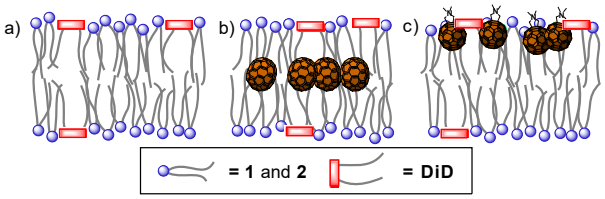


Figure 2. UV-vis absorption spectra of the C_{60}-**3**•γ-CDx complex (black line), liposome-DiD (blue line), LMIC_{60}-**3** (green line), and LMIC_{60}-**3**-DiD (red line) in water (1 mm cell, 25 °C, [**1**] = 1.0 mM, [C_{60}-**3**]/[**1**] = 5.0 mol%, [DiD]/[**1**] = 2.5 mol%).



Scheme 1. Schematic illustrations of a) C_{60}, b) C_{70}, and c) C_{60}-**3** in liposome-DiD

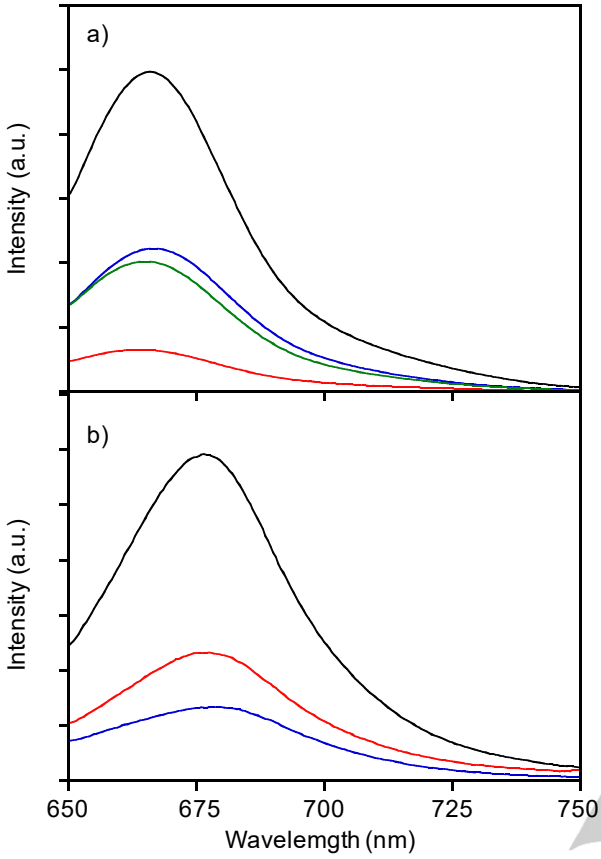


Figure 3. Fluorescence spectra (λ_{ex} 630 nm) of a) liposome-DiD (black line), $LMIC_{60}$-**3**-DiD (red line), $LMIC_{60}$-DiD (blue line), and $LMIC_{70}$-DiD (green line) ([**1**] = 0.1 mM, [C_{60}-**3**]/[**1**] = 5.0 mol%, [DiD]/[**1**] = 2.5 mol%), and b) DiD (black line), DiD with the C_{60}-**3**•γ-CDx complex (red line), and DiD with the C_{60}•γ-CDx complex (blue line) in water ([DiD] = 2.5 μM, [C_{60}] = [C_{60}-**3**] = 50.0 μM).

We investigated the levels of generated cytotoxic $^1O_2$ by liposomal PS under visible-light irradiation at a wavelength greater than 620 nm. We have reported that fullerenes and their derivatives transfer energy to $^3O_2$ to give singlet oxygen molecules ($^1O_2$) (energy-transfer pathway Type II).[6,9b] The $^1O_2$ generation ability was studied through the photoreaction between anthracene and $^1O_2$. As shown in Scheme 1, conversion to an endoperoxide from 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) by reaction with $^1O_2$ leads to a decrease in absorbance at 400 nm (Figures S3).[4e,12] The absorption level of ABDA at 400 nm (the absorption maximum of ABDA) was plotted as a function of the photoirradiation time of the liposomal PS in O_2-saturated aqueous solutions (Figure 4). Although we have reported that $LMIC_{60}$-**3** is capable of generating high levels of $^1O_2$, much higher levels of $^1O_2$ were generated in $LMIC_{60}$-**3**-DiD (Figure 4, red line) when compared with that of $LMIC_{60}$-**3** (Figure 4, black line). The result suggests that the energy transfer from photoactivated antenna molecules to C_{60}-**3** occurs efficiently within liposomes. To confirm the energy transfer, the level of $^1O_2$ generated by $LMIC_{60}$-**3**-DiI was measured under light-irradiation at a wavelength over 620 nm because DiI has a λ_{max} at 551 nm and barely absorbs light above 600 nm (Figure 4, purple line). The result shows that the level of $^1O_2$ generation by $LMIC_{60}$-**3**-DiI is equal to that of $LMIC_{60}$-**3**, indicating that DiI scarcely acted as the antenna molecule. When DiI absorbs light under irradiation over 500 nm, $LMIC_{60}$-**3**-DiI functioned as a dyad system (Figure 4, orange line). In contrast, the levels of $^1O_2$ generation by $LMIC_{60}$-DiD and $LMIC_{70}$-DiD (Figure 4, blue and green lines) were much lower than that of $LMIC_{60}$-**3**-DiI. The results are consistent with the fluorescence quenching results. Furthermore, we have reported that a large number of $^1O_2$ are produced by an effective energy transfer process from the excited C_{60}-**3** molecules to the dissolved oxygen molecules because of the large number of available collisions, and by the existence of C_{60}-**3** on the hydrophilic surface of liposomes (Scheme 1c).[9b]

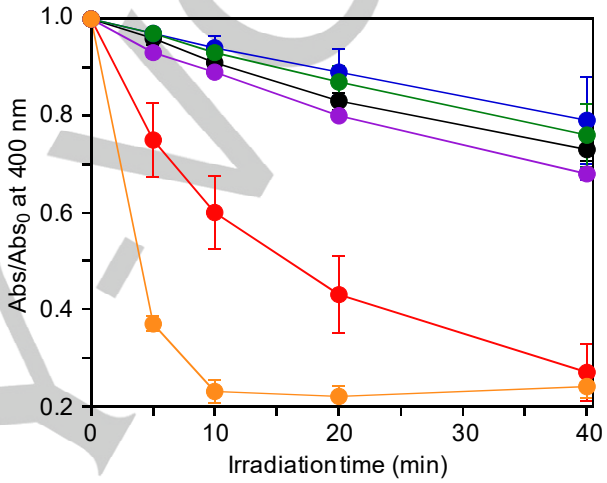


Figure 4. Time-dependent bleaching of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) caused by singlet oxygen generated from $LMIC_{60}$-**3** (black line), $LMIC_{60}$-**3**-DiD (red line), $LMIC_{60}$-DiD (blue line), $LMIC_{70}$-DiD (green line), $LMIC_{60}$-**3**-DiI (purple line) upon photoirradiation (> 620 nm, 15 mW cm^{-2}), and $LMIC_{60}$-**3**-DiI (orange line) upon photoirradiation (> 500 nm, 15 mW cm^{-2}). A DMSO solution of ABDA was injected into an aqueous solution of the liposomes. Changes in the ABDA absorption at 400 nm were monitored as a function of time (Abs_0: initial absorbance). [**1**] = 0.3 mM, [C_{60}-**3**, C_{60}, or C_{70}]/[**1**] = 5.0 mol%, [DiD or DiI]/[**1**] = 2.5 mol%, [ABDA] = 25 μM: under an oxygen atmosphere at 25 °C. All data represent the mean values of three independent experiments. Error bars represent the standard deviations.

The photodynamic activity of $LMIC_{60}$-**3**-DiD using human cervical cancer HeLa cells was evaluated. Following incubation with $LMIC_{60}$-DiD, $LMIC_{70}$-DiD, and $LMIC_{60}$-**3**-DiD, the cells were exposed to light with wavelengths longer than 610 nm (610–740 nm), at which the light was absorbed by DiD. Using the WST-8 assay, cell viability was measured in light irradiated and un-irradiated cells as a ratio (%) compared with untreated cells. The results showed that no samples had dark toxicity, even at the highest concentrations used (Figure 5). Moreover, $LMIC_{60}$-DiD, $LMIC_{70}$-DiD, and $LMIC_{60}$-**3**-DiD reduced the viability of HeLa cells in a photoirradiated-dependent manner. These photodynamic activities of $LMIC_{60}$-DiD, $LMIC_{70}$-DiD, and $LMIC_{60}$-**3**-DiD were drug dose-dependent and the medium inhibitory concentrations (IC_{50} value) were estimated to be ca. 8.0, 7.2, and 0.87 μM of fullerenes (Figure 5). The much higher photodynamic activity of $LMIC_{60}$-**3**-DiD compared with that of $LMIC_{60}$-DiD and $LMIC_{70}$-DiD is considered to be due to the high $^1O_2$ generation ability of

LMIC$_{60}$-**3**-DiD because the intracellular uptake by HeLa cells are similar as the same cationic liposomes were used as drug carriers. The IC_{50} value of LMIC$_{60}$-**3**-DiD was lower than that of photofrin (Figure S4a), which gave an IC_{50} value of 3.02 μM[13] under the same conditions (Figure S4b) when the number of moles was converted to the number of porphyrin units, because photofrin consists of porphyrin oligomers containing two to eight units (Figure S4a). These results therefore revealed that the photodynamic activity of LMIC$_{60}$-**3**-DiD was approximately 3.5 times higher than that of photofrin, currently the main drug in clinical use as a photosensitizer.[14] Furthermore, in the absence of the antenna molecule, LMIC$_{60}$-**3** gave an IC_{50} value of 1.24 μM[13] under the same conditions. Although the value of LMIC$_{60}$-**3** was higher than that of LMIC$_{60}$-**3**-DiD (1.4 times), the difference between the two values was lower than that predicted by the levels of $^1O_2$ generated (Figure 4). This observation is probably because C$_{60}$-**3** separates from DiD by collapse of a part of the liposomes in HeLa cells. If the separation between C$_{60}$-**3** and DiD occurs, the fluorescence of DiD will be observed in HeLa cells after intracellular uptake. We used DiI instead of DiD because the lamp wavelength is too short to excite DiD in a fluorescence microscope. After incubation of the cells with liposome-DiI or LMIC$_{60}$-**3**-DiI at a DiI concentration of 1.0 μM on a glass dish for 24 h in air with 5% CO_2 at 37 °C, the fluorescence intensity of LMIC$_{60}$-**3**-DiI was considerably weaker than that of liposome-DiI (Figure 6). This result suggests that the majority of LMIC$_{60}$-**3**-DiD remains in a stable formation in HeLa cells, with some minor release of DiD from the liposomes. In the future, more stable liposomes should be used to realize PDT systems with higher photodynamic activity. For example, liposomes composed of dipalmitoylphosphatidylcholine, which has a higher phase transition temperature than **1**.

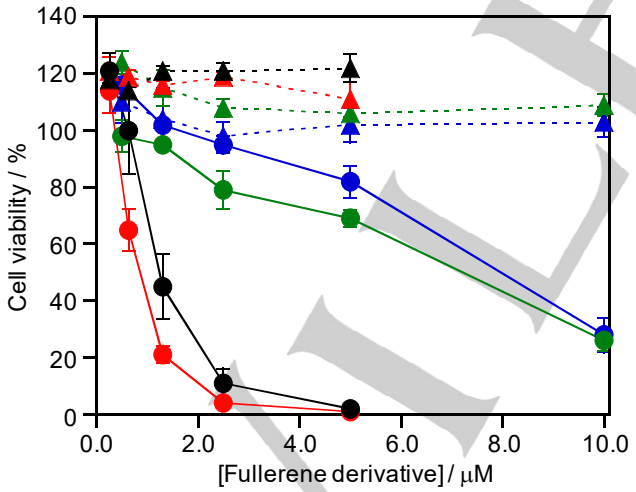


Figure 5. Cell viability with the LMIC$_{60}$-**3** (black dotted line), LMIC$_{60}$-**3**-DiD (red dotted line), LMIC$_{60}$-DiD (blue dotted line), and LMIC$_{70}$-DiD (green dotted line). In the dark and LMIC$_{60}$-**3** (black solid line), LMIC$_{60}$-**3**-DiD (red solid line), LMIC$_{60}$-DiD (blue solid line), and LMIC$_{70}$-DiD (green solid line). Samples were photoirradiation (610–740 nm) for 30 min at different concentrations. Cell viability was confirmed by the WST-8 method. Error bars represent the mean ± standard deviation (SD) for n = 3.

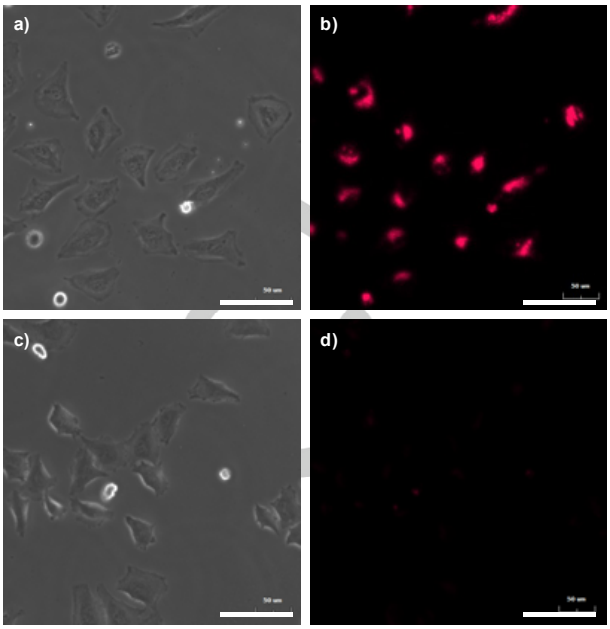


Figure 6. Phase contrast (a and c) and fluorescence (b and d) images of HeLa cells after treatment with (a and b) liposome-DiI and (c and d) LMIC$_{60}$-**3**-DiI for 24 h at 37 °C. The scale bar represents 100 μm.

In summary, we demonstrated that dyad systems comprising DiD as a light-harvesting pigment and fullerene as an energy transfer medium showed high photodynamic activities in liposomal membranes. We confirmed that LMIC$_{60}$-**3**-DiD acts as a dyad system because: (i) the fluorescence quenching of DiD by C$_{60}$-**3** was observed strongly in LMIC$_{60}$-**3**-DiD and (ii) LMIC$_{60}$-**3**-DiI without absorbance over 600 nm generates a much lower level of $^1O_2$ than LMIC$_{60}$-**3**-DiD under visible-light irradiation at a wavelength greater than 620 nm. The photodynamic activity of LMIC$_{60}$-**3**-DiD toward HeLa cells was much higher than those of LMIC$_{60}$-DiD and LMIC$_{70}$-DiD. The main reason for this higher photodynamic activity is probably because of the high $^1O_2$ generation ability of LMIC$_{60}$-**3**-DiD. That is, both energy transfers between fullerenes and light-harvesting molecules and between fullerenes and dissolved oxygen occur readily in LMIC$_{60}$-**3**-DiD because the distance between C$_{60}$-**3** and DiD or dissolved oxygen is shorter than those in C$_{60}$ or C$_{70}$ due to the location of C$_{60}$-**3** in the neighborhood of the liposomal surface.

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Keywords: fullerenes • liposomes • energy transfer • photodynamic therapy • photosensitizers

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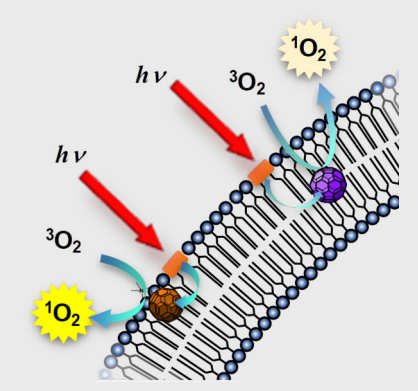
Entry for the Table of Contents

COMMUNICATION

A dyad system consisting of light-harvesting antenna molecules and a fullerene derivative coexisting in a lipid membrane bilayer displays high photodynamic activity toward human cancer cells, because both energy transfers between the fullerene derivative and the antenna molecules and between the fullerene derivative and dissolved oxygen occur readily in the photosensitizer using the dyad system.



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Page No. – Page No.

Improvement of Photodynamic Activity of Lipid-Membrane-Incorporated Fullerene Derivative by Combination with a Photo-Antenna Molecule