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<td><strong>Auther(s)</strong></td>
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<tr>
<td><strong>Citation</strong></td>
<td>Chemistry - A European Journal, 24 (29): 7335 - 7339</td>
</tr>
<tr>
<td><strong>Issue Date</strong></td>
<td>2018-05-23</td>
</tr>
<tr>
<td><strong>DOI</strong></td>
<td>10.1002/chem.201800674</td>
</tr>
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<td><strong>Self DOI</strong></td>
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<tr>
<td><strong>URL</strong></td>
<td><a href="http://ir.lib.hiroshima-u.ac.jp/00046213">http://ir.lib.hiroshima-u.ac.jp/00046213</a></td>
</tr>
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D. Antoku, S. Satake, T. Mae, K. Sugikawa, H. Funabashi, A. Kuroda, A. Ikeda, Improvement of Photodynamic Activity of Lipid-Membrane-Incorporated Fullerene Derivative by Combination with a Photo-Antenna Molecule, Chem.-Eur. J., 24, 7335-7339, 2018, which has been published in final form at https://doi.org/10.1002/chem.201800674. This article may be used for non-commercial purposes in accordance with Wiley Terms and Conditions for Use of Self-Archived Versions.  
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Improvement of Photodynamic Activity of Lipid-Membrane-Incorporated Fullerene Derivative by Combination with a Photo-Antenna Molecule


**Abstract:** The weak absorbance of pristine C₆₀, C₇₀, 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 C₆₀ 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 C₆₀ and C₇₀.

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 (¹O₂), which are produced by photochemical reactions between photoexcited photosensitizers (PS) and dissolved molecular oxygen.[1] Fullerene 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 C₆₀ (LMIC₆₀) showed high photodynamic activity toward HeLa cells under photoirradiation between 350‒500 nm,[5] the light absorption of C₆₀ between 600‒700 nm is too low to show photodynamic activity (PS) and dissolved molecular oxygen. [1] Fullerenes and their photochemical reactions between photoexcited photosensitizers have a potential approach of photosynthesis.[6] The photodynamic activity of the molecules and pristine C₆₀ in lipid bilayers by a biomimetic approach of photosynthesis.[8,9] The photodynamic activity of the dyad systems can be improved by suitable choice of fullerenes and/or antenna molecules. In lipid-membrane-incorporating C₆₀ and 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine (DiD: dialkylated carbocyanine lipid membrane probes) molecules (LMIC₆₀-DiD), the quantum yield of energy transfer between DiD and C₆₀ was less than 50% because fluorescence quenching of DiD by C₆₀ 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 C₆₀ to accept the energy from DiD is low, and (ii) the distance between C₆₀ and DiD is too far for the energy transfer to occur and molecular oxygen has to migrate into the membrane to contact the excited C₆₀, because C₆₀ is located in the hydrophobic core of the lipid bilayer. In this paper, we overcome these issues by employing C₇₀[7] and C₆₀ 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[8,9] and have an absorption maximum (λₘₐₓ) of 648 nm in liposomes, which matches the optimal wavelength range for PDT. In contrast, 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindodicarbocyanine (DiI), which is structurally similar to DiD, was employed as a reference antenna molecule because DiI has a λₘₐₓ 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.[9] 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 ¹H NMR peaks assigned to the γ-CDx×C₆₀ complex disappeared completely after the C₆₀ derivative-exchange reaction (Figures 1 and S2).[8,9] The result indicated that all of the C₆₀ 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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[Figure 1] was added to improve intracellular uptake of LMIFullerene-antenna molecule dyads.[9] 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 ¹H NMR peaks assigned to the γ-CDx×C₆₀ complex disappeared completely after the C₆₀ derivative-exchange reaction (Figures 1 and S2).[8,9] The result indicated that all of the C₆₀ molecules had been released from the γ-CDx cavities in the presence of the liposomes. Furthermore, peaks belonging to the guest molecules and the
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-DiD. The size distributions of the liposomes were studied using dynamic light scattering (DLS), which measures the average hydrodynamic diameters (D_{hy}) of the LMIC_{60}-3-DiD. Unfortunately the D_{hy} values of LMICfullerene-DiD could not be determined accurately because the absorption of DiD interferes with the laser equipped in the DLS instrument. Therefore, LMICfullerene-DiD 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.

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. 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 LMICfullerene-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), whereas C_{60}-3 is located at the surface of the lipid membrane (Scheme 1c).
photoactivated antenna molecules to C60-line). The result suggests that the energy transfer from much higher levels of 1O2 were generated in LMIC60-generated by LMIC 60- and barely absorbs light above 600 nm (Figure 4, purple line). The result shows that the level of 1O2 generation by LMIC 60-DiD is equal to that of LMIC60-3, indicating that DII scarcely acted as the antenna molecule. When DII absorbs light under irradiation over 500 nm, LMCIC60-3-Dill functioned as a dyad system (Figure 4, orange line). In contrast, the levels of 1O2 generation by LMCIC60-DID and LMCIC70-DID (Figure 4, blue and green lines) were much lower than that of LMCIC60-3-Dill. The results are consistent with the fluorescence quenching results. Furthermore, we have reported that a large number of 1O2 are produced by an effective energy transfer process from the excited C60-molecules to the dissolved oxygen molecules because of the large number of available collisions, and by the existence of C60 on the hydrophilic surface of liposomes (Scheme 1c).[6b]

We investigated the levels of generated cytotoxic 1O2 by liposomal PS under visible-light irradiation at a wavelength greater than 620 nm. We have reported that fullerences and their derivatives transfer energy to 1O2 to give singlet oxygen (1O2) (energy-transfer pathway Type II).[6a,b] The 1O2 generation ability was studied through the photoreaction between anthracene and 1O2. As shown in Scheme 1, conversion to an endoperoxide from 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) caused by singlet oxygen generated from LMIC60-3, LMIC60-3-DIff and LMIC70-3-DIff (blue line), LMIC60-3-DIff (green line), LMIC60-3-Dlff (purple line) upon phototiirradiation (> 620 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/[Abs0: initial absorbance]).[6e,12] The absorption level of ABDA at 400 nm, at which the light was absorbed by DiD, was measured under light-irradiation over 610 nm (610–740 nm), at which the light was absorbed by DiD. The results showed that no samples had dark toxicity, even at the highest concentrations used (Figure 5). Moreover, LMIC60-3-DID, LMIC70-3-DID, and LMCIC60-3-DID reduced the viability of HeLa cells in a phototiirradiated-dependent manner. These photodynamic activities of LMIC60-3-DID, LMIC70-3-DID, and LMCIC60-3-DID were drug dose-dependent and the medium inhibitory concentrations (IC50 value) were estimated to be ca. 8.0, 7.2, and 0.87 μM of fullerences.

The photodynamic activity of LMCIC60-3-DID using human cervical cancer HeLa cells was evaluated. Following incubation with LMCIC60-DID, LMIC70-DID, and LMCIC60-3-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, LMIC60-3-DID, LMIC70-3-DID, and LMCIC60-3-DID reduced the viability of HeLa cells in a phototiirradiated-dependent manner. These photodynamic activities of LMIC60-3-DID, LMIC70-3-DID, and LMCIC60-3-DID were drug dose-dependent and the medium inhibitory concentrations (IC50 value) were estimated to be ca. 8.0, 7.2, and 0.87 μM of fullerences (Figure 5). The much higher photodynamic activity of LMCIC60-3-DID compared with that of LMCIC60-DID and LMCIC70-DID is considered to be due to the high 1O2 generation ability of fullerences. We investigated the levels of generated cytotoxic 1O2 by liposomal PS under visible-light irradiation at a wavelength greater than 620 nm. We have reported that fullerences and their derivatives transfer energy to 1O2 to give singlet oxygen (1O2) (energy-transfer pathway Type II).[6a,b] The 1O2 generation ability was studied through the photoreaction between anthracene and 1O2. As shown in Scheme 1, conversion to an endoperoxide from 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) caused by singlet oxygen generated from LMIC60-3, LMIC60-3-DIff and LMIC70-3-DIff (blue line), LMIC60-3-DIff (green line), LMIC60-3-Dlff (purple line) upon phototiirradiation (> 620 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/[Abs0: initial absorbance]).[6e,12] The absorption level of ABDA at 400 nm, at which the light was absorbed by DiD, was measured under light-irradiation over 610 nm (610–740 nm), at which the light was absorbed by DiD. The results showed that no samples had dark toxicity, even at the highest concentrations used (Figure 5). Moreover, LMIC60-3-DID, LMIC70-3-DID, and LMCIC60-3-DID reduced the viability of HeLa cells in a phototiirradiated-dependent manner. These photodynamic activities of LMIC60-3-DID, LMIC70-3-DID, and LMCIC60-3-DID were drug dose-dependent and the medium inhibitory concentrations (IC50 value) were estimated to be ca. 8.0, 7.2, and 0.87 μM of fullerences (Figure 5). The much higher photodynamic activity of LMCIC60-3-DID compared with that of LMCIC60-DID and LMCIC70-DID is considered to be due to the high 1O2 generation ability of fullerences.
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\textsuperscript{[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.\textsuperscript{[14]} Furthermore, in the absence of the antenna molecule, LMIC_{60}-3 gave an IC_{50} value of 1.24 μM\textsuperscript{[13]} under the same conditions. The IC_{50} 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} separates from DiD by collapse of a part of the liposomes in HeLa cells. If the separation between C_{60} and DiD occurs, the fluorescence of DiD will be observed in HeLa cells after intracellular uptake. We used Dil instead of DiD because the lamp wavelength is too short to excite DiD in a fluorescence microscope. After incubation of the cells with liposome-Dil or LMIC_{60}-3-Dil at a Dil concentration of 1.0 μM on a glass dish for 24 h in air with 5% CO\textsubscript{2} at 37 °C, the fluorescence intensity of LMIC_{60}-3-Dil was considerably weaker than that of liposome-Dil (Figure 6). This result suggests that the majority of LMIC_{60}-3-Dil 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}-Dil (blue dotted line), and LMIC_{70}-Dil (green dotted line). In the dark and LMIC_{60}-3 (black solid line), LMIC_{60}-3-DiD (red solid line), LMIC_{60}-Dil (blue solid line), and LMIC_{70}-Dil (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-Dil and (c and d) LMIC_{60}-3-Dil 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} was observed strongly in LMIC_{60}-3-DiD and (ii) LMIC_{60}-3-Dil without absorbance over 600 nm generates a much lower level of 1O_{2} than LMIC_{60}-3-Dil under visible-light irradiation at a wavelength greater than 620 nm. The photodynamic activity of LMIC_{60}-3-Dil toward HeLa cells was much higher than those of LMIC_{60}-Dil and LMIC_{70}-Dil. 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 fullerences and light-harvesting molecules and between fullerences and dissolved oxygen occur readily in LMIC_{60}-3-Dil because the distance between C_{60} and DiD or dissolved oxygen is shorter than those in C_{60} or C_{70} due to the location of C_{60} in the neighborhood of the liposomal surface.

**Acknowledgments**

This work was supported by a JSPS KAKENHI Grant-in-Aid for Scientific Research (B) (Grant No. JP16H04133) and a Grant-in-Aid for Challenging Exploratory Research (Grant No. JP16K13982). We thank the Edanz Group (www.edanzediting.com/ac) for editing a draft of this manuscript.

**Keywords:** fullerences • liposomes • energy transfer • photodynamic therapy • photosensitizers
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