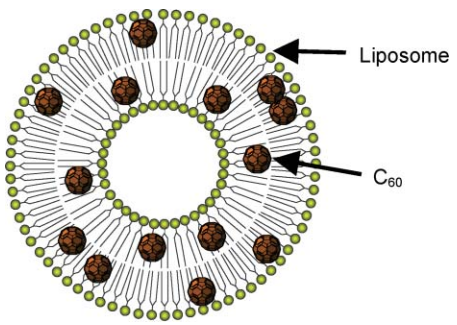
Induction of cell death by photodynamic therapy with water-soluble lipid-membrane-incorporated [60]fullerene†

Atsushi Ikeda,*[a] Yuki Doi,[a] Koji Nishiguchi,[a] Keiko Kitamura,[a] Mineo Hashizume,[a] Jun-ichi Kikuchi,[a] Keiichiro Yogo,[b] Takuya Ogawa[b] and Tatsuo Takeya[b]

Received 5th February 2007, Accepted 5th March 2007
First published as an Advance Article on the web 16th March 2007
DOI: 10.1039/b701767g

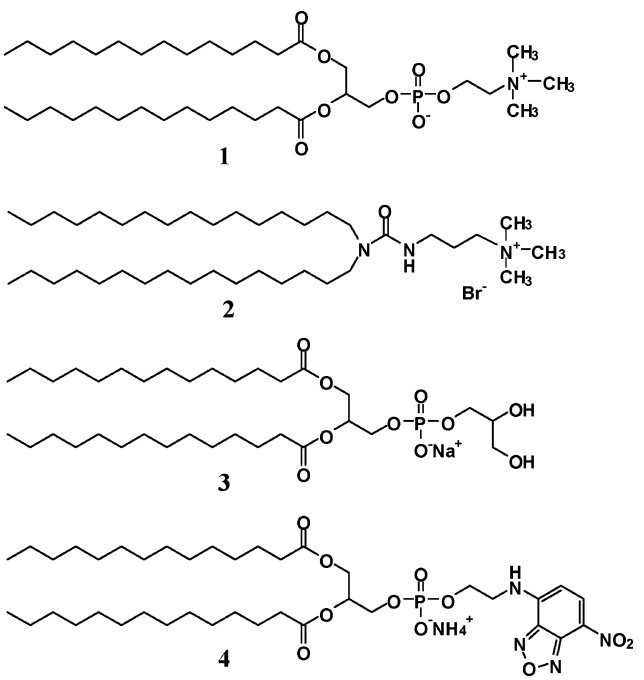
Intracellular uptake of a lipid-membrane-incorporated C_{60} with a cationic surface into HeLa cells was found to induce cell death under visible light irradiation in high efficiency.

Recently, liposomes have been of great interest as materials for drug delivery systems (DDSs), such as photodynamic therapy (PDT) and in gene therapy.[1–3] Advantages of utilising liposomes include the lack of immune and/or inflammatory responses, lower cost, and ease of large-scale manufacture. Moreover, because most photosensitising drugs (PSs) are hydrophobic, *e.g.* porphyrin derivatives,[4] the liposomes act as solubilising agents for the drugs. Although most sensitisers currently under clinical evaluation for PDT are porphyrins or porphyrin-based molecules,[3] the combination of absorption of visible light by fullerenes and the formation of a long-lived triplet state allows fullerenes to act as PSs.[5] Therefore, we employed lipid-membrane-incorporated C_{60}[6,7] (LMI[60]fullerene; Scheme 1) as PSs for three reasons: (i) unmodified C_{60} in vesicles can generate $^1O_2$ through energy transfer or a C_{60} anion radical by electron transfer more efficiently than other chemically modified C_{60} derivatives;[8,9] (ii) various vesicles with positively charged, negatively charged, non-ionic and zwitterionic surfaces can be prepared through a selection of lipids such as phospholipids, aminolipids and glycolipids, which might confer a function as a drug carrier;[10] and (iii) large vesicle formation is promising for enhanced permeability and retention (EPR) effects.[5,11] Recently, we reported that various types of LMI[60]fullerene with 'high C_{60} concentrations' are readily prepared in several hours using the C_{60} exchange method from the γ-cyclodextrin (γ-CDx) cavity to vesicles.[6] Furthermore, this method can be treated as a homogeneous system which requires no separate incorporated and insoluble C_{60} by gel exclusion chromatography because all C_{60} are transferred from the γ-CDx cavity to lipid membranes to yield vesicle-incorporated C_{60}. The DNA photocleaving activities of these LMI[60]fullerenes depend on the surface charges of their vesicles. Particularly, the photocleaving activity of cationic LMI[60]fullerenes was considerably higher than that of the C_{60}·γ-CDx complex. In this study, we assayed biological activities of various types of LMI[60]fullerenes under visible light irradiation.



Scheme 1 Scheme of the LMI[60]fullerene structure.

All liposomes were prepared through sonication of an aqueous dispersion of dimyristoylphosphatidylcholine (**1**) only, **1** and a cationic lipid **2** or an anionic lipid **3** in a 9 : 1 molar ratio with a cup-type sonicator at 50 W for 1 h. All LMI[60]fullerenes were prepared using an exchange reaction between the liposomes and the C_{60}·γ-CDx complex[12] by heating at 80 °C for 1 h, as described in previous works.[6,13] Size distributions of the liposomes were studied using dynamic light scattering (DLS). Table S1 (see ESI †) shows the average diameters of all liposomes before and after the exchange reaction of C_{60}. Final concentrations of the respective components were evaluated using integral intensities of their ^1H



[a]*Graduate School of Materials Science, Nara Institute of Science and Technology, Ikoma, Nara, 630-0192, Japan. E-mail: aikeda@ms.naist.jp; Fax: +81 743 72 6099; Tel: +81 743 72 6099*
[b]*Graduate School of Biological Sciences, Nara Institute of Science and Technology, Ikoma, Nara, 630-0192, Japan*

† Electronic supplementary information (ESI) available: Average particle sizes, detection of $^1O_2$ generation by a chemical method, cell% stained by PI, fluorescence microscopy. See DOI: 10.1039/b701767g

NMR spectra, where [γ-CDx] = 1.02 mM, [C_{60}] = 0.10 mM and [lipids] = 1.00 mM (γ-CDx–C_{60}–lipids = 10.2 : 1 : 10). On the other hand, lipid-membrane-incorporated fluorochromes (LMIF) were prepared using lipids containing 0.5 mol% **4**.[14]

We confirmed $^1O_2$ generation using a chemical method with the disodium salt of 9,10-anthracenedipropionic acid as a detector.[15] The decrease in absorbance at 400 nm (absorption maximum for disodium salt of 9,10-anthracenedipropionicacid) is shown in Fig. S1 (see ESI†), in [lipids] = 146 μM of LMI[60]fullerenes of **1** + **2**, **1** and **1** + **3**, as a function of the time of light exposure. The plots for the LMI[60]fullerenes of **1** + **2**, **1** and **1** + **3** showed decreased absorbance concomitant with the increased time of light exposure, indicating the generation of $^1O_2$ with efficiency in each of the LMI[60]fullerenes.[16]

The intracellular uptake of LMIFs by HeLa cells was observed with fluorescence microscopy at the emission wavelength of fluorochrome. The HeLa cells were incubated with LMIFs of **1** + **2**, **1** and **1** + **3** at a lipid concentration of 50 μM on 24-well culture plates for 24 h in air containing 5% CO_2 at 37 °C (Fig. 1). Consequently, one can conclude that only the 'cationic' LMIF of **1** + **2** delivers a fluorochrome into HeLa cells. On the other hand, the 'neutral' and 'anionic' LMIFs of **1** and **1** + **3** cannot deliver a fluorochrome into HeLa cells (Fig. 1d–1f). Efficiency of incorporation of LMIFs of **1** + **2** to HeLa cells was 100%, as judged by flow cytometric analysis (data not shown). Similar results were obtained in a study for the 'neutral' C_{60}·γ-CDx complex and the 'neutral' C_{60}·PVP complex. Effects of the surface densities were consistent with those described in previous reports.[17]

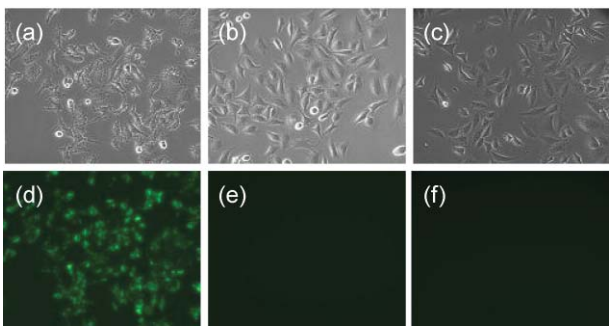


Fig. 1 Phase contrast (a–c) and fluorescence (d–f) images of LMIFs in HeLa cells. (a) and (d): LMIFs of **1** + **2**, (b) and (e): LMIFs of **1**, (c) and (f): LMIFs of **1** + **3**.

These LMI[60]fullerenes were evaluated in culture for photodynamic activity toward HeLa cells. Cell cultures were incubated for 24 h in the dark with [lipids] = 50 μM of LMI[60]fullerenes of **1** + **2**, **1** and **1** + **3** on 35 mm culture plates with 5% CO_2. After incubation, the plates were rinsed with sterile PBS and replaced by fresh medium. The plates were immediately exposed to light at 350–500 nm for 2 h each. The power at the cell level was 19 mW cm^{-2}. Cells treated with the LMI[60]fullerene of **1** + **2** showed abnormal shape after light irradiation, whereas other treatments induced no apparent change (Fig. 2a–2i). This morphological change was probably caused by cell death because the cell was unable to exclude the propidium iodide (Fig. 2d–2f). Hoechst 33342 was used to visualise all cells. The number of cells was the same for each set of conditions (Fig. 2g–2i). The quantitative data of cell viability are shown in Fig. 3, and Fig. S2 and Table S1 (see ESI†). In all cases, 84.8% of cells were stained with PI by treatment of the LMI[60]fullerene of **1** + **2** in combination with light irradiation, but few cells were stained by other neutral and anionic LMI[60]fullerenes (3.8% for **1** and 0.9% for **1** + **3**), or by the LMI[60]fullerene of **1** + **2** without light irradiation (0.1%). These results are consistent with those for the intracellular uptake of LMIFs, indicating that intracellular LMI[60]fullerenes can act as photosensitisers. No LMI[60]fullerene showed dark cytotoxicity to HeLa cells after 24 h incubation (Table S1; 0.0–1.3%†).

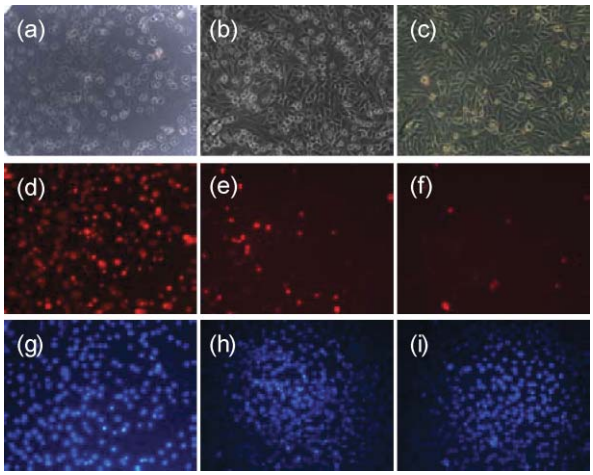


Fig. 2 Photodamage from LMI[60]fullerenes in HeLa cells. The cells were exposed to light (350–500 nm) for 2 h at 19 mW cm^{-2} and observed using microscopy: phase contrast images (a–c), exclusion of propidium iodide (d–f) and staining with Hoechst 33342 (g–i). For (a), (d) and (g): LMI[60]fullerene of **1** + **2**. For (b), (e) and (h): LMI[60]fullerene of **1**. For (c), (f) and (i): LMI[60]fullerene of **1** + **3**.

Apoptotic dead cells were evaluated using an Annexin V-Biotin apoptosis detection kit (R & D Systems, Inc.) to examine the mode of cell death in the LMI[60]fullerene of **1** + **2**. The procedure was conducted according to the manufacturer's recommendations. Analyses were done immediately after light irradiation for 0.25, 0.5, 1 and 2 h. Fig. S3e, 3j and 3o† show that, after light irradiation

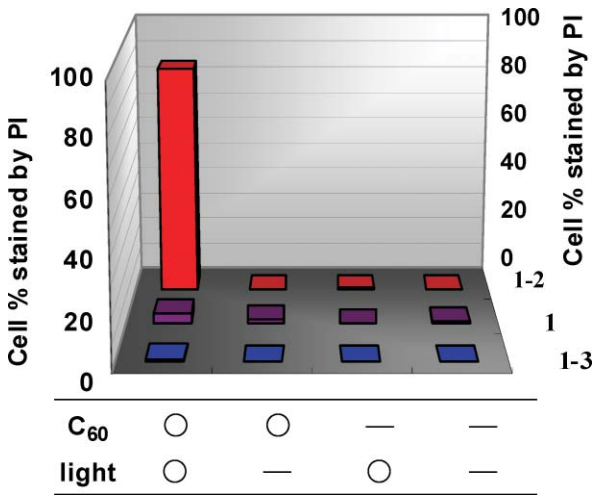


Fig. 3 Cell percentages stained by PI of HeLa cells treated with liposomes alone as a control and LMIFs of **1** + **2**, **1** and **1** + **3** before and after 19 mW cm^{-2} light irradiation for 2 h at 35 °C. Each experiment was repeated five times; the average is shown.

for 2 h, dead cells stained with propidium iodide were completely identical to those stained with Annexin V. The result suggests that almost all cells quickly undergo apoptosis by PDT treatment.

In conclusion, results of this study show that the LMI[60]fullerene of **1** + **2** with a cationic surface show a low dark toxicity that engenders cell death by photoirradiation. Furthermore, to the best of our knowledge, we demonstrated for the first time the photo-induced cell death of unmodified C_{60}.[18] These findings imply, therefore, that the concepts of cultivated lipid-membrane-incorporated C_{60} and its homologues can be applied more fruitfully to medicinal chemistry.

Notes and references

1 Y. Kaneda, *Adv. Drug Delivery Rev.*, 2000, **43**, 197; V. P. Torchilin, *Nat. Rev. Drug Discovery*, 2005, **4**, 145 and references therein.

2 H. I. Pass, *J. Natl. Cancer Inst.*, 1993, **85**, 443; T. J. Dougherty, C. Gomer, B. W. Henderson, G. Jori, D. Kessel, M. Korbelik, J. Moan and Q. Peng, *J. Natl. Cancer Inst.*, 1998, **90**, 889 and references therein.

3 K. Lang, J. Mosinger and D. M. Wagnerová, *Coord. Chem. Rev.*, 2004, **248**, 321 and references therein.

4 A. S. L. Derycke and P. A. M. de Witte, *Adv. Drug Delivery Rev.*, 2004, **56**, 17 and references therein.

5 Y. Tabata and Y. Ikada, *Pure Appl. Chem.*, 1999, **71**, 2047; F. Rancan, M. Helmreich, A. Mölich, N. Jux, A. Hirsch, B. Röde, C. Witt and F. Böhm, *J. Photochem. Photobiol., B*, 2005, **80**, 1; M. E. Milanesio, M. G. Alvarez, V. Rivarola, J. J. Silber and E. N. Durantini, *Photochem. Photobiol.*, 2005, **81**, 891; C. Yu, T. Canteenwala, L. Y. Chiang, B. Wilson and K. Pritzker, *Synth. Met.*, 2005, **153**, 37.

6 A. Ikeda, T. Sato, K. Kitamura, K. Nishiguchi, Y. Sasaki, J. Kikuchi, T. Ogawa, K. Yogo and T. Takeya, *Org. Biomol. Chem.*, 2005, **3**, 2907.

7 H. Hungerbühler, D. M. Guldi and K.-D. Asmus, *J. Am. Chem. Soc.*, 1993, **115**, 3386.

8 T. Hamano, K. Okuda, T. Mashino, M. Hirobe, K. Arakane, A. Ryu, S. Mashiko and T. Nagano, *Chem. Commun.*, 1997, 21.

9 A. Ikeda, T. Hatano, M. Kawaguchi, H. Suenaga and S. Shinkai, *Chem. Commun.*, 1999, 1403; T. Hatano, A. Ikeda, T. Akiyama, S. Yamada, M. Sano, Y. Kanekiyo and S. Shinkai, *J. Chem. Soc., Perkin Trans. 2*, 2000, 909; A. Ikeda, T. Hatano, S. Shinkai, T. Akiyama and S. Yamada, *J. Am. Chem. Soc.*, 2001, **123**, 4855; A. Ikeda, T. Hatano, T. Konishi, J. Kikuchi and S. Shinkai, *Tetrahedron*, 2003, **59**, 3537; T. Konishi, A. Ikeda, M. Asai, T. Hatano, S. Shinkai, M. Fujitsuka, O. Ito, Y. Tsuchiya and J. Kikuchi, *J. Phys. Chem. B*, 2003, **107**, 11261; A. Ikeda, A. Ejima, K. Nishiguchi, J. Kikuchi, T. Matsumoto, T. Hatano, S. Shinkai and M. Goto, *Chem. Lett.*, 2005, **34**, 308.

10 *Liposomes: A Practical Approach*, ed. V. P. Torchilin and W. Weissig, Oxford University Press, Oxford, 2nd edn, 2003.

11 Y. Matsumura and H. Maeda, *Cancer Res.*, 1986, **46**, 6387; H. Maeda, L. Seymour and Y. Miyamoto, *Bioconjugate Chem.*, 1992, **3**, 351.

12 K. Komatsu, K. Fujiwara, Y. Murata and T. Braun, *J. Chem. Soc., PerkinTrans. 1*, 1999, 2963.

13 ^{1}H NMR spectroscopy was used to confirm that all C_{60} were transferred from the γ-CDx cavity to lipid membranes to yield vesicle-incorporated C_{60}. ^{1}H NMR spectra for all LMI[60]fullerenes show that the peaks at 5.41 ppm, assignable to the aggregation of the C_{60}·γ-CDx complex by itself, did not appear and that the peaks assignable to the C_{60}·γ-CDx complex (4.19 and 5.05 ppm) disappeared by heating at 80 °C for 4 h after addition of the **1** + **2**, **1** and **1** + **3** vesicles.

14 Compound **2** was prepared as described in a previous paper.[19] **1**, **3** and γ-CDx were purchased from NOF Corp. (Tokyo, Japan), Avanti Polar Lipids Inc. (Birmingham, AL) and Aldrich Chemical Co. Inc., respectively. C_{60} (>99.5%) was purchased from MER Co. (Tucson, AZ).

15 B. A. Lindig, M. A. J. Rodgers and A. P. Schaap, *J. Am. Chem. Soc.*, 1980, **102**, 5590; I. Roy, T. Y. Ohulchanskyy, H. E. Pudavar, E. J. Bergey, A. R. Oseroff, J. Morgan, T. J. Dougherty and P. N. Prasad, *J. Am. Chem. Soc.*, 2003, **125**, 7860.

16 The order of the generation of $^1O_2$ was **1** + **3** > **1** > **1** + **2**. Absorbance of LMI[60]fullerenes of **1** and **1** + **2** increased due to light scattering at several hours after the addition of the disodium salt of 9,10-anthracenedipropionic acid. The reason considered is that the anionic disodium salt of 9,10-anthracenedipropionicacid induced the self-assembly of cationic and zwitterionic liposomes by electrostatic interactions. Therefore, the absorption of C_{60} at 440 nm is partially prevented by overlap with the light scattering and the generation of $^1O_2$ in the LMI[60]fullerenes of **1** and **1** + **2** is lower than that in the LMI[60]fullerenes of **1** + **3**.

17 C. R. Miller, B. Bondurant, S. D. McLean, K. A. McGovern and D. F. O'Brien, *Biochemistry*, 1998, **37**, 12875.

18 Chen *et al.* observed that HeLa cell density, when a HeLa cell is treated with an LMI[60]fullerene of lecithin (the lecithin contains **1**) at 4 days after photoirradiation, increased to two times that of a similar treatment without LMI[60]fullerene. These apparent discrepancies between our results and those of Chen *et al.* are explained by the fact that the neutral LMI[60]fullerene of lecithin cannot deliver a fluorochrome into HeLa cells (see Fig. 1e); consequently, it cannot engender apoptosis; see: W. Z. Li, K. X. Qian, W. D. Huang, X. X. Zhang and W. X. Chen, *Chin. Phys. Lett.*, 1994, **11**, 207.

19 M. Hashizume, S. Kawanami, S. Iwamoto, T. Isomoto and J. Kikuchi, *Thin Solid Films*, 2003, **438–439**, 20.

