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# Efficient photocleavage of DNA utilising water-soluble lipid membrane-incorporated [60]fullerenes prepared using a [60]fullerene exchange method†

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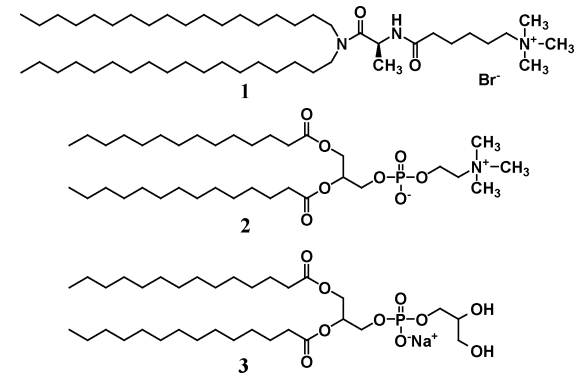
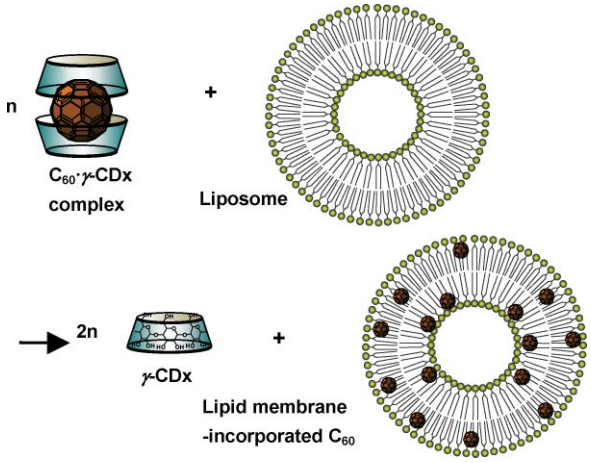
***Received 6th June 2005, Accepted 7th July 2005***
***First published as an Advance Article on the web 19th July 2005***

**Various types of lipid membrane-incorporated $C_{60}$ with high $C_{60}$ concentrations can be prepared easily in several hours using the $C_{60}$ exchange method and the photocleaving activity of cationic lipid membrane-incorporated $C_{60}$ was appreciably higher than that of the $C_{60}$·γ-CDx complex.**

The ready availability of [60]fullerene ($C_{60}$) and its homologues has increasingly invited exploration of their unique physical and chemical properties. Their ready excitation by visible light irradiation suggests their potential for application as water-soluble fullerenes for various medicinal uses. For example, they act as a singlet oxygen ($^1O_2$) photosensitiser[1,2] to cleave DNA[3–8] and as a photoinactivator of viruses.[9,10] Despite their potential, however, their poor water-solubility has limited their applications extremely. Several research efforts have attempted to compensate for that drawback: by introduction of water-soluble substituents;[3–7] mixing with water-soluble polymers[11] or lipid membranes;[12,13] and solubilisation in γ-cyclodextrin (γ-CDx)[14–17] or water-soluble calixarenes.[18,19] Of these methods, we employed lipid membrane-incorporated $C_{60}$ (LMI[60]fullerenes), which form vesicles, for three reasons: (i) unmodified $C_{60}$ in vesicles can generate $^1O_2$ by energy transfer or $C_{60}$ anion radical by electron transfer more efficiently than other chemically-modified $C_{60}$ derivatives;[20] (ii) various vesicles with positively charged, negatively charged, nonionic and zwitterionic surface can be prepared by a selection of lipids such as phospholipids, aminolipids and glycolipids and may confer a function as a drug carrier;[21] and (iii) relatively large vesicle formation is promising for the enhanced permeability and retention (EPR) effect because macromolecular compounds or aggregates can preferentially accumulate in solid tumors by virtue of the enhanced permeability of tumor vascular endothelia and the lack of functional lymphatic drainage in tumor tissues.[7,22] Nevertheless, two obstacles remain regarding the Bangham method:[12,13,23] (a) the difficulty in preparing LMI[60]fullerenes in several types of lipids; and (b) low concentrations of incorporated $C_{60}$.[12] We report a novel method to prepare LMI[60]fullerenes with high $C_{60}$ concentrations and to enable the treatment as a homogeneous system. Moreover, we assayed biological activities of the LMI[60]fullerenes under visible light irradiation, finding that the lipid membranes may serve as photoinduced DNA cleavers.

All LMI[60]fullerenes were prepared using an exchange reaction between the vesicle formed by the lipids and the $C_{60}$·γ-CDx complex[15] by heating at 80 °C for 4 h (Scheme 1).[24] Size distributions of the liposomes were studied using dynamic light scattering (DLS). Table S1 summarizes the average diameters




**Scheme 1** Exchange reaction between the liposome and the $C_{60}$·γ-CDx complex.

of all liposomes before and after the exchange reaction of $C_{60}$. Nondestruction of liposomes after the exchange reaction was confirmed using transmission electron microscopy (TEM) images (Fig. S1).† An initial concentration of $C_{60}$ in the $C_{60}$·γ-CDx complex, determined by measuring the absorbance of the solution at 332 nm (a specific extinction coefficient for the water-soluble $C_{60}$·γ-CDx complex of $\varepsilon_{332} = 4.27 \times 10^4$ cm$^2$ g$^{-1}$),[15] was 0.20 mM in an aqueous solution (1.0 ml). After an aqueous solution of lipids (ten equivalents of $C_{60}$) was added to the solution (1.0 ml, 2.00 mM), final concentrations of the respective components were evaluated using integral intensities of the $^1$H NMR spectrum, 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). The formation of the LMI[60]fullerenes from the $C_{60}$·γ-CDx complex was indicated by a change in the UV-vis absorption spectra with time. Figs. S2a–c† show that when the mixture of the **1**, **2** or **3** vesicles and the $C_{60}$·γ-CDx complex was heated at 80 °C, peak

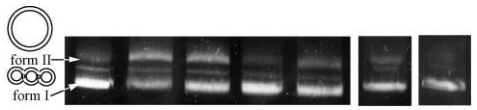
† Electronic supplementary information (ESI) available: DLS measurements, zeta potential measurements, TEM images, UV-vis absorption spectra and $^1$H NMR spectra. See http://dx.doi.org/10.1039/b507954c

DOI: 10.1039/b507954c

broadening at 337 nm was observed and a new broad absorption band appeared in the 400–550 nm region. The final spectrum of this mixture was similar to that of the previously reported lecithin vesicle.[12] However, two questions remain: (i) whether the reaction is the exchange or the aggregation of the $C_{60}$·γ-CDx complex by itself[15] and (ii) whether or not the equilibrium of the reaction shifts completely to the right-hand side.

The $^1$H NMR spectroscopic method was used to answer these questions. Fig. S3† shows that the peaks at 5.41 ppm assignable to the aggregation of the $C_{60}$·γ-CDx complex by itself[25] did not appear and 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** and **3** vesicles. These results indicate that all $C_{60}$ were transferred from the γ-CDx cavity to lipid membranes to yield vesicle-incorporated $C_{60}$. These results also show that concentrations of $C_{60}$ in all the vesicles are equal to the initial concentration of the $C_{60}$·γ-CDx complex (0.10 mM). The stoichiometries of [$C_{60}$]–[lipids] are each 10%, which is considerably higher than that in a previous study (less than 3%).[13] This reason implied that in the Bangham method, the self-aggregation of $C_{60}$ should prevent the formation of the liposome when dry thin membrane of $C_{60}$ and lipids mixtures are prepared and extracted in an aqueous solution. These results indicate that the exchange reaction of $C_{60}$ is suitable for various types of vesicles.

These LMI[60]fullerenes were applied to the photocleavage of the ColE1 supercoil plasmid. DNA was cleaved neither under dark conditions in the presence of these reagents nor under visible light irradiation in the absence of $C_{60}$ (Fig. 1: lanes 6 and 7). Under visible light irradiation, the **1**-incorporated $C_{60}$ and the **2**-incorporated $C_{60}$ showed a distinct DNA-cleaving activity (lanes 2 and 3). In lanes 2 and 3, about 44% and 24% of supercoiled DNA (form I) were converted to nicked DNA (form II).[26] In contrast, the **3**-incorporated $C_{60}$ (lane 4) was markedly lower than that of the **1**-incorporated $C_{60}$ and the **2**-incorporated $C_{60}$ under visible light irradiation (2%). Consequently, one can conclude that $C_{60}$ included in the 'cationic' vesicle of **1** is transported onto 'anionic' DNA with the aid of electrostatic interactions. The $C_{60}$ cleaves it with the aid of photoirradiation.[18,27,28] In contrast, $C_{60}$ included in the 'anionic' vesicle of **3** is not transportable onto 'anionic' DNA due to the electrostatic repulsions; consequently, it showed little DNA-cleaving activity.[28] For comparison, we conducted a control experiment using the $C_{60}$·γ-CDx complex.[8] The DNA-cleaving activity of **1**-incorporated $C_{60}$ (lane 2; 44%) was appreciably higher than that of the $C_{60}$·γ-CDx complex (lane 5; 6%).




| lane | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
| lipids | – | **1** | **2** | **3** | – | **1** | **1** |
| γ-CD | – | – | – | – | ○ | – | – |
| $C_{60}$ | – | ○ | ○ | ○ | ○ | ○ | – |
| light | – | ○ | ○ | ○ | ○ | – | ○ |

**Fig. 1** Agarose gel electrophoretic patterns of DNA nicked by the LMI[60]fullerenes. Reaction samples contained 1.3 mg $L^{-1}$ of ColE1 supercoiled plasmid. Lane 1: no chemicals were in the distilled water. Lanes 2, 3 and 4: 200 μM of **1**, **2** and **3** and 20 μM of $C_{60}$. Lane 5: 204 μM of γ-CD and 20 μM of $C_{60}$. Lane 6: 200 μM of **1** and 20 μM of $C_{60}$. Lane 7: 200 μM of **1**. Lanes 2–5 and 7: incubated under visible light irradiation at a distance of 10 cm using a 500 W Xe-arc lamp (UI-502Q; Ushio, Inc.) at 25 °C for 3 h under the aerobic conditions. Lanes 1 and 6: incubated in the dark for 3 h under the aerobic conditions. After the addition of 5 μL of 10% SDS solution and loading buffer (Wako Pure Chemical Industries, Ltd.) in this order, electrophoresis was performed using 0.9% agarose gel. The gel was stained with SYBR Gold (1 : 10000 dilution of stock supplied by Molecular Probes Inc., Eugene, Ore.) and viewed on a UV transilluminator.

In conclusion, this study revealed that LMI[60]fullerenes with high $C_{60}$ concentrations are easily prepared in several hours using the exchange method and that DNA photocleavage abilities depend on the surface charges of their vesicles. These findings have important implications for various applications in biological and medicinal chemistry, and in material chemistry because the materials-incorporated $C_{60}$ can be prepared easily by the $C_{60}$ exchange method using the $C_{60}$·γ-CDx complex. Applications of these systems are being studied in our laboratories.

This work was supported by Grants-in-Aid for Scientific Research from the Ministry of Education. We thank Professor Koichi Komatsu and Dr Yasujiro Murata (Kyoto University) for helpful comments about HSVM treatment and Mr Robert Wright (Nara Institute of Science and Technology) for reading the entire text in its original form.

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24 Compound **1** was prepared as described in a previous paper.[29] **2**, **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).
25 In the absence of lipids, the $C_{60}$·γ-CDx complex slowly aggregates by itself.[15] A new peak assignable to the aggregation of the $C_{60}$·γ-CDx complex (5.41 ppm) appeared separately from that assignable to the free γ-CDx (5.12 ppm); a peak assignable to the $C_{60}$·γ-CDx complex (5.05 ppm) disappeared in the $^1$H NMR spectrum after heating for 12 h.
26 The efficiency of photocleavage is not quantitative but qualitative. Therefore, these efficiencies change by experimental conditions; for example, photoirradiation power, photoirradiation time, *etc*. In this paper, we employed 3 h as the photoirradiation time because we intended to compare the efficiency among LMI[60]fullerenes with cationic, zwitterionic, anionic surface charges. When we employed 6 h, the cationic **1**-incorporated $C_{60}$ was able to cleave ~100% of DNA.
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