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Photodynamic Activity of C_{70} Caged within Surface-Cross-Linked Liposomes

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Abstract: [70]Fullerene (C_{70}) encapsulated into a surface-cross-linked liposome, a so-called cerasome, was prepared by an exchange reaction incorporating C_{70}·γ-cyclodextrin complexes into lipid membranes. Fullerene exchange in a cerasome-incorporated C_{70} (CIC_{70}), as well as in a lipid-membrane-incorporated C_{70} ($LMIC_{70}$), was completed within 1 min with stirring at 25 °C. CIC_{70} was more resistant to lysis than $LMIC_{70}$ towards lysing agents such as surfactants. Furthermore, the photodynamic activity of CIC_{70} in HeLa cells was similar to that of $LMIC_{70}$, indicating that C_{70} can act as a photosensitizing drug (PS) without release from cerasome membranes. Thus, in contrast with general drug-delivery systems (DDSs), which require the drug to be released from the interior of liposomes, carriers for PSs for use in photodynamic therapy (PDT) do not necessarily need to release the drug. These results indicate that DDSs with high morphological stability can increase the residence time in blood and achieves tumor-selective drug delivery by the enhanced permeability and retention (EPR) effect.

Keywords: cyclodextrins · exchange reactions · fullerenes · lipid bilayers · photodynamic activities

Introduction

Liposomes have attracted considerable attention as materials for drug-delivery systems (DDSs), which incorporate small drugs such as anticancer agents inside a vesicle or polymers such as DNA outside the vesicle.[1–3] Advantages of using liposomes include the lack of immune or inflammatory responses, lower cost, and ease of large-scale manufacture. However, materials for general DDSs need to combine the opposing properties of morphological stability for drug delivery and lability for drug release from the interior of liposomes.[1] In contrast with general DDSs, carriers for photosensitizing drugs (PSs) for use in photodynamic therapy (PDT)[4] do not need to release the drugs, because the generation of active oxygen species from photoexcited PSs in liposomes leads to cell death (Scheme 1). Thus, if this hypothesis is correct, we should be able to design carriers for PSs with sufficiently durable structures to lengthen their bioavailability and circulation in the blood. To prove our hypothesis, we developed a so-called cerasome, which has a

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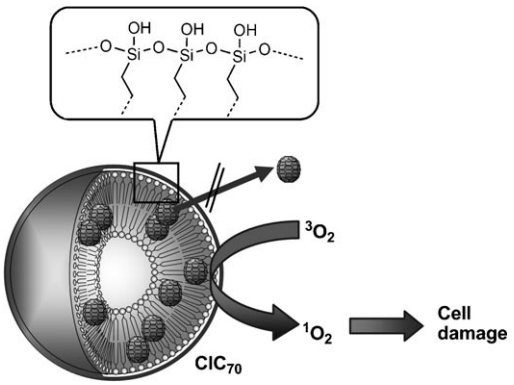


Scheme 1. Cell damage by CIC_{70} through the oxygen molecule.

liposomal bilayer structure and a polysiloxane surface.[5,6] Because the vesicular bilayer is covered with a silicate surface, the cerasome is morphologically more stable than general liposomes, such as the ones formed with dimyristoylphosphatidylcholine (DMPC, **4**).[5,6] We recently reported that lipid-membrane-incorporated fullerenes ($LMIC_x$: $x=60$ or 70) can be easily prepared by transferring fullerenes from water-soluble host–guest complexes to lipid membranes.[7–10] $LMIC_{60}$ and $LMIC_{70}$ were obtained through the exchange reaction of C_{60}·γ-cyclodextrin (γ-CDx) and C_{70}·γ-CDx complexes, respectively, with liposome. The lipid-membrane-incorporated fullerenes had higher DNA cleavage ability than C_{60}·γ-CDx and C_{70}·γ-CDx complexes.[7,9] Furthermore, $LMIC_{60}$ and $LMIC_{70}$ have low toxicity toward HeLa cells in the dark and engender cell death by photoirradiation. The photodynamic activity of $LMIC_{70}$ was found to be much higher than that of $LMIC_{60}$ in the same photon flux ($\lambda > 400$ nm).[10]

Herein, we report an advanced preparation with a high morphological stability and resistance to lysis as well as the photodynamic activity of a cerasome-incorporated C_{70} (CIC_{70}). CIC_{70} was produced by using an exchange reaction from the unstable water-soluble complex of fullerene C_{70}·γ-CDx to the cerasome. The morphological stability of CIC_{70} against a surfactant such as a nonionic Triton X-100 (TX-100) was much higher than that of the cerasome itself and of a liposome formed with DMPC. Furthermore, the photodynamic activity of CIC_{70} toward HeLa cells was assayed under visible-light irradiation ($\lambda > 400$ nm), and CIC_{70} has a photodynamic activity comparable with that of $LMIC_{70}$.

Results and Discussion

Selection of the Cerasome-Forming Lipids

Cerasome-forming lipids were selected to act as a C_{70} carrier. Because the intracellular uptake of cationic nanoparticles by cells is higher than that of anionic or neutral nanoparticles, 10 mol% of cationic cerasome-forming lipid **2** was added (Figure 1).[8,10–12] Moreover, the addition of **2** was limited because cationic compounds are typically very cytotoxic. The cationic cerasome of **2** alone was cytotoxic in the dark (data not shown); therefore, 89 or 90 mol% of **1** was added for the cerasome from 1, 2, and 3 or 1 and 2, respectively. Also, 1 mol% of alkyltrialkoxysilane **3** was added as a reinforcing agent. Although the morphological stabilities of the cerasomes from **1** and **2** are superior to those of the liposomes from **4** and **5**, the siloxane network that forms between the cerasome-forming lipids on the cerasome surface was incomplete because the distance between the Si atoms in the Si–O–Si unit is much shorter than the diameter of the cross-section of the dialkyl tail (Scheme 2a).[5,6] Owing to its small cross-sectional area, **3**, which has a monoalkyl tail, should be able to form connections between the siloxane networks of **1** and **2** which could not develop by using the cerasome-forming lipid alone (Scheme 2b). The morphological stabilities and lysis resistance of these cerasomes with or without C_{70} will be discussed in detail in the following sections.

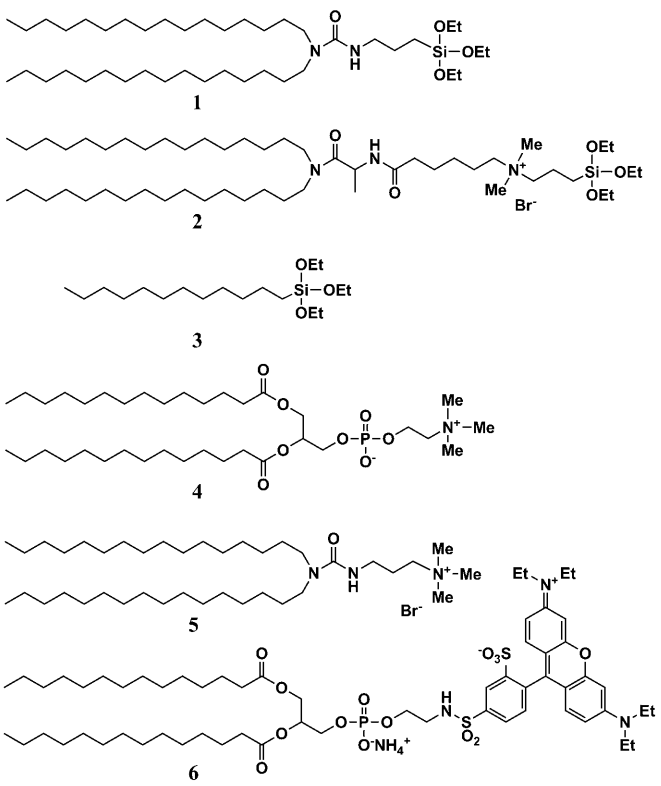
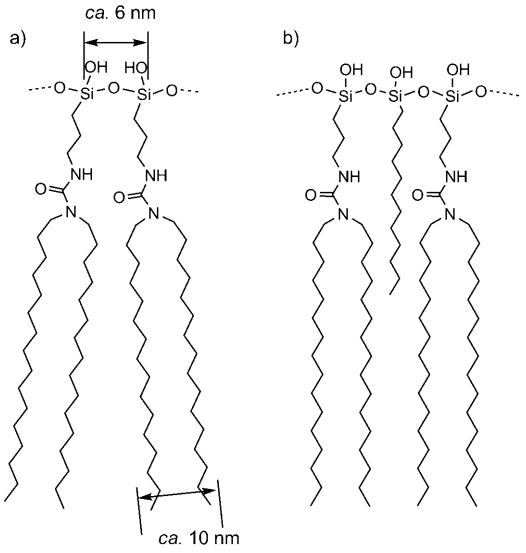


Figure 1. Chemical structures of the compounds **1**–**6**.

If the cerasomes were prepared in salt-free solution, the reduced osmotic pressure of the inner phase owing to the flow of the inner aqueous phase out through the membrane crushes the cerasomes in the culture medium. Although the cerasome must be prepared in physiological saline (0.9% w/v NaCl solution) for isotonic conditions between the inner aqueous phase and culture medium, it proved difficult to control the sizes of the cerasome vesicles in solution, and a turbid solution was obtained. We then measured the size distributions of the liposomes and the cerasomes. Measurements by dynamic light scattering (DLS) revealed that average diameters of the cerasomes were approximately 250 and 450 nm after sonication for 1 h and 2 h, respectively (Table 1). The reason for the increased size of the cerasomes was not clear. Therefore, the cerasomes were prepared in a

Abstract in Japanese:

我々は高い形態安定を持つセラソームと呼ばれる表面架橋型リポソーム中に取り込まれたC_{70}をC_{70}·γ-CDx錯体から脂質膜への交換反応により調製した。交換反応は可視-紫外吸収スペクトル、蛍光スペクトル、^{1}H NMRスペクトルにより、25℃、1分間の撹拌により終了していることが確認された。その反応速度は、C_{70}含有リポソームのときと同様、十分早いものであった。C_{70}含有セラソームはC_{70}含有リポソームに比べ、界面活性剤のような溶解剤に対して高い形態安定性を有した。さらに、C_{70}含有セラソームのHeLa細胞に対する光線力学活性はC_{70}含有リポソームと同様であり、この結果はC_{70}がセラソームの膜から放出されなくても光増感剤として働くことを示す。今後、光増感治療における光増感剤の輸送体は光増感剤を必ずしも放出する必要がないことから、高い血中安定性をもつDDSを設計できるため癌指向性をより高められるものと期待される。



Scheme 2. Siloxane network a) between **1** and b) between **1** and **3**.

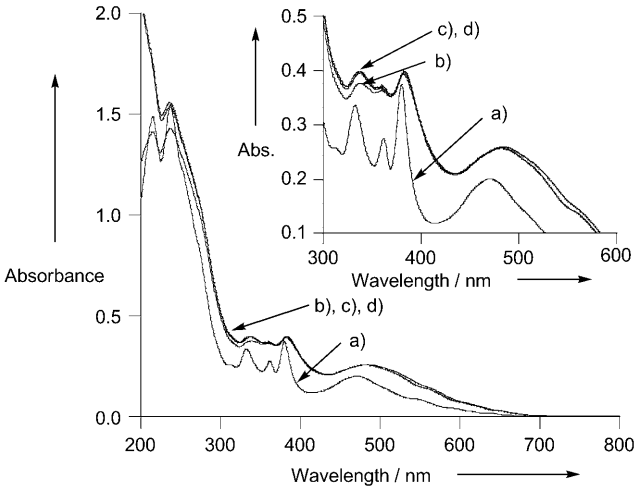


Figure 2. UV/Vis absorption spectra of the C_{70}·γ-CDx complex a) before and after the addition of b) liposome **4**–**5**, c) cerasome **1**–**2**, and d) cerasome **1**–**2**–**3** (1 mm cell). Insert: Expansion of the region 300–600 nm.

Table 1. Average particle sizes determined by a light-scattering method at 25 °C before and after the exchange reaction.

	Solvent	Average particle size [nm][a] Before addition of C_{70}	After addition of C_{70}
$LMIC_{70}$ **4**–**5**	0.9 % w/v NaCl solution	91 ± 33	80 ± 27
CIC_{70} **1**–**2**–**3**	0.9 % w/v NaCl solution	456 ± 15[b]	–
CIC_{70} **1**–**2**–**3**	5 % w/v glucose solution	178 ± 10	170 ± 15

[a] Each experiment was performed three times. The reported values of particle sizes are the average of these separate tests. [b] This value was obtained after sonication for 2 h. After sonication for 1 h, the value was 251 ± 24 nm.

5 % w/v glucose solution. The average diameters were estimated to be 178 nm, fulfilling the requirements for the enhanced permeability and retention (EPR) effect.[13]

Preparation of CIC_{70} by a C_{70} Exchange Reaction

Figure 1 shows the chemical structures of the compounds used in the current study. CIC_{70} was prepared by an exchange reaction between the cerasomes and the C_{70}·γ-CDx complex[14] in a manner similar to that for $LMIC_{70}$.[9] After mixing the C_{70}·γ-CDx complex with a cerasome at 25 °C, peak broadening was observed in the region 300–700 nm in the UV/Vis absorption spectrum (Figure 2). The spectra for the cerasomes from **1** and **2** and from **1**, **2**, and **3** were similar to that for the liposome, which implies that C_{70} was completely transferred from two γ-CDx cavities to the cerasomes to yield vesicle-incorporated C_{70}. This transfer was evident from the complete precipitation of C_{70} after heating an aqueous solution of the C_{70}·γ-CDx complex in the absence of liposomes or cerasomes. Furthermore, the exchange reaction was confirmed by ^{1}H NMR spectroscopy. Figure 3 a shows the peaks at $\delta = 4.00$ and 5.05 ppm, which were assigned to the C_{70}·γ-CDx complex. After the aqueous solution of the C_{70}·γ-CDx complex was added to the cerasome at 25 °C, the peaks at $\delta = 4.00$ and 5.05 ppm disappeared (Figure 3 b, c), indicating that C_{70} was completely released from two γ-CDx cavities. To confirm that the released C_{70} was transferred into lipid membranes of the cerasome, we measured fluorescence quenching of rhodamine B/dipalmitoylphosphatidylethanolamine (**6**) by C_{70} in the cerasome-incorporated fluorochrome (CIF) of **1**, **2**, and **3** prepared using lipids containing 0.25 mol % **6** (Figure 4 a).[15] After adding the C_{70}·γ-CDx complex, the exchange reaction with C_{70} led to fluorescence

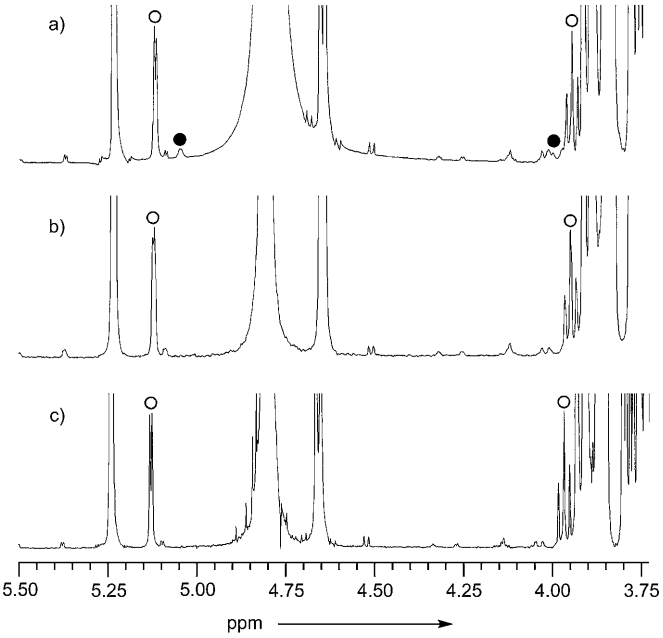


Figure 3. ^{1}H NMR spectra (600 MHz, D_2O, 25 °C) of the C_{70}·γ-CDx complex a) before and after the addition of b) cerasome **1**–**2**, or c) cerasome **1**–**2**–**3** (○: free γ-CDx, ●: the C_{70}·γ-CDx complex).

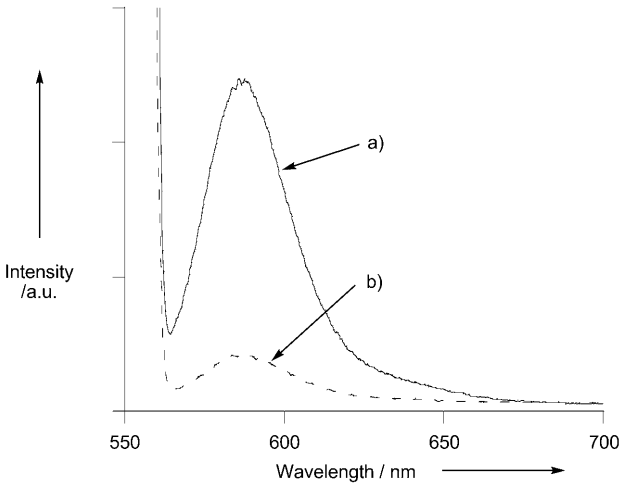


Figure 4. Fluorescence spectral change of the cerasome **1–2–3** containing **6** a) before and b) after the addition of the C_{70}·γ-CDx complex.

quenching of 83%, indicating that C_{70} and **6** coexist in the cerasome and C_{70} acts as a quencher. That is, the released C_{70} from two γ-CDx cavities was transferred into the lipid membranes of the cerasome. These results also confirm that the concentration of C_{70} in all vesicles was equal to the initial concentration of the C_{70}·γ-CDx complex (0.10 mM).

Because the reaction was completed after only 1 min with stirring at 25 °C, the exchange rate in CIC_{70} **1–2–3**, as well as $LMIC_{70}$ **4–5**, was sufficiently rapid because the exchange reaction was carried out before the cerasomal surfaces were covered by the siloxane network. After the exchange reaction, CIC_{70} was incubated for 24 h at room temperature to develop the siloxane network by the hydrolysis of the triethoxysilyl head groups. Table 1 summarizes the average diameters measured by DLS analysis of the cerasome before and after the exchange reactions of C_{70}. The diameter of cerasome from **1**, **2**, and **3** hardly changed before and after the exchange reaction (before: 178 nm; after: 170 nm) because the process did not involve heating. This result is similar to that for $LMIC_{70}$ **4–5**, as previously reported (Table 1).[9] Furthermore, CIC_{70} **1–2–3** could be stored for at least 3 weeks at room temperature without changes in the average diameter or absorbance at $\lambda = 482$ nm (Tables S1 and S2 in the Supporting Information).

The intracellular uptake of nanoparticles depends primarily on their surface densities because cationic nanoparticles will adhere to the anionic surface of cellular membrane by electrostatic interactions.[7,10–12] The surface charges of liposomes and cerasomes with and without C_{70} was determined from the zeta-potential (Table 2). The zeta potential of CIC_{70} **1–2–3** was slightly lower than that of $LMIC_{70}$ at pH 7.0. The lower potential of CIC_{70} results from the partial counteraction of the positive charge of **2** by deprotonation of the silanol groups on the cerasome surface.[6]

Table 2. Zeta potentials for $LMIC_{70}$ **4–5** and CIC_{70} **1–2–3** at 25 °C before and after the exchange reaction.

	Zeta potential/mV	
	Before addition of C_{70}	After addition of C_{70}
$LMIC_{70}$ **4–5**	+59.8±1.1	+57.2±1.5
CIC_{70} **1–2–3**	+51.0±0.1	+50.8±0.8

Morphological Stability of CIC_{70} and $LMIC_{70}$ Against a Nonionic TX-100

We evaluated the morphological stability of CIC_{70} **1–2**, CIC_{70} **1–2–3**, and $LMIC_{70}$ **4–5** using a method based on surfactant solubilization.[6,16,17] Davidson and Regen reported that polymerized liposomes were more morphologically stable and lysis resistant than unpolymerized liposomes against lysing agents such as ethanol or surfactants.[16] Figure 5 shows the resistance of CIC_{70} **1–2**, CIC_{70} **1–2–3**, and $LMIC_{70}$ **4–5** against the nonionic surfactant TX-100 determined by the intensity of light scattering of the vesicles. Cerasomes and liposomes in the absence of C_{70} were used as references. After adding two equivalents of TX-100, the light scattering intensity of the liposome from **4** and **5** in the absence of C_{70} was greatly decreased, indicating collapse of the vesicle. In contrast, the cerasome from **1** and **2** exhibited remarkable morphological resistance against TX-100; however, the addition of 20 equivalents of TX-100 collapsed the vesicle. The addition of alkyltrialkoxysilane **3** further improved the morphological stability of the cerasome, indicating that **3** can connect the siloxane networks (Scheme 2b). Therefore, we used the cerasome **1–2–3** (**1/2/3** 89:10:1 mol%) for all of the following experiments. The morphological stabilities in the presence of C_{70} were superior to those in its absence for all cerasomes and liposomes. Although it is not clear why this is so, this type of stabilization would be a particularly advantageous characteristic for PS carriers.

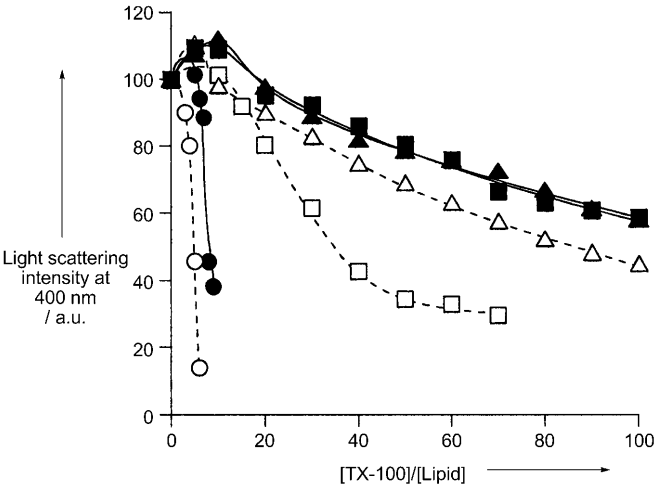


Figure 5. Light scattering intensities of the cerasomes **1–2** and **1–2–3**, and the liposome **4–5** in the absence and presence of C_{70} as a function of added equivalents of TX-100 at 25 °C: liposome **4–5** without C_{70} (○, dashed line) and with C_{70} (●, solid line); cerasome **1–2** without C_{70} (□, dashed line) and with C_{70} (■, solid line); cerasome **1–2–3** without C_{70} (△, dashed line) and with C_{70} (▲, solid line).

Intracellular Uptake of CIF and LMIF by HeLa Cells

CIF **1–2–3** and the lipid-membrane-incorporated fluorochrome (LMIF) **4–5** using lipids containing **6** were prepared to use for the intracellular uptake measurements. Figure S1 in the Supporting Information shows that the fluorescence intensity was nearly identical with CIF **1–2–3** and LMIF **4–5**. The efficiency of incorporation of CIF **1–2–3** and LMIF **4–5** to HeLa cells was found by flow cytometry analysis to be 100% (Figure S2 in the Supporting Information). Moreover, cells treated with LMIF **4–5** showed higher geometric mean fluorescence intensity (344) than those treated with CIF **1–2–3** (273), indicating that the intracellular uptake of LMIF **4–5** is slightly higher than that of CIF **1–2–3**. The intracellular uptake of CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5** should be similar to that of CIF from **1–2–3** and LMIF **4–5** owing to the similar surface charges of the liposomes and cerasomes with and without C_{70} (Table 2).

Comparison of Photodynamic Activity between CIC_{70} and $LMIC_{70}$

CIC_{70} **1--2--3** and $LMIC_{70}$ **4–5** were evaluated in culture for photodynamic activity in HeLa cells. The cell viability, normalized to the value obtained in the absence of C_{70}, was measured by staining nonviable cells with propidium iodide (PI) because nonviable cells cannot exclude PI. After incubation with 5 μM fullerenes of CIC_{70} **1–2–3** or $LMIC_{70}$ **4–5** for 24 h, the cells were exposed to light ($\lambda = 400$–740 nm) for 60 min. The power of light at the cellular level was 57 mW cm^{-2}. Whereas no cytotoxicity in the dark was observed for CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5** after 24 h incubation (Table S3 in the Supporting Information; percentages of cell viability: each 99%), the cells treated with CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5** had abnormal shapes (Figure 6b,f) and were stained with PI after light irradiation (Figure 6d,h). Figure 7 shows the percentages of cell viability of CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5** treatments as a function of irradiation time (Figure S3 in the Supporting Information). Despite the higher intracellular uptake in the LMIF **4–5** relative to CIF **1–2–3**, similar curves were observed for CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5** both immediately after light irradiation and after light irradiation followed by 24 h of incubation. These similar results for CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5** indicate that C_{70}, which is not released from cerasome membranes, can act as a PS.

Analysis of the Mode of Cell Death Induced by CIC_{70} and $LMIC_{70}$

To analyze the mode of cell death induced by CIC_{70} **1–2–3** and $LMIC_{70}$ **4–5**, cell surface-exposed phosphatidyl serine, which is present in the earlier stages of apoptosis, was visualized by using fluorescein isothiocyanate (FITC) conjugated annexin V. When FITC-conjugated annexin V is combined with with PI, the different labeling patterns identify the different cell fractions: namely, PI-negative/annexin V-negative viable cells, PI-negative/annexin V-positive early apoptotic cells, and PI-positive/annexin V-positive late apop-

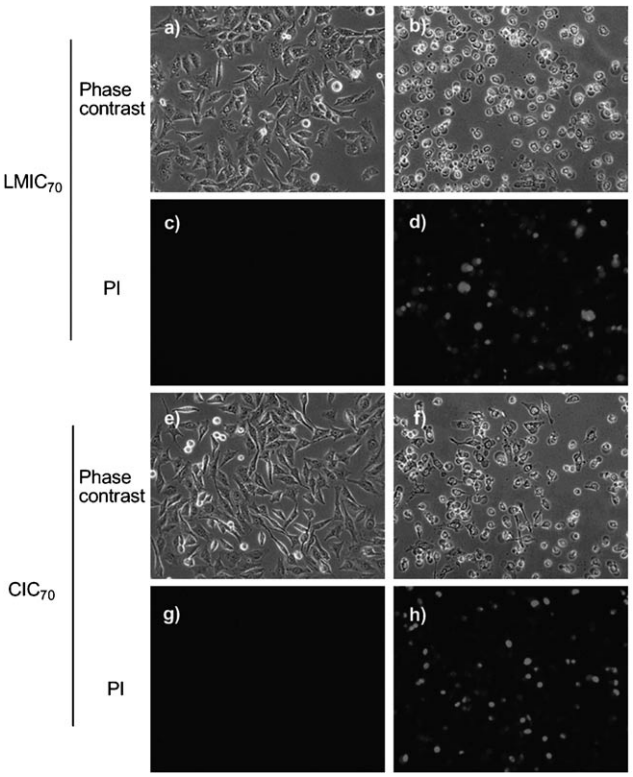


Figure 6. Photodamage from $LMIC_{70}$ **4–5** and CIC_{70} **1–2–3** in HeLa cells. $LMIC_{70}$ and CIC_{70} treated cells were exposed to light ($\lambda > 400$ nm) for 60 min at 35°C (b, d, f, and h) or incubated at 35°C without light irradiation as a control (a, c, e, and g). Following irradiation, the cells were stained with PI and observed by fluorescence microscopy: phase contrast images (a, b, e, and f), PI (c, d, g, and h): a–d) $LMIC_{70}$; e–h) CIC_{70}.

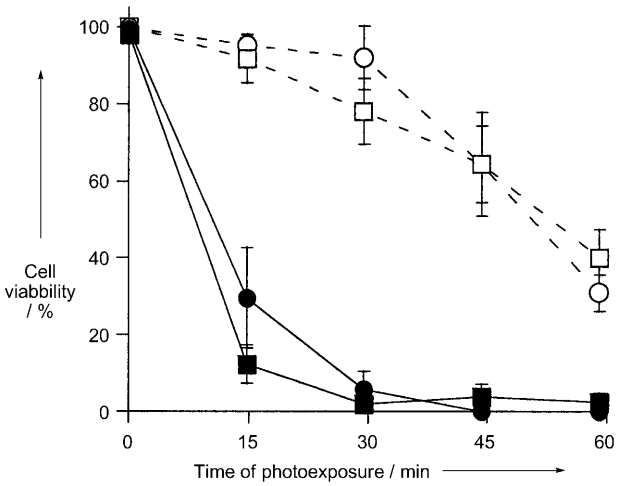


Figure 7. Cell viability (%) as a function of photoexposure time of cells stained with PI immediately after irradiation ($LMIC_{70}$ **4–5** ○; CIC_{70} **1–2–3** □; dashed lines) and after irradiation and subsequent incubation for 24 h ($LMIC_{70}$ **4–5** ●; CIC_{70} **1–2–3** ■; solid lines). HeLa cells treated with $LMIC_{70}$ and CIC_{70} after irradiation at $\lambda > 400$ nm at 35°C. Error bars represent the standard deviation of three measurements.

totic or necrotic cells. After irradiation for 30 min, the cells treated with $LMIC_{70}$ and CIC_{70} were stained only with annexin V and not with PI (Figure 8). This result suggests that CIC_{70} **1**–**2**–**3** and $LMIC_{70}$ **4**–**5** induced apoptosis.[18]

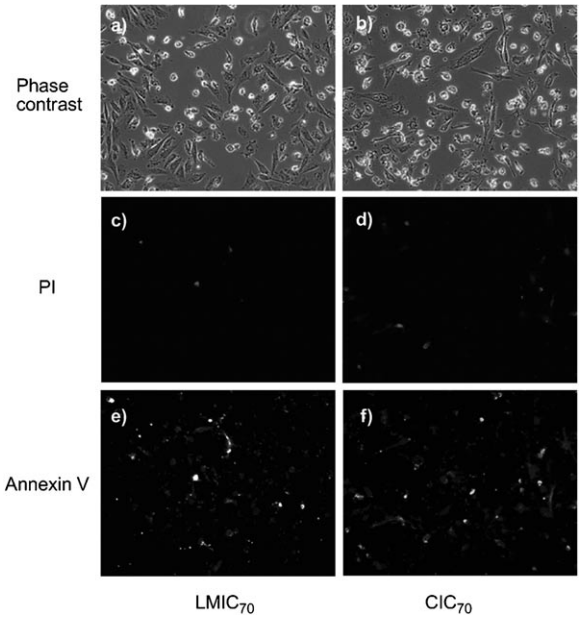


Figure 8. Photodamage from $LMIC_{70}$ **4**–**5** (a, c, and e) and CIC_{70} **1**–**2**–**3** (b, d, and f) in HeLa cells detected by using phase contrast images (a and b), by fluorescence microscopy using PI (c and d), and by using an annexin V apoptosis kit (e and f) and fluorescent staining after irradiation for 30 min at 35 °C.

Conclusions

The encapsulation of C_{70} within the cerasomes was achieved within only 1 min by an exchange reaction at 25 °C using the cerasomes uncovered by the siloxane network. The cerasome from **1**, **2**, and **3** was more stable and resistant against lysing agents such as surfactants than the liposome from **4** and **5** and cerasome from only **1** and **2**. Furthermore, after the exchange reaction, $LMIC_{70}$ and CIC_{70} were morphologically more stable than the liposome and cerasomes in the absence of C_{70}. The photodynamic activity in HeLa cells was similar to that of $LMIC_{70}$, indicating that the drug release of C_{70} from a cerasome does not need to occur for PDT. The use of stable carriers for PSs can increase the residence time and bioavailability of the drug in the blood and can achieve tumor-selective drug delivery through the EPR effect.[13]

Experimental Section

General

UV/Vis spectra were obtained on a UV-2550 spectrophotometer (Shimadzu Corporation). Fluorescence measurements of individual cells were performed by using a FACS calibur flow cytometer (Becton–Dickinson) equipped with an argon ion laser (excitation at 488 nm, emission at 585/42 nm band-pass filters). Light irradiation was performed with a xenon lamp (MAX-301, 300 W; Asahi Spectra Co. Ltd.) equipped with a Vis mirror module (385–740 nm; Asahi Spectra Co. Ltd.) and a long-pass filter with cutoff at 400 nm (Asahi Spectra Co. Ltd.). Fluorescence microscopy was performed with an inverted Axiovert 135M (Carl Zeiss Inc.), equipped with an AxioCam CCD camera (Carl Zeiss Inc.) and AxioVision 3.0 software (Carl Zeiss Inc.). The following sets of filters were used PI and rhodamine B (BP546, FT580, and LP 590) and FITC (450–490, FT 510, and 515–565).

Materials

Compounds **1**,[19] **2**,[20] and **5**[19] were prepared as described previously. Compounds **3**, **4**, **6**, and γ-CDx were purchased from NOF Corp., Avanti Polar Lipids, Inc. and Aldrich Chemical Co., Inc., respectively. C_{70} (>95%) was purchased from MER Co.

Preparation of $LMIC_{70}$ and CIC_{70} by a C_{70} exchange reaction: $LMIC_{70}$ **4**–**5**, composed of **4** and cationic lipid **5** in a 9:1 molar ratio, was prepared according to a previously described procedure.[9] The initial concentration of C_{70} in the C_{70}·γ-CDx complex, determined by measuring the absorbance of the solution at $\lambda = 381$ nm (molecular extinction coefficient for the C_{70}·γ-CDx complex $\varepsilon_{381} = 3.80 \times 10^4$ $dm^3 mol^{-1} cm^{-1}$),[14] was 0.20 mM in an aqueous solution (1.0 mL). After an aqueous solution of lipids (10 equiv of C_{70}) was added to the solution (1.0 mL, 2.00 mM), the final concentrations of the respective components were measured by using integral intensities from the ^{1}H NMR spectra: [γ-CDx] = 1.35 mM, [C_{70}] = 0.10 mM, and [lipids] = 1.00 mM (γ-CDx/C_{70}/lipids = 19:1:10).

Cerasomes from **1** and **2** in a 9:1 molar ratio and from **1**, **2**, and **3** in an 89:10:1 molar ratio were prepared according to a previously described procedure.[6] CIC_{70} **1**–**2** and CIC_{70} **1**–**2**–**3** were prepared by an exchange reaction between the cerasomes and the C_{70}·γ-CDx complex at 25 °C for 1 min. The final concentrations of the respective components were measured by using the integral intensities from the ^{1}H NMR spectra: [γ-CDx] = 1.35 mM, [C_{70}] = 0.10 mM, and [lipids] = 1.00 mM (γ-CDx/C_{70}/lipids = 19:1:10). After the exchange reaction, CIC_{70} **1**–**2** and CIC_{70} **1**–**2**–**3** was incubated for 24 h at room temperature.

Dynamic light scattering (DLS) analysis: The hydrodynamic diameters of $LMIC_{70}$ **4**–**5** and CIC_{70} **1**–**2**–**3** were measured by a dynamic light-scattering spectrophotometer (DLS-6000HL, Otsuka Electronics). The instrument consisted of a He/Ne laser operated at 633 nm and 10 mW. The data obtained were analyzed by using a cumulant method.

Zeta-potential measurements: The zeta potentials of $LMIC_{70}$ **4**–**5** and CIC_{70} **1**–**2**–**3** were measured on an instrument for electrophoretic light scattering with a laser Doppler system (Nano ZS90, Malvern Instruments Ltd., UK).

Cell cultures: HeLa cells were maintained in CO_2-independent medium (Gibco BRL) supplemented with 10% fetal calf serum at 37 °C in 5% CO_2. For experiments to determine the biological activities of CIC_{70}, the cells were seeded on culture plates at a density of 2.13×10^4 cells cm^{-2}. After growing overnight, the cells were used for the experiments.

Flow cytometry analysis: To analyze the intracellular uptake of cerasome and liposome, CIF and LMIF were prepared by supplying 0.25 mol% of **6** relative to the total lipids. Cells were incubated with CIF and LMIF at a concentration of 50 μM lipids for 24 h at 37 °C in 5% CO_2. After incubation, the cells were washed with phosphate-buffered saline (PBS), detached with 0.05% trypsin/0.02% EDTA-PBS (EDTA = ethylenediaminetetraacetic acid), and then suspended in PBS. The suspended cells were added directly to a FACS Calibur flow cytometer. Analysis was gated to include single cells on the basis of forward and side light scattering and was based on the acquisition of data from 10000 cells. Log fluorescence was determined and displayed as single-parameter histograms. The geometric mean fluorescence intensity was calculated by using the CellQuest 3.0 program (Becton–Dickinson).

Photodynamic activity experiments: The cells were incubated with 5 μM fullerenes of CIC_{70} or $LMIC_{70}$ for 24 h in the dark at 37 °C in 5% CO_2. After incubation, the cells were washed with PBS and exposed to light, and fresh medium was then supplied. Photoirradiation was carried out under 57 mW cm^{-2} light power at the cell level at 35 °C. To visualize the nonviable cells, the cells were stained with PI (1 μg mL^{-1}; Sigma–Aldrich)

for 10 min at room temperature following $LMIC_{70}$ or CIC_{70} treatment, photoirradiation, and 24 h incubation after photoirradiation. The cells were washed with PBS, the medium was replaced, and the cells were monitored by fluorescence microscopy.

Cell staining with PI and FITC-conjugated annexin V: For analysis of the mode of cell death induced by $LMIC_{70}$ or CIC_{70}, the cells were seeded on glass coverslips. After photoirradiation, the cells were stained with PI (1 $\mu g mL^{-1}$) and FITC-conjugated annexin V (0.5 $\mu g mL^{-1}$; BioVision) according to the manufacturer's instructions. After staining with PI and annexin V, the cells were mounted in a Permafluor instrument (Beckman Coulter Inc.) and monitored by fluorescence microscopy.

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