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Electronic supplementary information

Improved photodynamic activities of liposome-incorporated [60]fullerene derivatives bearing a polar group

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Experimental section

Materials: C_{60}-**1**, C_{60}-**2**, C_{60}-**3**, and **5** were prepared as described in previous papers.[11,12,S1] *γ*-CDx was purchased from Wako Pure Chemical Industries Ltd (Tokyo, Japan). 1,2-Dimyristoyl-*sn*-glycero-3-phosphocholine (**4**) was purchased from Funakoshi Co., Ltd (Tokyo, Japan). Rhodamine B-dipalmitoyl phosphatidylethanolamine (**6**) was obtained from Molecular Probes, Inc. (Eugene, OR, USA). Photofrin and 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) were purchased from Takeda Pharmaceutical Co., Ltd (Osaka, Japan) and Sigma-Aldrich (Milwaukee, WI, USA), respectively.

Preparation of liposomes: An appropriate amount of **4** or a mixture of **4** and **5** ([**4**]:[**5**] = 9:1 mol/mol) was dissolved in chloroform. The solvent was evaporated under a gentle stream of nitrogen, followed by a period under vacuum to remove any trace solvent. The resulting thin lipid films were hydrated on the wall of the vial above the phase transition temperature with an appropriate amount of water. The hydrated materials were subjected to five freeze-thaw cycles (–195 and 50 °C) to give unilamellar vesicles, which were extruded 11 times through 0.05 μm pores using a LiposoFast miniextruder from Avestin above the phase transition temperature. The resulting liposomes were uniform in size with a diameter of approximately 80 nm.

Preparation of the C_{60}-1, C_{60}-2 and C_{60}-3·*γ*-CDx complexes

C_{60}-**1**, C_{60}-**2** or C_{60}-**3** (6.94×10^{-6} mol) and *γ*-CDx (36.0 mg, 3.77×10^{-5} mol) were placed in an agate capsule with two agate-mixing balls. The materials were then mixed vigorously at 30 Hz for 20 min using a high-speed vibration mill (MM 200; Retsch Co., Ltd, Haan, Germany). The resulting solid mixture was suspended in H_2O or D_2O (1.5 mL) to produce a black emulsion, which was centrifuged for 20 min (18,000 ×*g*, 25 °C),

allowing for the removal of the non-dispersed C_{60} derivative from the solution. The concentrations of C_{60}-**1**, C_{60}-**2**, and C_{60}-**3** in the γ-CDx complex were determined based on the absorbance characteristics of the corresponding solutions at 324, 322, and 380 nm, respectively (the molar absorption coefficients for the water-soluble C_{60}-**1**, C_{60}-**2**, and C_{60}-**3**•γ-CDx complexes were $\varepsilon_{324} = 4.11 \times 10^4$, $\varepsilon_{322} = 4.31 \times 10^4$, and $\varepsilon_{380} = 3.60 \times 10^4$ dm^3 mol^{-1} cm^{-1}, respectively). These values were found to be 1.11, 1.27, and 1.79 mM in aqueous solution, respectively. These aqueous solutions were diluted to a final concentration of 1.00 mM.

Preparation of LMIC_{60}-1, LMIC_{60}-2 and LMIC_{60}-3 using a fullerene exchange reaction: LMIC_{60}-**1**, LMIC_{60}-**2**, and LMIC_{60}-**3** were prepared using an exchange reaction between the liposomes and the C_{60}-**1**·γ-CDx, C_{60}-**2**·γ-CDx, and C_{60}-**3**·γ-CDx complexes, as described in previous works.[3] The final concentrations of the respective components were [C_{60}-**1**, C_{60}-**2**, or C_{60}-**3**] = 0.05 mM and [lipids] = 1.00 mM ([C_{60}-**1**, C_{60}-**2**, or C_{60}-**3**]/[lipids] = 5 mol%).

Preparation of liposome-4-6, liposome-4-5-6, RhB-LMIC_{60}-1, RhB-LMIC_{60}-2 and RhB-LMIC_{60}-3: Liposomes containing 0.25 mol% **6** were prepared by the injection of a methanol solution of **6** ([**4**]:[**6**] = 1:0.0025 mol/mol) into an aqueous solution of liposome-**4**, liposome-**4**+**5**, LMIC_{60}-**1**, LMIC_{60}-**2**, or LMIC_{60}-**3**.

Spectrophotometric assay: The absorbance spectra of the LMIFullerenes prepared in the current study were scanned using a UV-2550 spectrophotometer (Shimadzu Corporation, Kyoto, Japan). Fluorescence spectra of **6** in liposomes were obtained using an F-4500 fluorescence spectrophotometer (Hitachi Ltd, Tokyo, Japan). The excitation and emission wavelengths were set to 555 and 560–700 nm, respectively.

Dynamic light scattering (DLS) analysis and zeta-potential measurements: The hydrodynamic diameters and the zeta potentials of the liposomes were measured on an

electrophoretic light scattering instrument equipped with a laser Doppler system (Zetasizer Nano ZS, Malvern Instruments Ltd., Malvern, UK).

Photodynamic activity experiments: 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 aimed at determining the photodynamic activities of the LMIFullerenes, the cells were seeded into 48-well culture plates at a density of 8.55×10^4 cells per well. After growing overnight, the cells were incubated with the LMIfullerenes for 24 h in the dark. The cells were washed with PBS and exposed to light for 30 min at room temperature. Light irradiation was achieved using a xenon lamp (MAX-301, 300 W; Asahi Spectra Co., Ltd, Tokyo, Japan) equipped with a VIS mirror module (385−740 nm) and a long-pass filter with a cut-off at 610 nm. The power of the light was 9 mW cm^{-2} (610–740 nm) at the cell level. The viability of the cells was measured as the ratio (%) of viable cells in the treatment groups compared with the number of viable cells in the untreated group. A WST-8 assay was carried out 24 h after photoirradiation using the Cell Counting Kit-8 (Dojindo Laboratories, Kumamoto, Japan) according to the manufacturer's instructions.

Identification of reactive oxygen: The ROS generated as a consequence of the ABDA bleaching method (Sigma-Aldrich Corp.) were detected according to a previously reported method.[16] ABDA was used as a DMSO solution ([ABDA] = 2.50 mM). The concentrations of the fullerene and ABDA in the mixture were determined to be 15 and 25 μM, respectively. Oxygen was bubbled through all of the sample solutions for 30 min before photoirradiation to generate aerobic conditions. Photoirradiation was achieved using a xenon lamp (SX-UID500X, 500 W; Ushio Inc., Tokyo, Japan) equipped with a long-pass filter with a cut-off at 620 nm. The light was cooled by passing it through a water filter. The power of the light was 16 mW cm^{-2} (over 610 nm) at the sample level.

Reference

S1 Y. Murakami, A. Nakano, A. Yoshimatsu, K. Uchitomi, and Y. Matsuda, *J. Am. Chem. Soc.,* 1984, **106**, 3613.

Table S1 Average hydrodynamic diameters D_{hy} (nm), as determined using a light-scattering method at 25°C in the absence and presence of the fullerene derivatives.

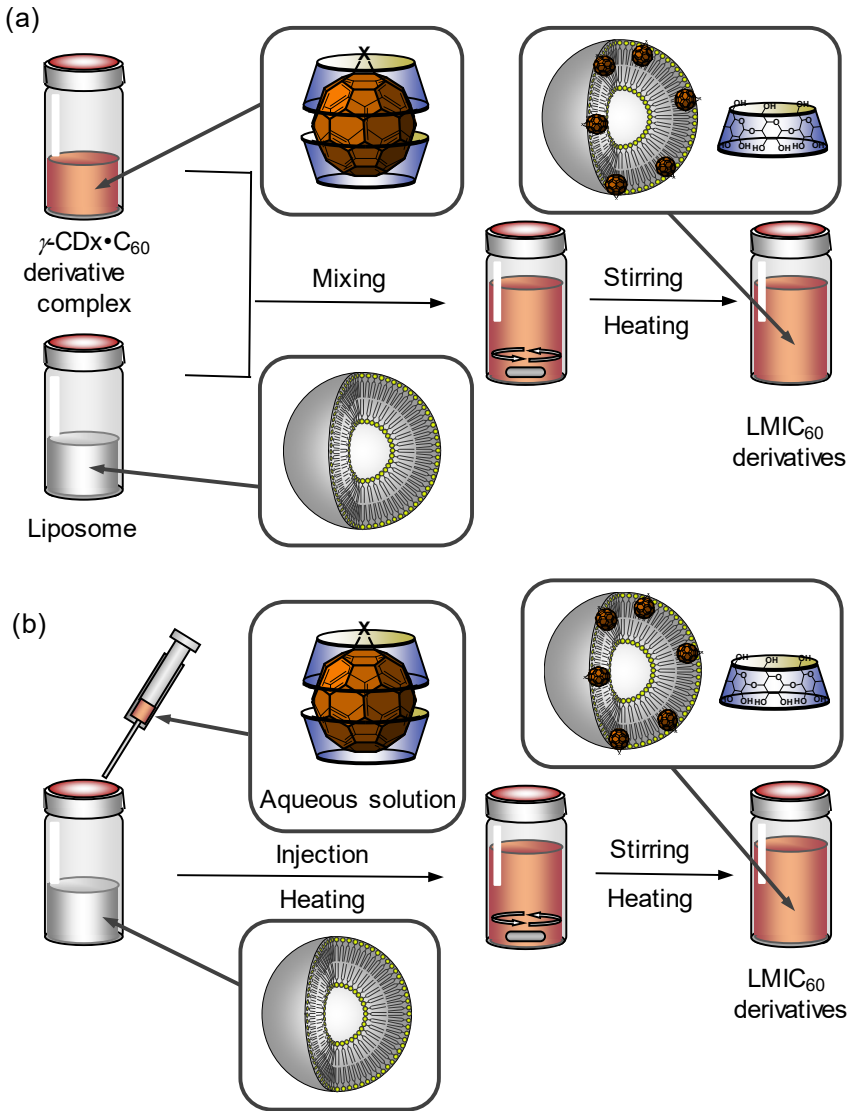
	Before addition of the γ-CDx complex		After addition of the γ-CDx complex	
	Average D_{hy}/nm	PDI[a]	Average D_{hy}/nm	PDI[a]
$LMIC_{60}$-**1**	119	0.11	105	0.26
$LMIC_{60}$-**2**	143	0.16	138	0.12
$LMIC_{60}$-**3**	129	0.08	112	0.21

[a]PDI: Polydispersity index.

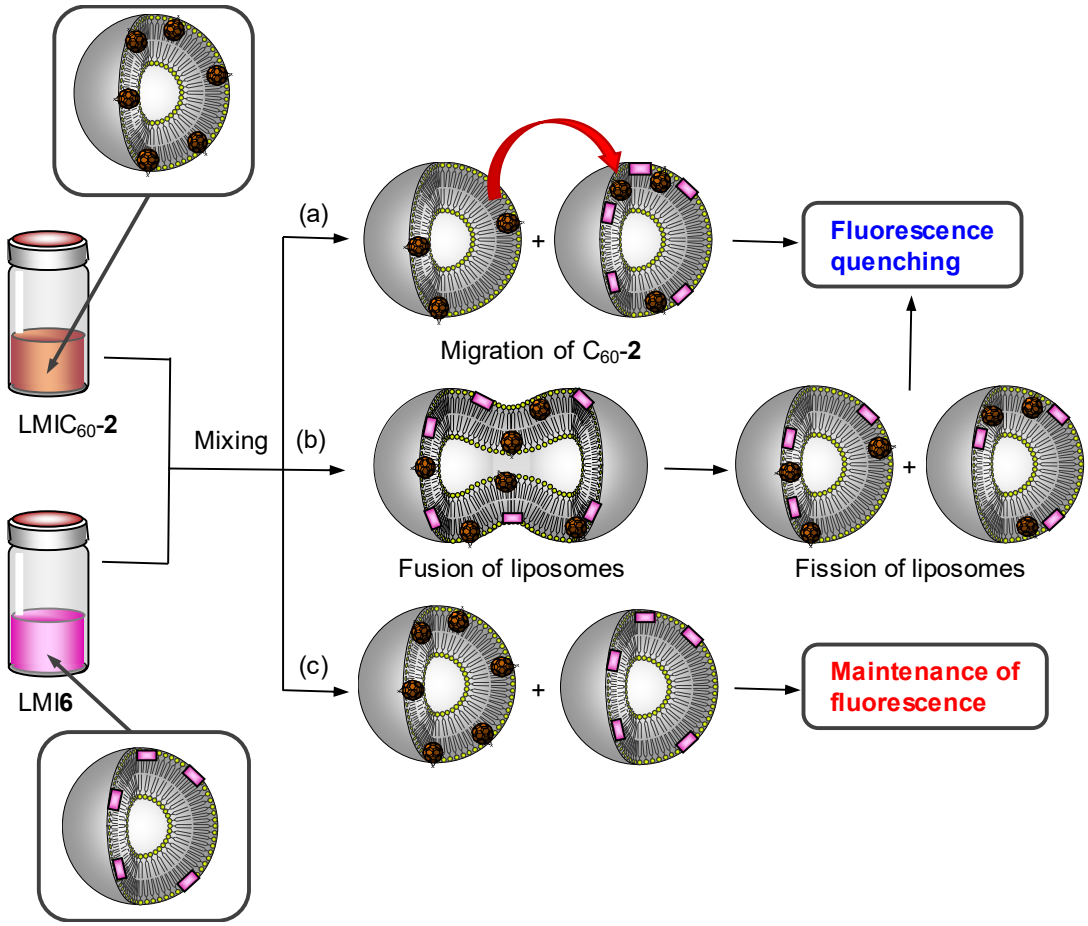
Table S2 Zeta potentials (mV) of $LMIC_{60}$, $LMIC_{70}$, $LMIC_{60}$-**1** and $LMIC_{60}$-**2** at 25 °C before and after the exchange reaction.

LMIFullerene	Liposomes	Zeta potential (mV)	
		Before addition of the γ-CDx·fullerene·complex	After addition of the γ-CDx·fullerene·complex
$LMIC_{60}$	**4** + **5**[a]	40.6	31.9
$LMIC_{60}$-**1**	**4** + **5**[a]	40.6	24.8
$LMIC_{60}$-**2**	**4** + **5**[a]	40.6	34.9
$LMIC_{60}$-**3**	**4** + **5**[a]	40.6	33.1

[a][**5**]/[**4** + **5**] = 10 mol%



Scheme S1 Exchange method used for the preparation of the $LMIC_{60}$ derivatives. (a) Rapid mixing of two solutions and (b) Injection.



Scheme S2 Changes in the fluorescence properties after the mixing of aqueous solutions of liposome-**4**-**6** or liposome-**4**-**5**-**6** and LMIC$_{60}$-**2** consisting of liposome-**4** or liposome-**4**-**5**. (a) Migration of C$_{60}$-**2**, (b) fusion and fission of liposomes and (c) retention of liposome-**4**-**6** or liposome-**4**-**5**-**6** and the LMIC$_{60}$-**2** consisting of liposome-**4** or liposome-**4**-**5**.

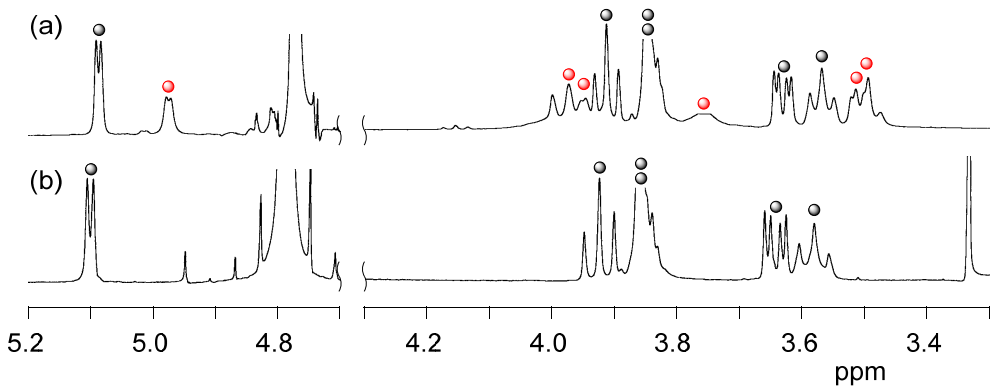


Fig. S1 Partial ^{1}H NMR spectra of the γ-CDx•C_{60}-**3** complex (e) before and (f) after the addition of liposome-4-5 (●: free γ-CDx, ●: γ-CDx in the γ-CDx• C_{60}-**3** complex, [C_{60}-**3**]/[**4** + **5**] = 5 mol%, [**4** + **5**] = 2.0 mM).

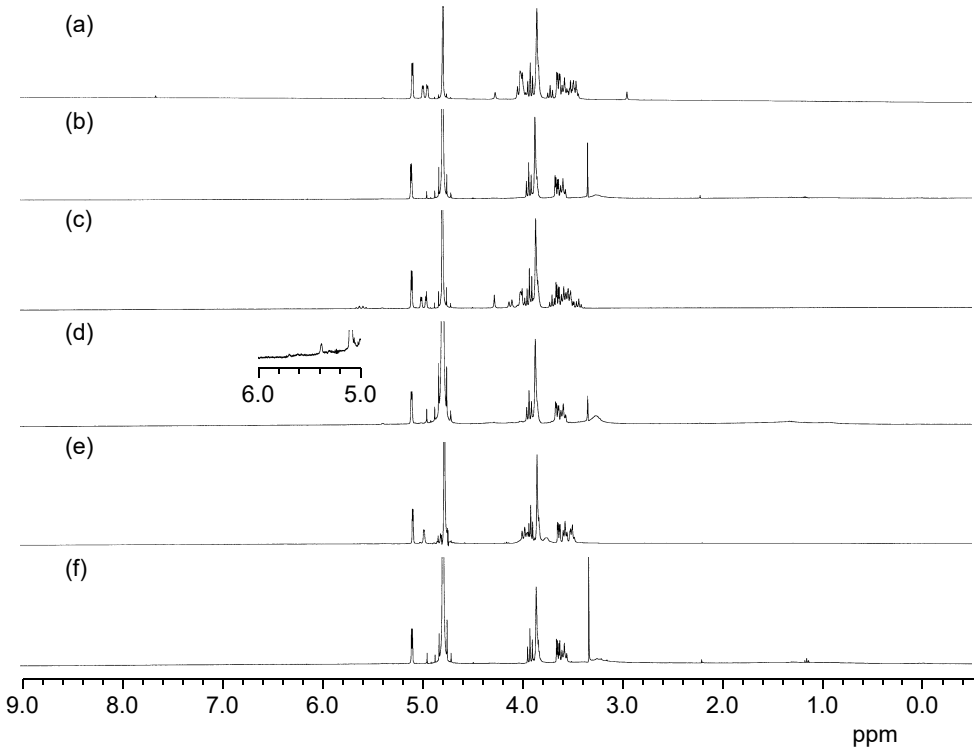


Fig. S2 Complete ^{1}H NMR spectra of the *γ*-CDx•C_{60}-**1** complex (a) before and (b) after the addition of liposome-**4**-**5**; *γ*-CDx•C_{60}-**2** complex (c) before and (d) after the addition of liposome-**4**-**5** (the inset shows the 5.0–6.0 ppm region.); and *γ*-CDx•C_{60}-**3** complex (e) before and (f) after the addition of liposome-**4**-**5** ([C_{60}, C_{60}-**1**, C_{60}-**2** or C_{60}-**3**]/[**4** + **5**] = 5 mol%, [**4** + **5**] = 2.0 mM).

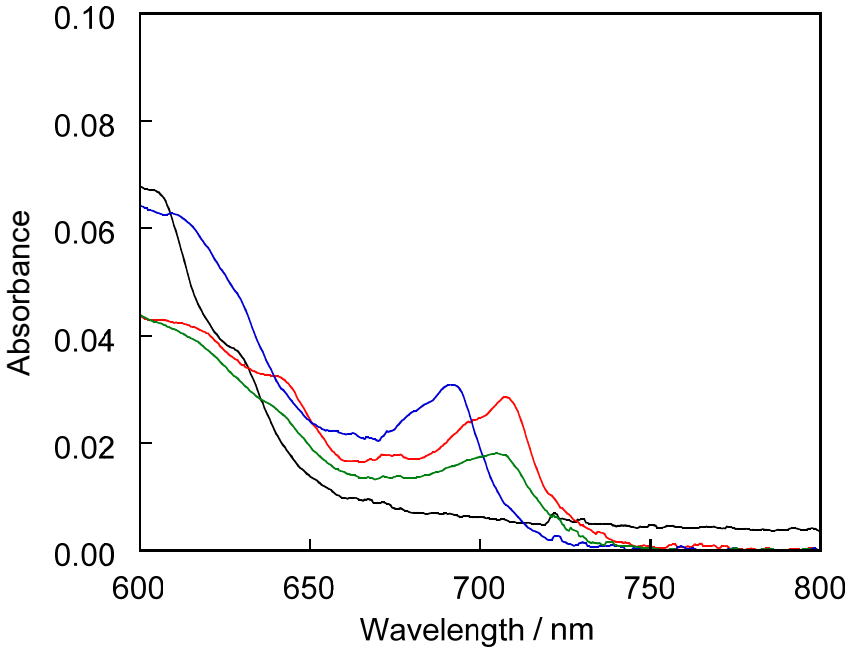


Fig. S3 UV-vis absorption spectra of the γ-CDx•fullerene complexes. UV-vis absorption spectra of the γ-CDx•C_{60} (black line), γ-CDx•C_{60}-**1** (red line), γ-CDx•C_{60}-**2** (blue line) and γ-CDx•C_{60}-**3** (green line) complexes ([γ-CDx complex] = 1.0 mM). All spectra were recorded at 25 °C with a 1 mm cell.

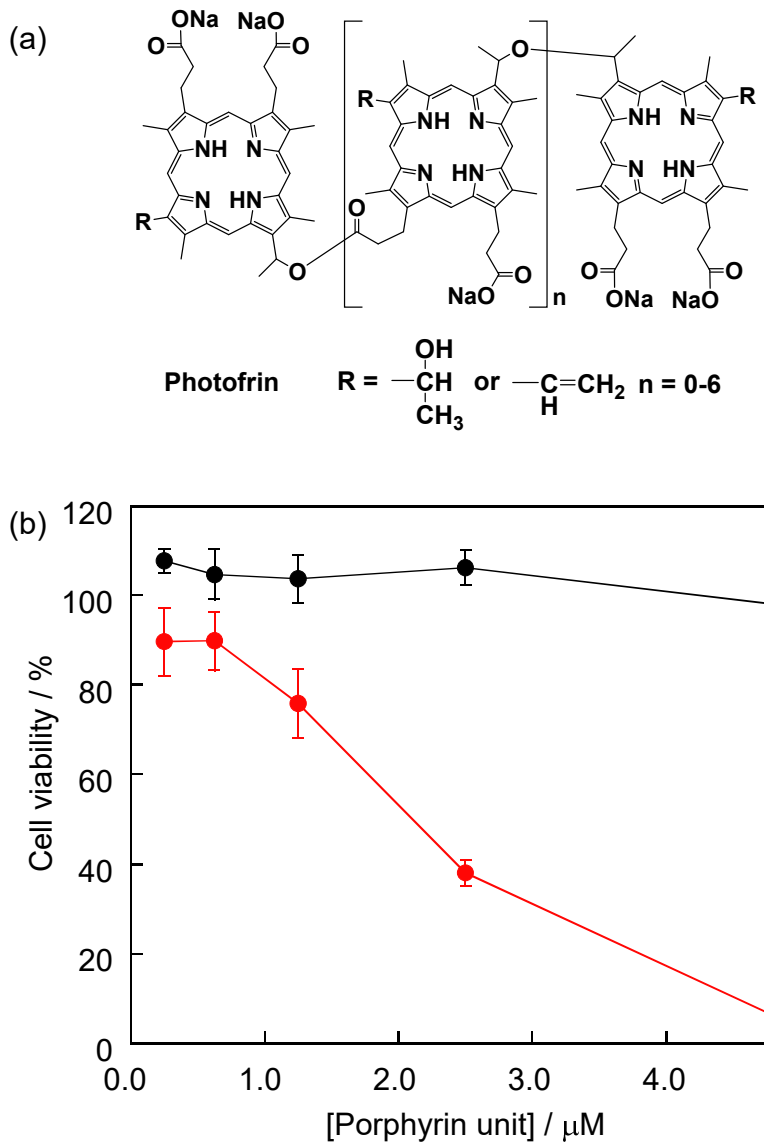


Fig. S4 (a) Structure of photofrin and (b) the cytotoxicity of photofrin in the absence (black line) and the presence of light irradiation (red line) (610–740 nm, 30 min). Cell viability was evaluated according to the WST-8 method. Each value represents the mean ± standard deviation (SD) of three experiments (n = 3).