Supporting Information

Preparation and characterization of phospholipid-conjugated indocyanine green as a near-infrared probe

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Materials and Methods

Molecular Modeling

The three-dimensional structure of ICG was constructed using MOE (version 2009, CCG Inc., Montreal, Canada) according to PubChem Compound CID 11967809 (National Center for Biotechnology Information, Bethesda MD, USA). The three-dimensional structure of DOPE was constructed using VMD (http://www.ks.uiuc.edu/). Then, the three-dimensional structures of ICG and DOPE were used to construct that of iDOPE using the build command in MOE. Molecular mechanics calculations were performed to obtain the local minimum structures of ICG, DOPC, and iDOPE, using the AMBER99 force field in MOE. Then, the lipophilic colors of the surfaces of ICG, DOPC, and iDOPE were calculated from the Wildman and Crippen SlogP parameter in MOE.

Syntheses

iDOPE was prepared as shown in Scheme 1. The ICG fluorophore was synthesized from a single starting material, 2,3,3-trimetyl-4,5-benzo-3*H*-indole (**1**). **1** (3.1 g, 15 mmol) was added to acetonitrile (10 mL) with 3-bromo-1-propionic acid (1.5 g, 9.8 mmol), and the reaction solution was stirred at 65 °C for 16 h and then cooled to room temperature. Ethyl acetate (50 mL) was then added to the solution and the product, 2,3,3-trimetyl-1-(2-carboxyethyl)-4,5-benzoindolium bromide (**2**), was separated by filtration and rinsed with acetone, giving **1** as gray crystals (1.68 g, 47%). Then **1** (3.1 g, 15 mmol) and 1,4-butane sultone (2.1 g, 15 mmol) were mixed in a round-bottomed flask (25 mL) under a nitrogen atmosphere, and the reaction mixture was stirred at 80 °C for 4 h and then cooled to room temperature. On addition of acetone to the reaction

mixture the residue dissolved, and the product was crystallized. The reaction product, 2,3,3-trimethyl-1-(sulfobutyl)-4,5-benzoindolium inner salt (**3**), was separated by filtration and rinsed with acetone to give **3** as gray crystals (1.17 g, 23%). A mixture of **3** (1.04 g, 3.0 mmol) and glutaconaldehyde dianil hydrogen chloride salt (0.94 g, 3.3 mmol) was stirred at 120 °C for 1 h and then cooled to room temperature to crystallize the product, 2-(6-acetoanilido-1,3,5-hexatrienyl)-3,3-dimethyl-1-(sulfobutyl)-4,5-benzo[*e*]indolium inner salt (**4**). This salt was separated by filtration and rinsed with acetone to give **4** as dark-purple crystals (0.97 g, 58%). A pyridine solution (8 mL) of **2** (0.55 g, 1.5 mmol) and **4** (0.80 g, 1.5 mmol) was stirred at 50 °C for 45 min, and then cooled to room temperature. The reaction mixture was condensed under reduced pressure, and then distilled water (20 mL) and 10% HCl were added to adjust the pH to 3–4. The crude product was filtered from the reaction mixture and dried. After crystallization using methanol/chloroform (volume ratio: 5/1, 20 mL) and recrystallization in methanol/chloroform, the product, 4-(2-((1*E*,3*E*,5*E*,7*E*)-7-(3-(2-carboxyethyl)-1,1-dimethyl-1*H*-benzo[*e*]indol-2(3*H*)-ylide ne)hepta-1,3,5-trienyl)-1,1-dimethyl-1*H*-benzo[*e*]indolium-3-yl)butane-1-sulfonic acid (**5**), was obtained as dark-green crystals (0.38 g, 37%). In a mixed organic solvent of acetonitrile (3 mL) and chloroform (10 mL), **5** (0.5 g, 0.73 mmol) and *N*-hydroxysuccinimide (0.17 g, 1.5 mmol) were dissolved, and a chloroform solution (2 mL) of *N*,*N*′-dicyclohexylcarbodiimide (0.30 g, 1.5 mmol) was added dropwise at 2 °C under a nitrogen atmosphere. The reaction solution was stirred at room temperature for 30 min. The residue was separated by filtration and the reaction solution was condensed

under reduced pressure, with the temperature kept below 25 °C. The crude product was rinsed with ethyl acetate and acetone.

The crude product (0.66 g) was dissolved in chloroform (10 mL) in a round-bottomed flask (25 mL) and a chloroform solution (5 mL) of 1,2-dioleoyl-*sn*-glycero-3-phosphoethanolamine (0.54 g, 0.73 mmol) and triethylamine (0.10 g, 1.0 mmol) was added dropwise to the reaction solution over 20 min. The reaction mixture was stirred at room temperature for 2.5 h and the residue was separated by filtration. The obtained solution was condensed and purified using silica column chromatography with methanol/chloroform (volume ratio: 1/4-1/3). The product **6** was obtained as a green powder (0.37 g, 36%).

5: ^{1}H NMR (DMSO-d_6): δ (ppm) 1.90 (s, 14H); 2.54 (t, 2H); 2.75 (t, 2H); 4.25 (br, 2H); 4.38 (br, 2H); 6.34 (d, 1H); 6.52~6.68 (m, 3H); 7.44~7.59 (m, 6H); 7.76~7.97 (m, 3H); 8.01~8.10 (m, 6H); 8.20~8.29 (m, 2H); 12.6 (br, 1H).

6: ^{1}H NMR (DMSO-d_6): δ (ppm) 0.83 (t, 6H), 1.15–1.22 (m, 52H), 1.48 (t, 4H), 1.82 (q, 4H), 2.22 (t, 4H), 2.55 (m, 4H), 3.03 (q, 6H), 3.16 (t, 4H), 3.66 (quintet, 2H), 3.75 (t, 2H), 4.07 (m, 1H), 4.25 (t, 3H), 4.41 (t, 2H), 5.07 (m, 1H), 5.29 (m, 4H), 6.36 (d, 1H), 6.52–6.62 (m, 3H), 7.47 (m, 2H), 7.61–7.66 (m, 3H), 7.65 (m, 2H), 7.90–8.07 (m, 6H), 8.22 (d, 2H), 8.73 (t, 1H), 10.0 (br, 1H).

NMR Measurements

The ^{1}H NMR spectra of iDOPE (**6** in Scheme 1) and intermediate (**5** in Scheme 1) were run at 300 MHz in DMSO-d_6 using a Lambda-300 NMR spectrometer (JEOL, Tokyo, Japan).

FTIR Measurements

Infrared spectra of iDOPE (**6** in Scheme 1) and intermediate (**5** in Scheme 1) were recorded as potassium bromide pellets using an IRPrestige-21 spectrometer (Shimadzu, Kyoto, Japan) in the 4000–400 cm^{-1} region.

HPLC Analysis

iDOPE was analyzed by HPLC using a Shimadzu LC-20A chromatography system (Shimadzu, Kyoto, Japan) equipped with CHEMCOSORB 7-ODS-L (7 μm, 4.6 mm × 150 mm, Chemco Scientific, Osaka, Japan). The column temperature was controlled at 45 °C using a CTO column oven (Shimadzu).

Eluent: [0.02M KH_2PO_4 (pH3.0 with H_3PO_4)/CH_3OH (95/5)]/CH_3OH/THF: 21/50/29. Flow rate: 1.2 mL/min.

UV-vis-NIR and Fluorescence Spectroscopy

The absorption spectra of iDOPE and ICG dissolved in organic solvents were obtained using a UV-vis-NIR spectrometer (UV-3500, Shimadzu, Kyoto, Japan).

Fluorescence emissions from iDOPE and ICG were observed using a fluorescence spectrometer (F-4500, Hitachi, Tokyo, Japan).

Singlet Oxygen Measurements

The luminescence from singlet oxygen was measured using a high-sensitivity detection method (NIR-PII System, Hamamatsu Photonics, Hamamatsu, Japan). The excitation pulse was obtained using an optical parametric oscillator (L5996, Hamamatsu Photonics), which was excited using an Nd:YAG laser (Surelite I-20, Continuum, Santa Clara, CA, USA). The pulse width and intensity were approximately 5 ns and 300 μJ/pulse, respectively, and the repetition rate was 20 Hz. The singlet oxygen luminescence was monitored using an IR-gated image intensifier (NIR-PII, Hamamatsu Photonics) after passage through a polychromator (MS257, Oriel Instruments, Stratford, CT, USA). We began measurements at 5 μs after excitation, and the exposure time was 100 μs. The signals were accumulated by repeated detection (2000 times). The calibration of the wavelength was performed using a spectral calibration lamp (Krypton type, Oriel Instruments, Stratford, CT, USA).

Measurement of Photothermal Properties

iDOPE and ICG in a mixed organic solvent of $CH_3OH/CHCl_3$ (volume ratio: 1/9, 1.5 mL) in a quartz cuvette were irradiated using an 808-nm laser (LU0808T020 - E105N12A, Hanamura Optics, Yokohama, Japan) at 0.5 W/cm^2. The solution temperature was measured at 1-s intervals using an SF Series Temperature Probe (AM-8000, Anritsu Meter, Tokyo, Japan) under stirring.

Preparation of Liposomes

1. LP-iDOPE

Cholesterol (1.0 mM, Nippon Fine Chemical Co., Ltd., Tokyo, Japan), 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC, 10 mM, NOF Corporation, Tokyo, Japan), *N*-(carbonyl-methoxypolyethyleneglycol 5000)-1,2-distearoly-*sn*-glycero-3-phosphoethanolamine sodium salt (DSPE-PEG, 0.5 mM, NOF Corporation), and iDOPE (5.0×10^{-5}, 1.0×10^{-4}, 5.0×10^{-4}, and 1.0×10^{-3} mM, iDOPE/DOPC molar ratio of 0.005, 0.01, 0.05, and 0.1) were dissolved in a mixed organic solvent of $CH_3OH/CHCl_3$ (volume ratio: 1/9). A thin lipid film was formed by removal of the solvent under reduced pressure. After addition of an aqueous buffered solution (phosphate buffered saline [PBS], pH = 7.4) at room temperature, the liposome dispersion was filtered through a 0.1-mm pore polycarbonate filter attached to a LiposoFast-Stabilizer (Avestin Inc., Ottawa, Canada).

2. LP-ICG

Cholesterol (1.0 mM, NOF Corporation), DOPC (10 mM, NOF Corporation), and DSPE-PEG (0.5 mM, NOF Corporation) were dissolved in a mixed organic solvent of $CH_3OH/CHCl_3$ (volume ratio: 1/9). A thin lipid film was formed by removal of the solvent under reduced pressure. The dried thin lipid film, consisting of cholesterol, DOPC, and DSPE-PEG, was hydrated with PBS containing ICG (1.0×10^{-4}, 2.5×10^{-4}, and 5.0×10^{-4} mM, ICG/DOPC molar ratio of 0.01, 0.025, and 0.1) and the liposome dispersion was filtered through a 0.1-mm pore polycarbonate filter attached to a LiposoFast-Stabilizer (Avestin Inc.)

Gel-filtration Chromatography

LP-iDOPE and LP-ICG were separated from free iDOPE and ICG, respectively, using PD-10 Desalting Columns containing Sephadex G-25 (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The absorbance of the obtained fraction (1 mL) was measured using a UV-vis-NIR spectrometer (UV-1800, Shimadzu, Kyoto, Japan).

Measurement of Particle Size

Particle size analysis of LP-iDOPE was performed by dynamic light-scattering measurements (SZ-100, HORIBA Ltd., Kyoto, Japan).

Biodistribution of iDOPE by Liposome Formulas

Female BALB/c Slc (nu/nu) mice that were 5-6 weeks old were purchased from the Japan SLC, Inc. (Hamamatsu, Japan). Tumors were initially established by subcutaneously injecting a mixture of 1×10^{-7} A549 cells (adenocarcinomic human alveolar basal epithelial cells), matrigel, and DMEM. Tumor volume was calculated $\pi/6ab^2$, where a is the length and b is the width of the tumor. Mice received an intravenous injection of LP-iDOPE, when the tumors reached a volume of 170 mm^3. The biodistribution of LP-iDOPE was studied by injecting LP-iDOPE, containing 14.0 mg/kg of iDOPE, intravenously through a tail vein of mice bearing A549 tumors and imaged 24, 48, and 144 h after the injection with an In-Vivo MS FX PRO imaging system (Carestream Health, New York, USA)

Results

Molecular Modeling

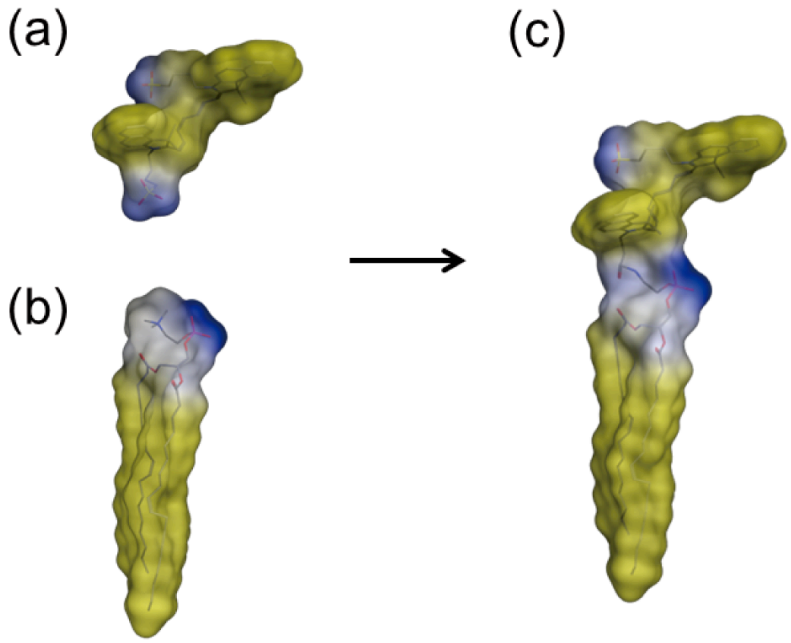

Figure S1. Predicted three-dimensional structures of (a) ICG, (b) DOPC, and (c) iDOPE. Blue: hydrophilic region; yellow: hydrophobic region.

NMR Measurements

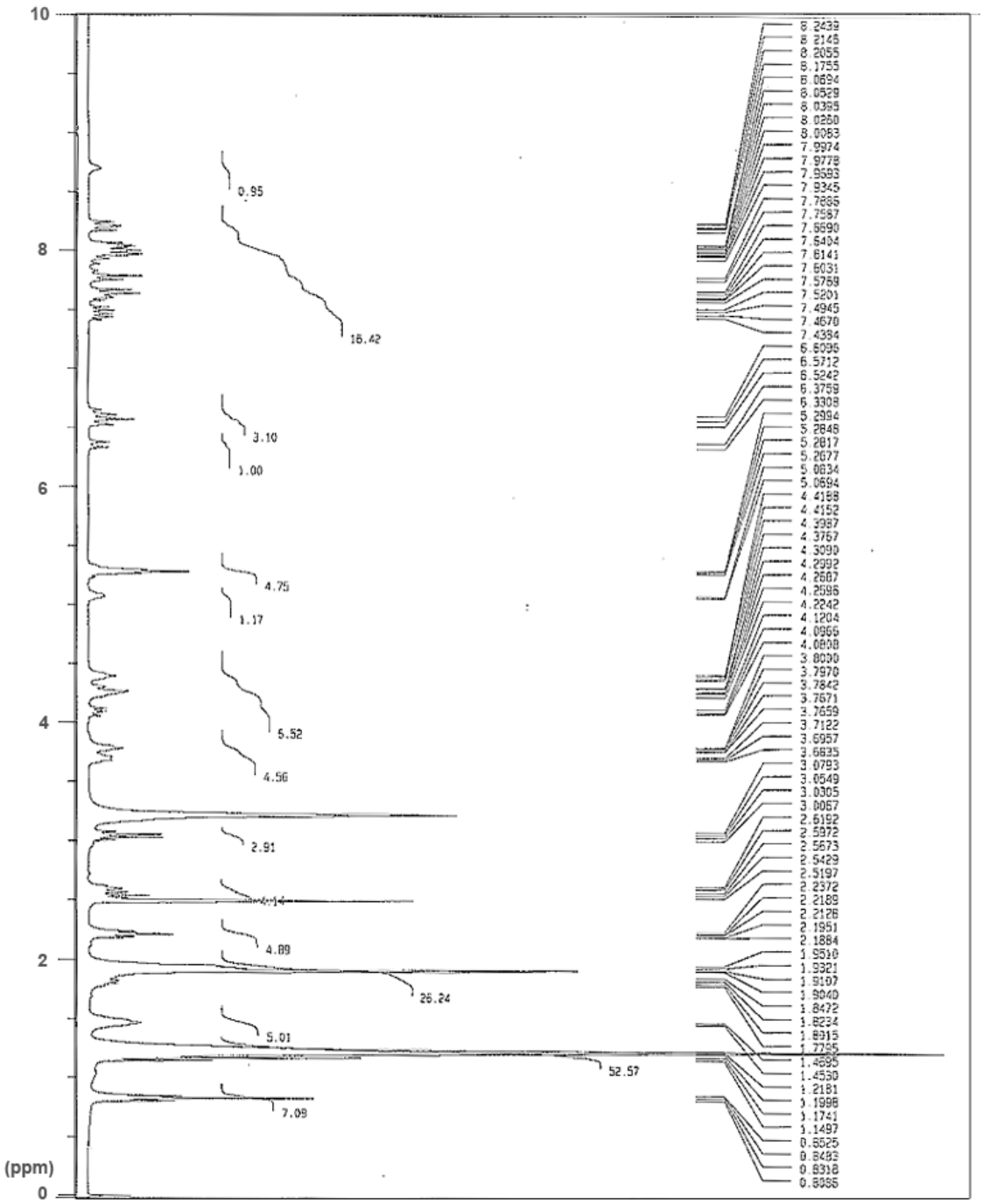

Figure S2. ^{1}H-NMR spectrum of iDOPE.

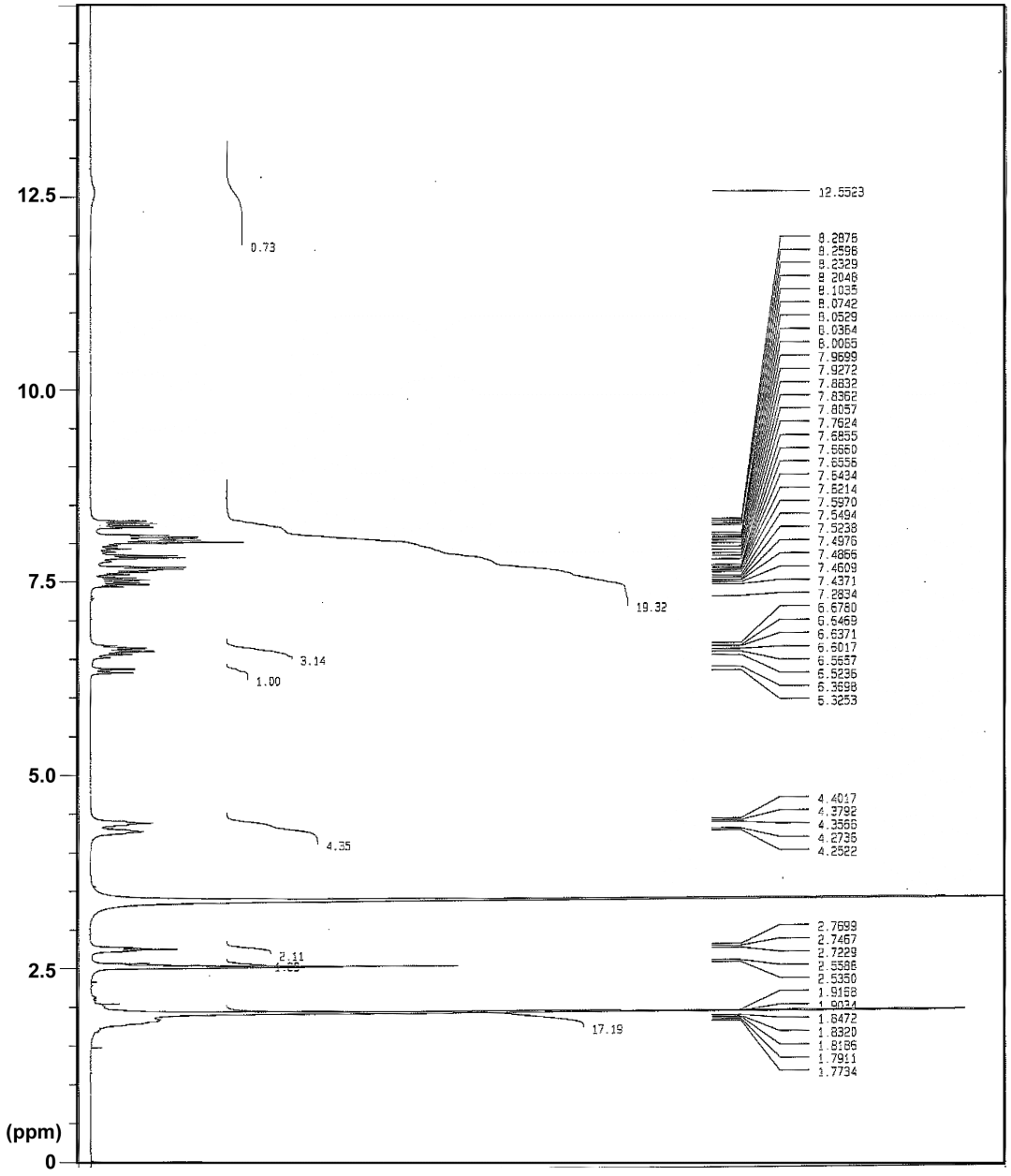

Figure S3. ^{1}H-NMR spectrum of intermediate **5** in Scheme 1.

FTIR Measurements

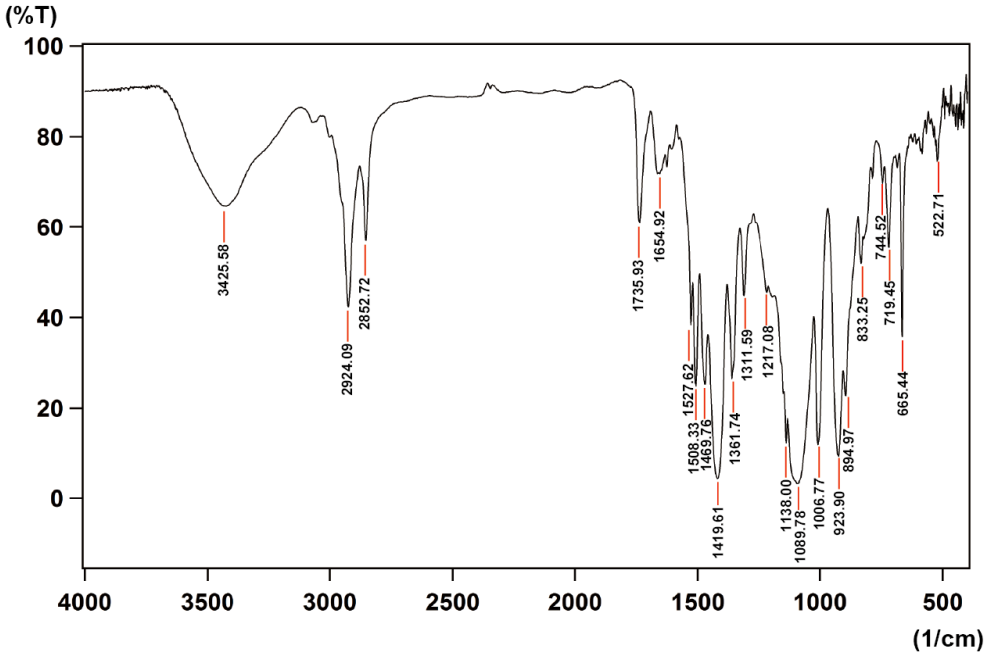

Figure S4. FTIR spectrum of iDOPE.

Table S1

Observed assignments of iDOPE

Experimental Frequency (cm^{-1})	Vibrational Assignments
2924.09	νCH
2852.72	νCH
1753.93	νC=O
1654.92	νC=C, γCN +γNH + νC=O
1527.62	νC=C
1508.33	νC=C
1089.78	νS-O

ν: stretching, γ: out-of-plane bending

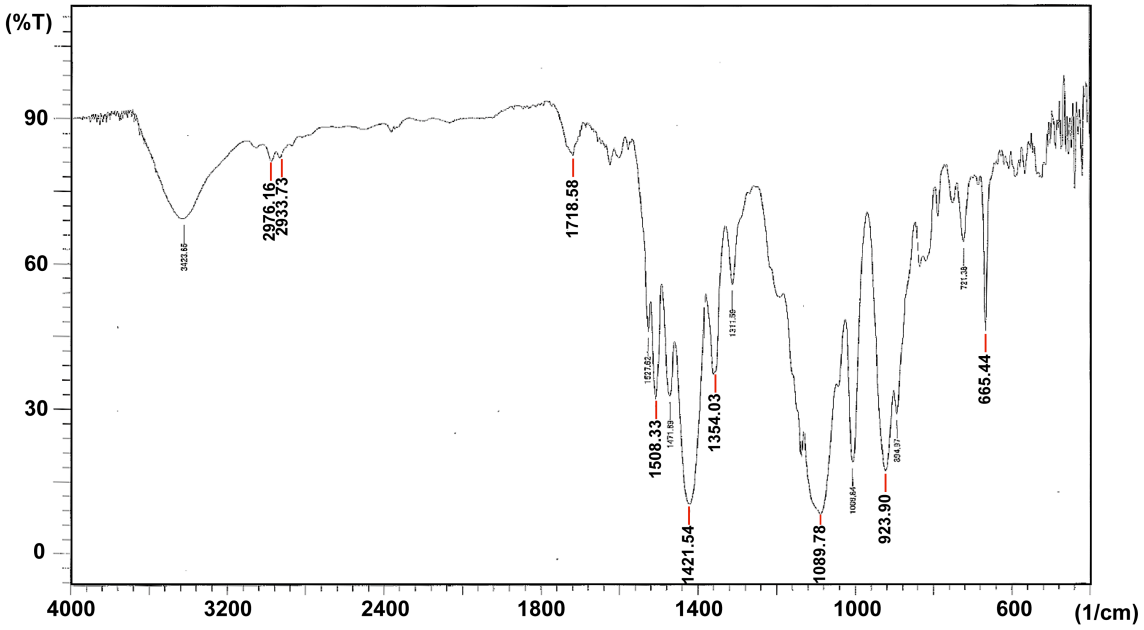

Figure S5. FTIR spectrum of intermediate **5** in Scheme 1.

Table S2

Observed assignments of intermediate **5** in Scheme 1

Experimental Frequency (cm^{-1})	Vibrational Assignments
2976.16	νCH
2933.73	νCH
1718.58	νC=O
1508.33	νC=C
1421.54	νC=C
1354.03	νS(=O)2
1089.78	νS-O
923.90	γCH
665.44	γCC

ν: stretching, γ: out-of-plane bending

HPLC Analysis

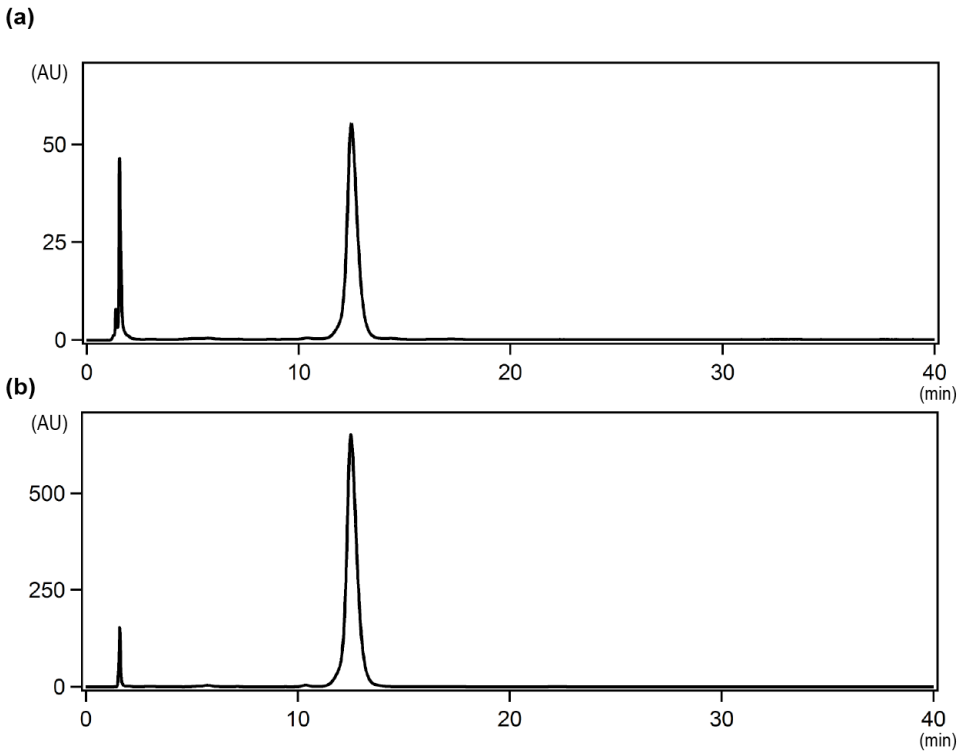

Figure S6. HPLC chromatograms of iDOPE: (a) 254 nm and (b) 780 nm.

Singlet Oxygen Measurements

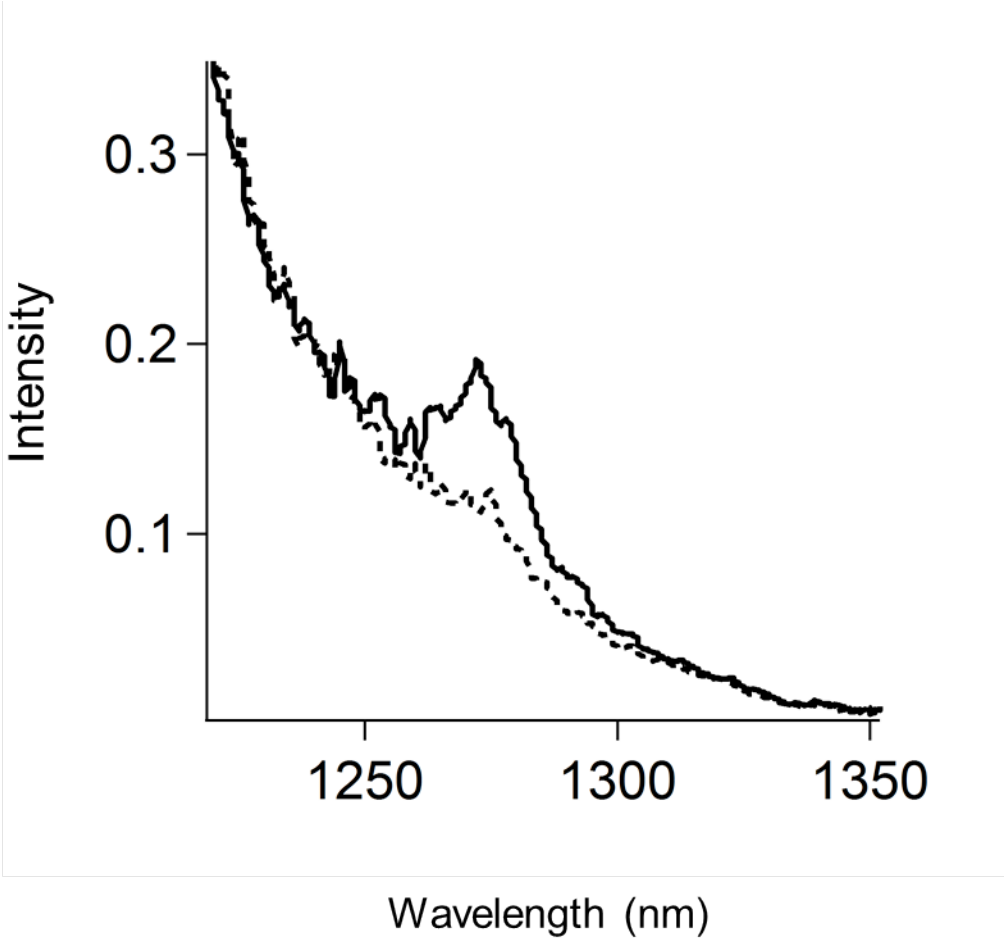

Figure S7. Luminescence spectrum of singlet oxygen in oxygen-saturated $CHCl_3$ solution of iDOPE (32 μM, solid line). The dotted line represents the luminescence spectrum of iDOPE in the presence of β-carotene (6 μM).

The excitation wavelength was 690 nm. The pulse width and intensity were approximately 10 ns and 300 μJ/pulse, respectively, and the repetition rate was 30 Hz. Measurements were begun at 30 μs after excitation, and the exposure time was 200 μs. The signals were accumulated by repeated detection (1500 times).

Dynamic Light-scattering Measurements

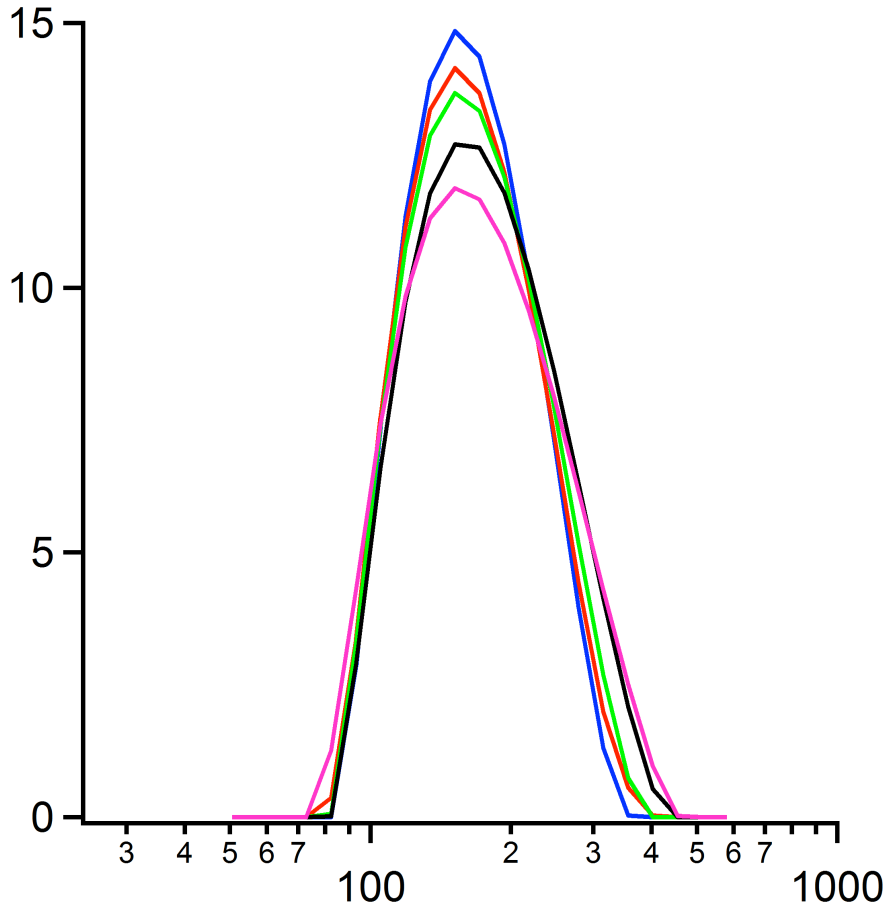

Figure S8. Diameter distribution of LP-iDOPE. iDOPE/DOPC molar ratios are 0 (blue line), 0.005 (red line), 0.01 (green line), 0.05.(black line), and 0.1 (magenta line).

Gel-filtration Chromatograph

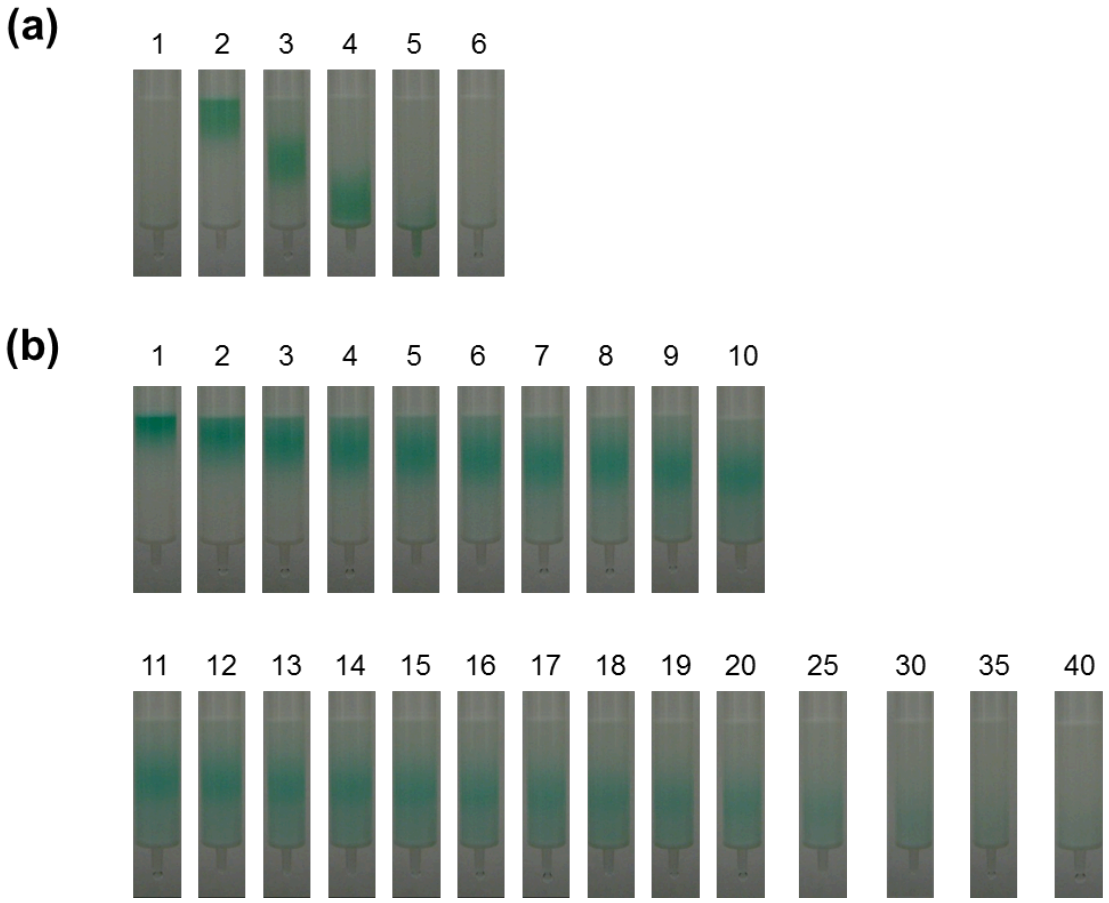

Figure S9. Gel-filtration chromatograph of (a) LP-ICG (ICG/DOPC molar ratios 0.01 and 0.1 mM of ICG) and (b) ICG (0.1 mM) on PD-10 Desalting Columns containing Sephadex G-25. Photographs of gel-filtration column were taken during the fraction tube exchange at every 1 ml elusion.