Electronic Supplementary Information

Guanidinium-appended γ-Cyclodextrin: Efficient Fullerene Solubiliser for Enhanced Photodynamic Therapy

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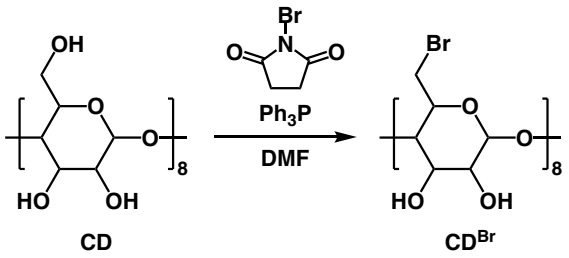
1. General

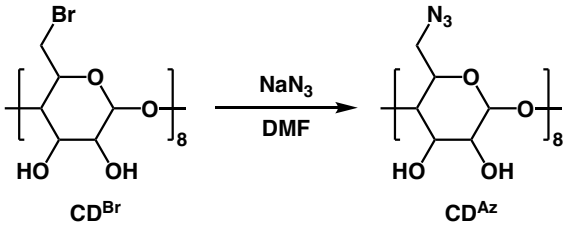
^{1}H and ^{13}C NMR spectra were recorded on a Bruker type AVANCE III 400 or 500 spectrometer (400 MHz and 500 MHz for ^{1}H; 101 MHz or 126 MHz for ^{13}C), where chemical shifts for ^{1}H NMR spectroscopy were determined with respect to non-deuterated solvent residues; DMSO (δ 2.50) and H_2O (δ 4.79), and those for ^{13}C NMR spectroscopy were determined with respect to DMSO (δ 39.5). Electrospray ionisation mass (ESI-MS) spectrometry was performed on a Bruker Daltonics Impact II QTOF spectrometer. Photoirradiation was carried out with Hangzhou Hengli Electronic Technology monochromatic LED light source model OR-GYD70 (λ = 370 nm, 525 nm and 630 nm, and 8300 K white light). Electronic absorption spectra were recorded on an IMPLEN nanophotometer model NP80 or a Molecular Devices model SpectraMax iD5 multi-mode microplate reader. Fluorescence spectra were recorded on a Molecular Devices model SpectraMax iD5 multi-mode microplate reader. Dynamic light scattering (DLS) analyses were performed using a Malvern model Zetasizer Pro equipped with He-Ne (633 nm) laser.

Unless otherwise noted, reagents and solvents were used as received from commercial sources without further purification. Triphenylphosphine (PPh_3), *N*-bromosuccinimide (NBS), fullerene C_{60} and fullerene C_{70} were purchased from Macklin. γ-Cyclodextrin, sodium methoxide and Rose Bengal were purchased from Dieckmann. Sodium azide was purchased from Sigma Aldrich. Concentrated aqueous ammonia (NH_3) was purchased from Scharlau. 1*H*-pyrazolecarboxamidine hydrochloride was purchased from Bidepharm. *N*,*N*-diisopropylethylamine (DIPEA) was purchased from Energy. Dulbecco's Modified Eagle Medium (DMEM, low glucose, GlutaMAX Supplement), Fetal Bovine Serum (FBS), Dulbecco's phosphate buffer saline (D-PBS) and MitoTracker Red CMX Ros Dye were purchased from Thermo Fisher Scientific. HeLa cells, Cell Counting Kit-8 (CCK-8), Hanks' Balanced Salt Solution (with Ca^{2+} and Mg^{2+}), Hoechst 33342 Staining Solution and LysoTracker Green were purchased from Beyotime Biotechnology. 1,1'-Dioctadecyl-3,3,3',3'-tetramethylindocarbocyanine perchlorate (DiI) was purchased from Aladdin. Zirconium dioxide (ZrO_2, diameter = 0.5 mm) beads were purchased from Ruirui Technology.

2. Synthesis of CD^{Gu}

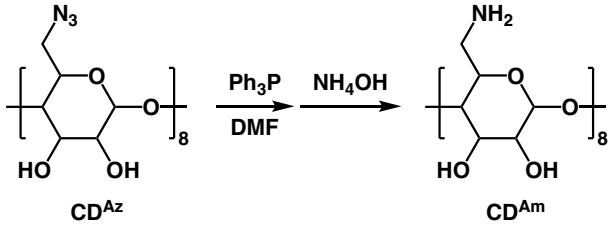


Octakis(6-bromo-6-deoxy)-γ-cyclodextrin (CD^{Br}).[S1] To an anhydrous DMF (65 mL) solution of Ph_3P (32.4 g, 12.3 mmol) was added dropwise an anhydrous DMF (65 mL) solution of NBS (21.9 g, 12.3 mmol) at 0 °C under Ar, and the mixture was stirred at room temperature for 30 min. The resulting mixture was added dropwise to an anhydrous DMF (130 mL) solution of γ-cyclodextrin (CD, 9.97 g, 7.69 mmol) and then stirred at 80 °C for 12 h under Ar. After being cooled to room temperature, MeOH (45 mL) was added to the resulting mixture and then stirred for 30 min. The reaction mixture was cooled on ice, and the pH was adjusted to 9 using sodium methoxide, followed by stirring for 1 h on ice. Then, the mixture was added to iced water (1 L) under stirring. The precipitate was collected, dissolved in DMF, and then reprecipitated with MeOH to allow the isolation of **CD^{Br}** as a white solid (8.32 g, 60%). ^{1}H NMR (500 MHz; DMSO-d_6; ppm): δ 6.00 (d, J = 6.92 Hz, 8H, 2-OH), 5.97 (d, J = 1.88 Hz, 8H, 3-OH), 5.01 (d, J = 3.52 Hz, 8H, 1-H), 3.98 (d, J = 10.00 Hz, 8H, 6-H), 3.81 (dd, J_1 = 8.82, J_2 = 8.82 Hz, 8H, 5-H), 3.68 (dd, J_1 = 7.74, J_2 = 10.82 Hz, 8H, 6'-H), 3.61 (dd, J_1 = 9.44, J_2 = 9.44 Hz, 8H, 3-H), 3.40–3.30 (m, 16H, 2-H and 4-H). ^{13}C NMR (126 MHz; DMSO-d_6; ppm): δ 131.45, 128.82, 102.02, 84.05, 72.26, 72.17, 71.02, 34.37. HRMS (ESI-QTOF): m/z found: 1800.7374 ([M + H$^+$] calcd: 1800.7377).

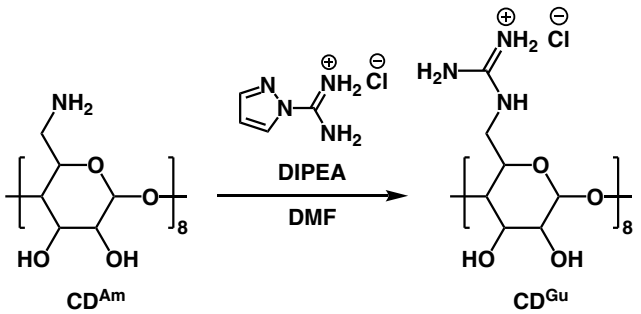


Octakis(6-azido-6-deoxy)-γ-cyclodextrin (CD^{Az}).[S2] To an anhydrous DMF (58 mL) solution of **CD^{Br}** (6.79 g, 3.77 mmol) was added sodium azide (3.92 g, 60.3 mmol), and the mixture was stirred at 70 °C under Ar for 24 h. The reaction mixture was evaporated to dryness under reduced pressure, and water was added to

the residue. The precipitate was collected and washed with water to allow the isolation of **CD^{Az}** as a white solid (5.48 g, 97%). ^{1}H NMR (400 MHz; DMSO-d_6; ppm): δ 5.93 (d, J = 6.53 Hz, 8H, 2-OH), 5.87 (d, J = 1.75 Hz, 8H, 3-OH), 4.94 (d, J = 3.63 Hz, 8H, 1-H), 3.80–3.67 (m, 16H, 6-H and 5-H), 3.64–3.50 (m, 16H, 3-H and 6'-H), 3.43–3.30 (m, 16H, 2-H and 4-H). ^{13}C NMR (101 MHz; DMSO-d_6; ppm): δ 102.06, 82.68, 72.46, 72.25, 70.47, 51.16. HRMS (ESI-QTOF): m/z found: 1497.4834 ([M + H$^+$] calcd: 1497.4817).



Octakis(6-amino-6-deoxy)-γ-cyclodextrin (CD^{Am}).[S2] To a DMF (85 mL) solution of **CD^{Az}** (5.48 g, 3.66 mmol) was added Ph_3P (15.4 g, 58.5 mmol), and the mixture was stirred for 2 h at room temperature until bubbles ceased to form. Then, aqueous NH_3 (~28%, 26.5 mL) was added dropwise to the reaction mixture. After being stirred for 18 h at room temperature, the resulting mixture was evaporated to dryness under reduced pressure. Then, EtOH (400 mL) was added to the residue, and the precipitate was washed with EtOH to allow the isolation of **CD^{Am}** as a white solid (3.99 g, 85%). Before NMR measurement, **CD^{Am}** was dissolved in aqueous HCl, reprecipitated with 1,4-dioxane, and dried under reduced pressure for 1 d. ^{1}H NMR (500 MHz; D_2O; ppm): δ 5.28 (d, J = 3.40 Hz, 8H, 1-H), 4.20 (m, J = 4.99 Hz, 8H, 5-H), 4.00 (t, J = 9.50 Hz, 8H, 3-H), 3.70 (dd, J_1 = 3.42, J_2 = 9.98 Hz, 8H, 2-H), 3.62 (dd, J_1 = 9.44, J_2 = 9.44 Hz, 8H, 4-H), 3.48 (dd, J_1 = 2.08, J_2 = 12.85 Hz, 8H, 6-H), 3.29 (dd, J_1 = 8.02, J_2 = 13.27 Hz, 8H, 6'-H). ^{13}C NMR (126 MHz; D_2O; ppm): δ 100.53, 80.90, 71.90, 71.70, 67.64, 40.30. HRMS (ESI-QTOF): m/z found: 430.5236 ([M + 3H$^+$] calcd: 430.5241).



Octakis(6-guanidino-6-deoxy)-γ-cyclodextrin (CD^{Gu}).[S2] To an anhydrous DMF (39 mL) suspension of **CD^{Am}** (3.27 g, 2.54 mmol) were added 1*H*-pyrazolecarboxamidine hydrochloride (6.85 g, 46.7 mmol) and DIPEA (12.1 g, 93.4 mmol), and the mixture was stirred at 70 °C for 12 h under Ar. Then, 1*H*-pyrazolecarboxamidine hydrochloride (6.85 g, 46.7 mmol) and DIPEA (12.1 g, 93.4 mmol) were added to the reaction mixture, and the mixture was stirred at 70 °C for 24 h under Ar. After being cooled to room temperature, Et_2O was added dropwise to the reaction mixture until suspension formed, and the resulting suspension was stirred for 2 h at room temperature. After decanting the supernatant, the precipitate was dissolved in water and then reprecipitated with EtOH to allow the isolation of **CD^{Gu}** as a white solid (2.06 g, 42%). ^{1}H NMR (500 MHz; D_2O; ppm): δ 5.14 (d, J = 2.85 Hz, 8H, 1-H), 4.04 (t, J = 8.80, 8H, 5-H), 3.94 (dd, J = 9.45, 8H, 3-H), 3.72 (dd, J_1 = 3.79, J_2 = 9.89 Hz, 8H, 2-H), 3.68 (d, J = 15.19 Hz, 8H, 6-H), 3.60–3.40 (m, 16H, 4-H, 6'-H). ^{13}C NMR (101 MHz; D_2O; ppm): δ 165.53, 99.67, 80.06, 70.99, 70.69, 67.73, 39.35. HRMS (ESI-QTOF): m/z found: 325.9530 ([M + 5H$^+$] calcd: 325.9522).

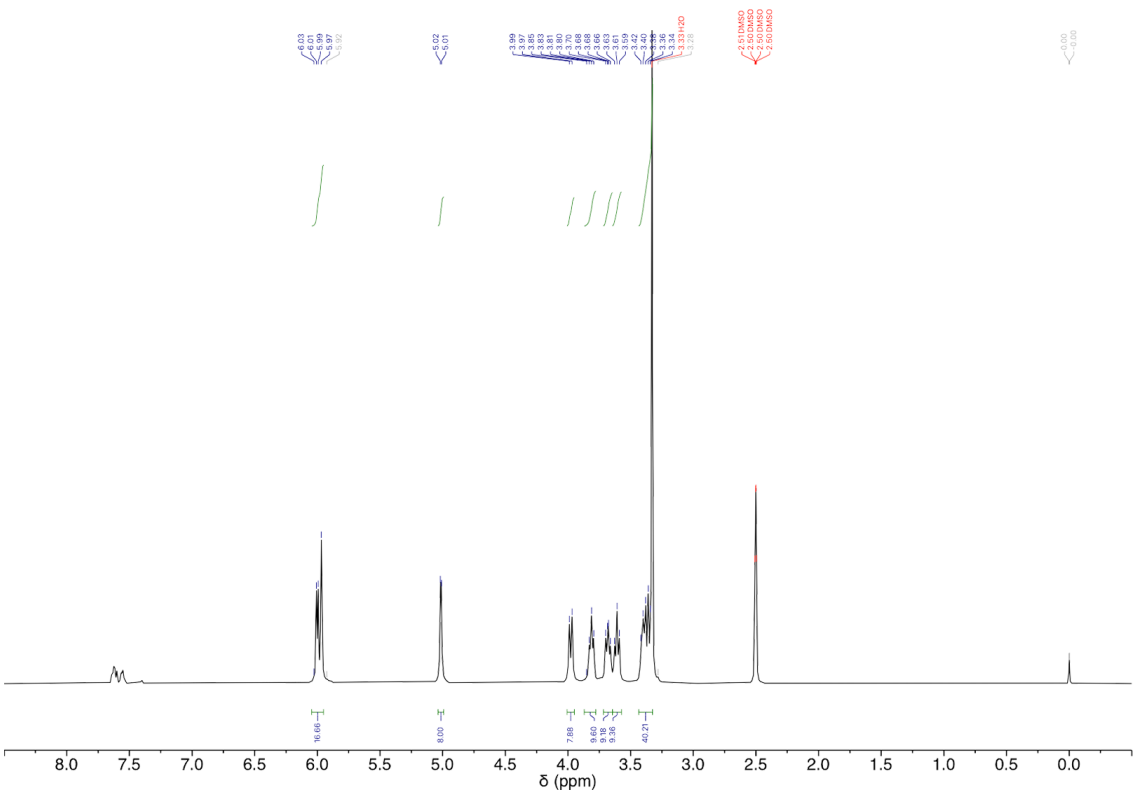


Fig. S1 ^{1}H NMR spectrum (500 MHz) of **CD^{Br}** in DMSO-d_6 at 25 °C.

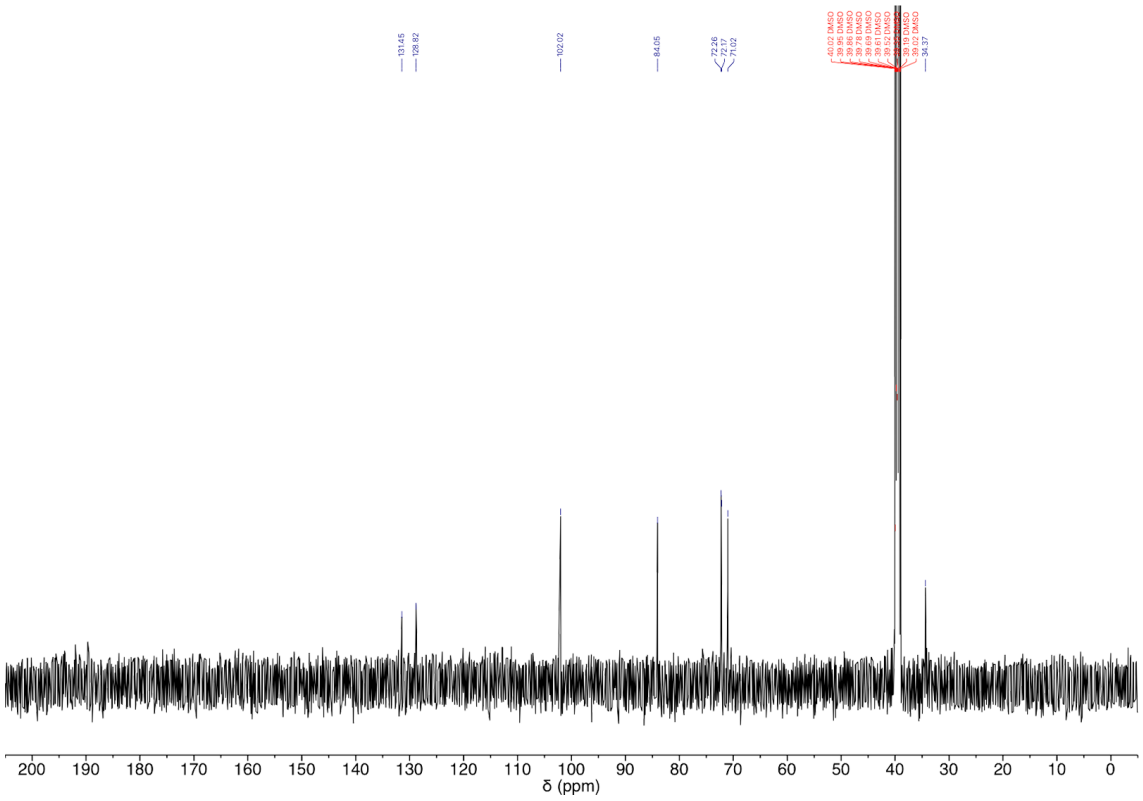


Fig. S2 ^{13}C NMR spectrum (126 MHz) of **CD^{Br}** in DMSO-d_6 at 25 °C.

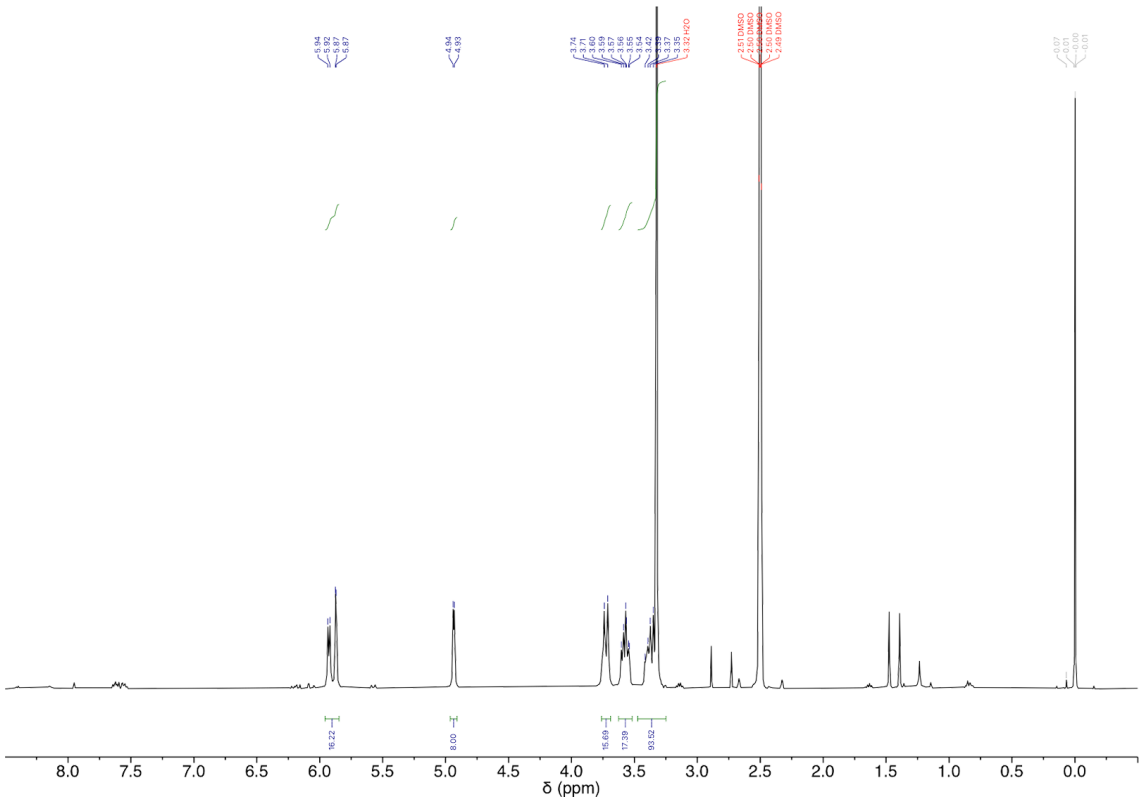


Fig. S3 ^{1}H NMR spectrum (400 MHz) of **CD^{Az}** in DMSO-d_6 at 24 °C.

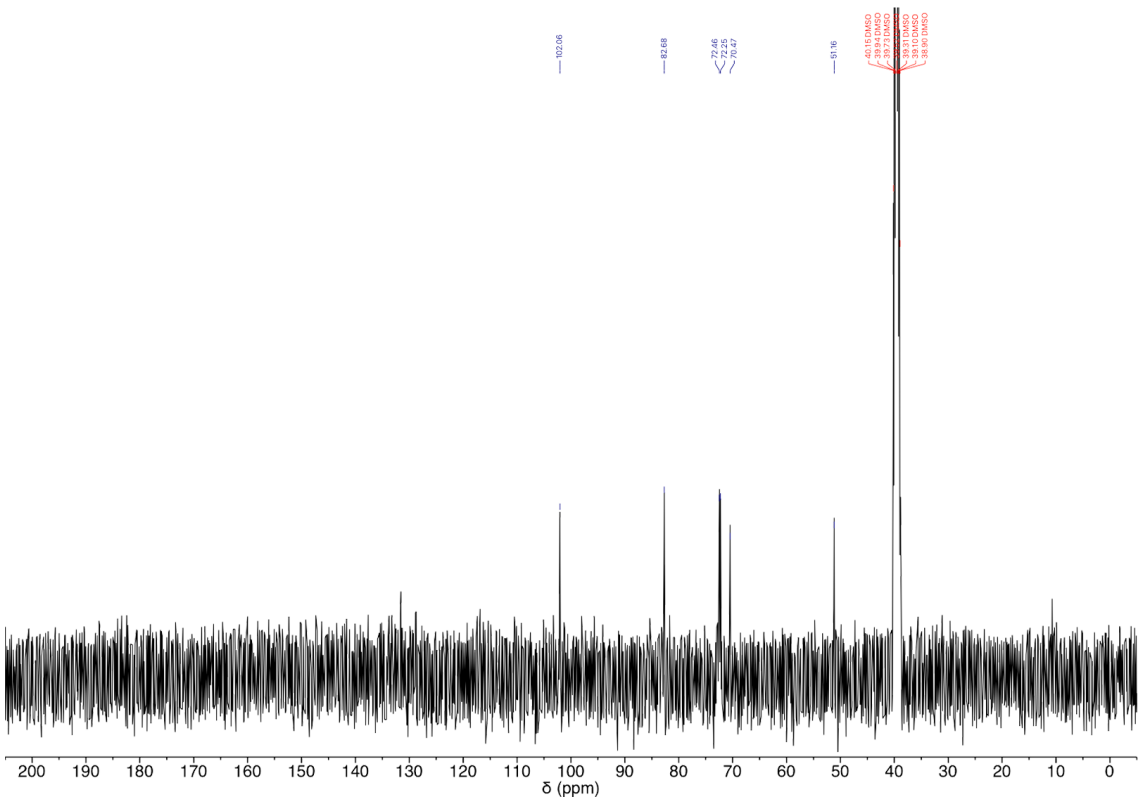


Fig. S4 ^{13}C NMR spectrum (101 MHz) of **CD^{Az}** in DMSO-d_6 at 24 °C.

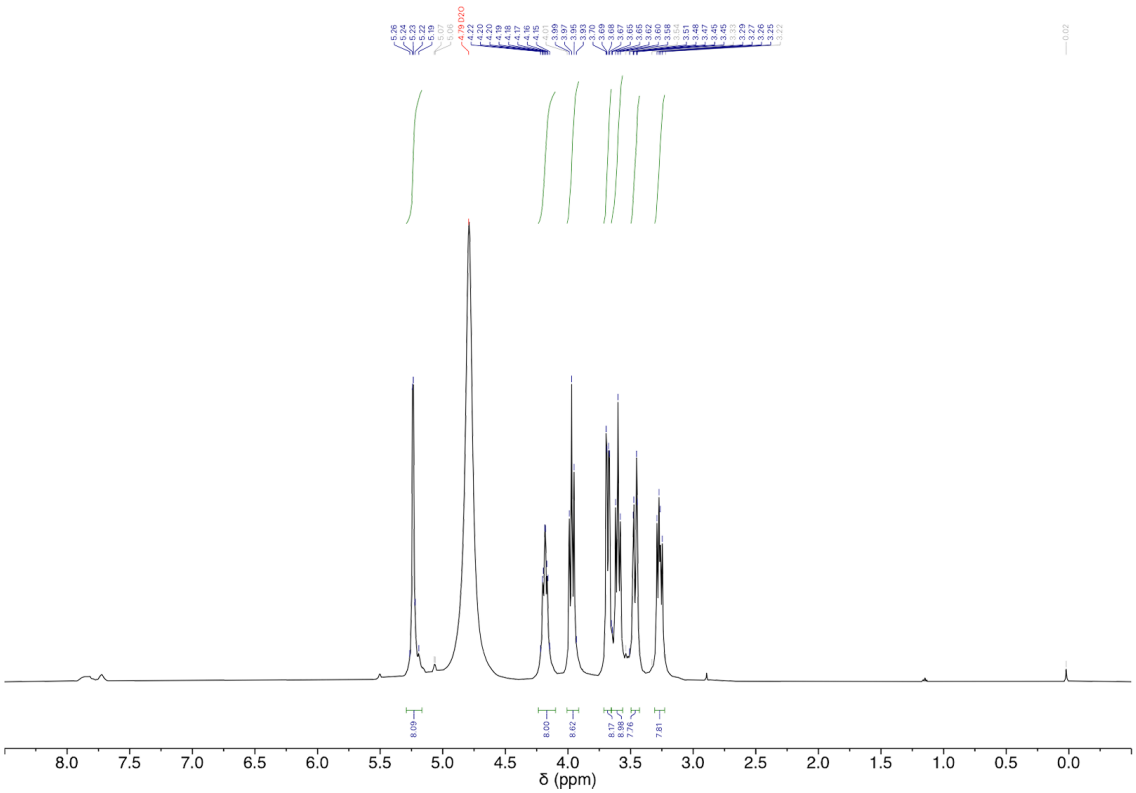


Fig. S5 ^{1}H NMR spectrum (500 MHz) of **CD^{Am}** in D_2O at 25 °C.

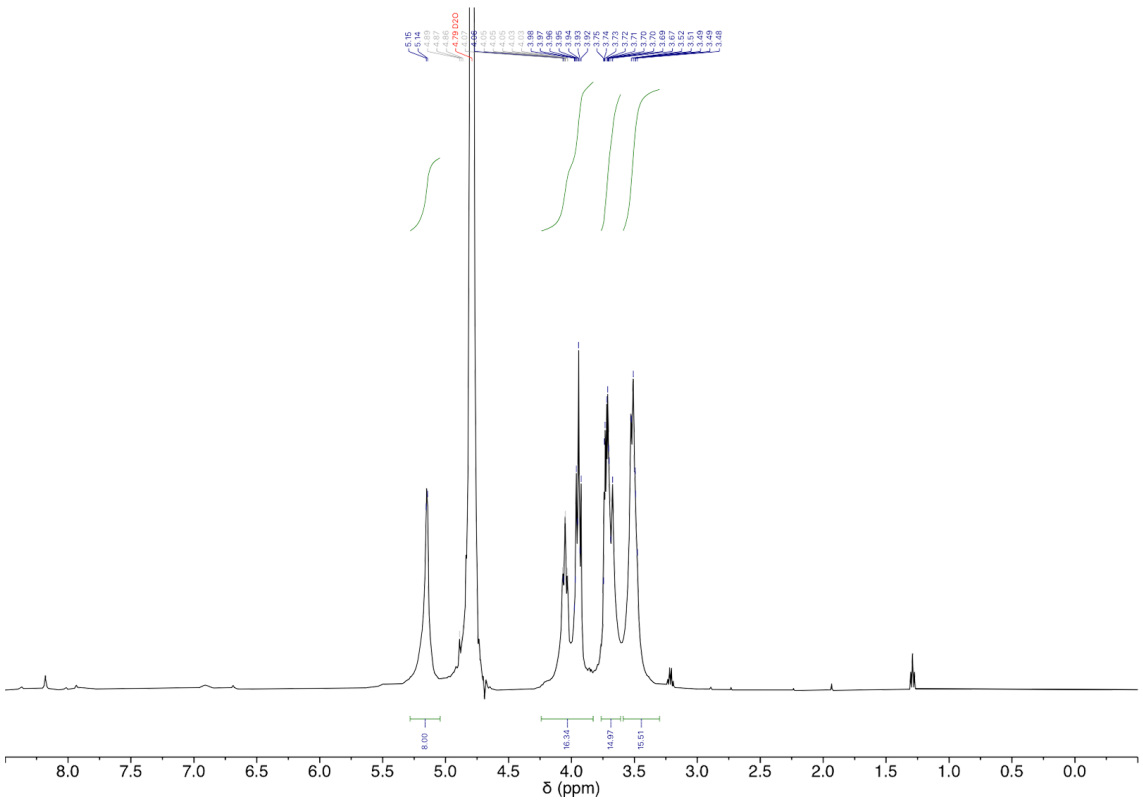


Fig. S6 ^{13}C NMR spectrum (126 MHz) of **CD^{Am}** in D_2O at 25 °C.

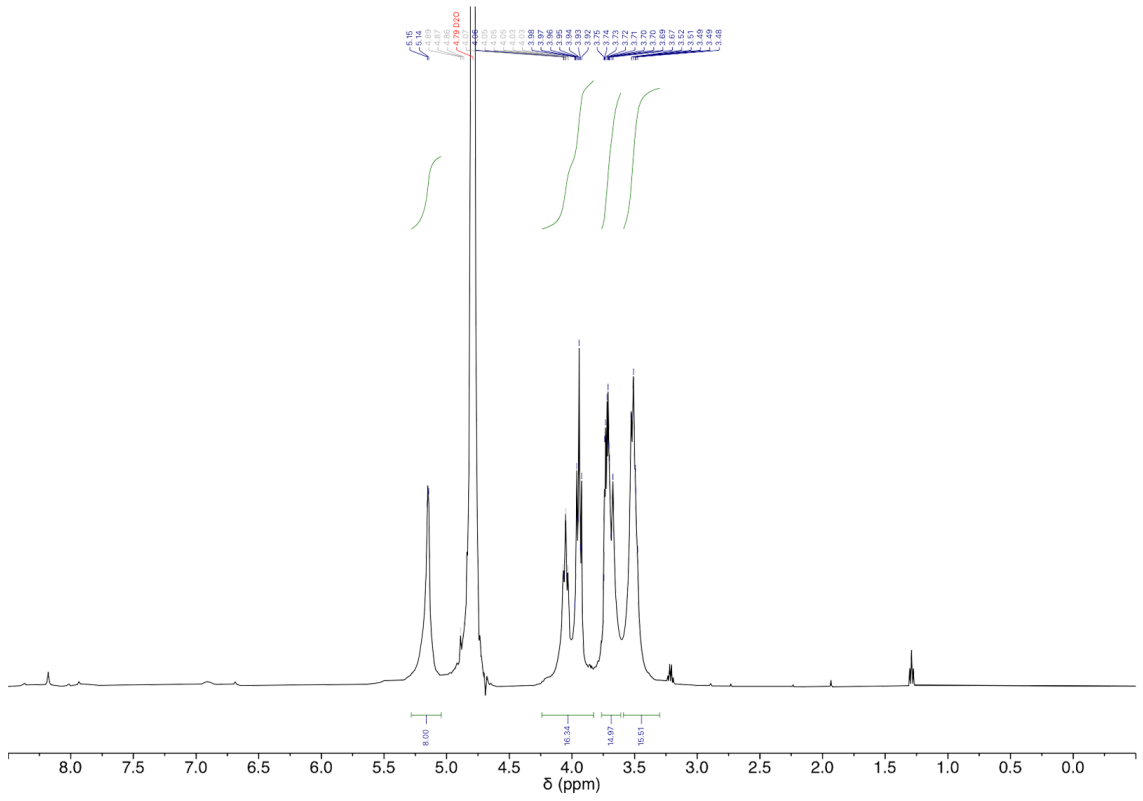


Fig. S7 ^{1}H NMR spectrum (500 MHz) of **CD^{Gu}** in D_2O at 25 °C.

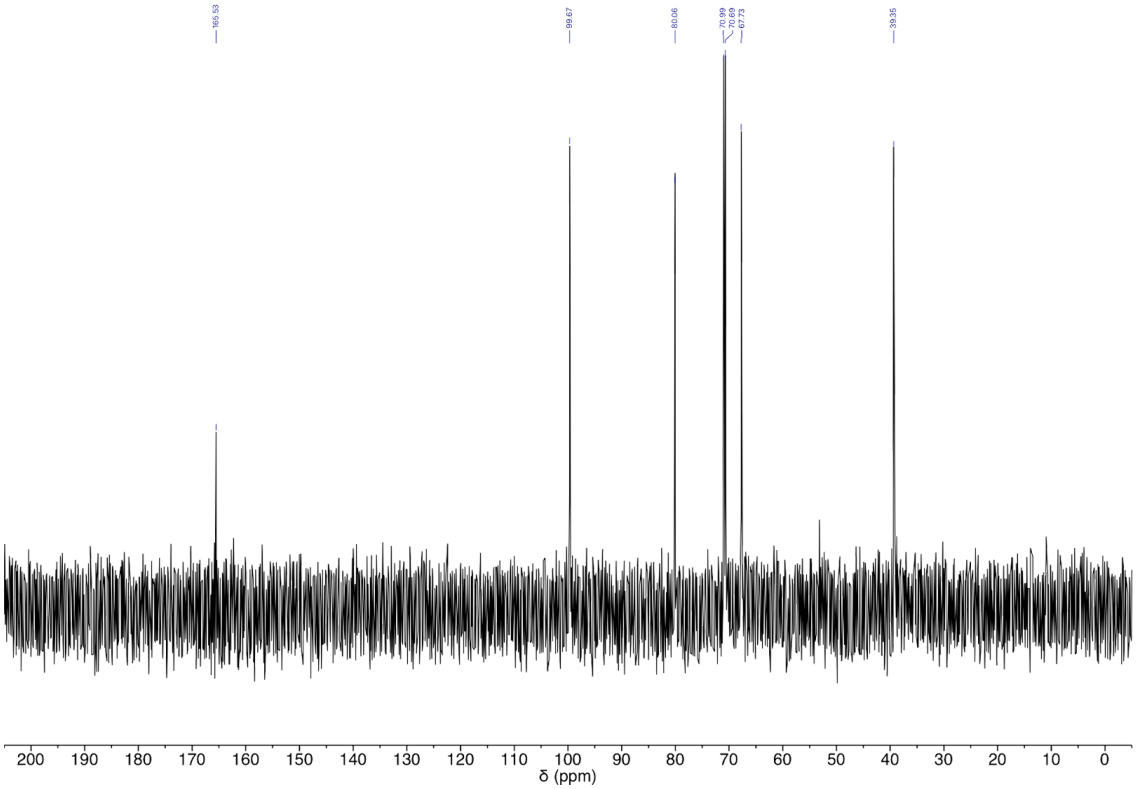


Fig. S8 ^{13}C NMR spectrum (101 MHz) of **CD^{Gu}** in D_2O at 22 °C.

3. Absorption Spectroscopy

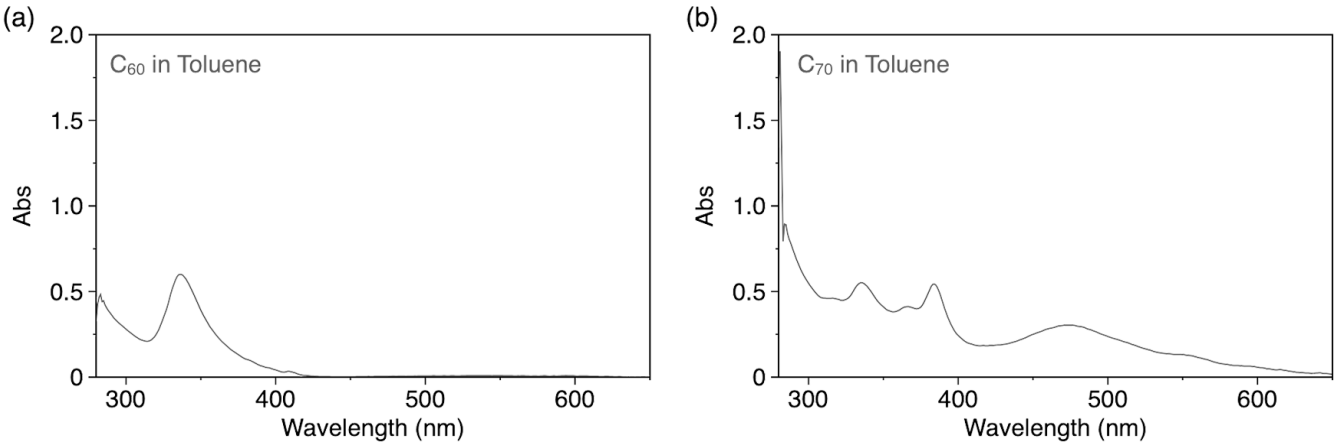


Fig. S9 (a, b) Absorption spectra in toluene of fullerene (a) C_{60} (12 µM) and (b) C_{70} (23 µM).

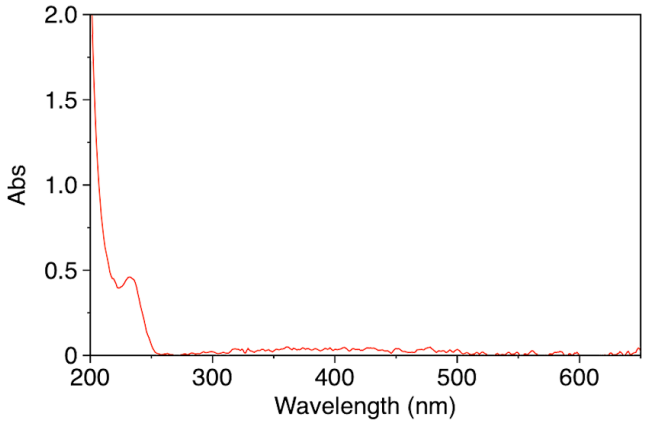


Fig. S10 Absorption spectrum of CD^{Gu} (100 μM) in water.

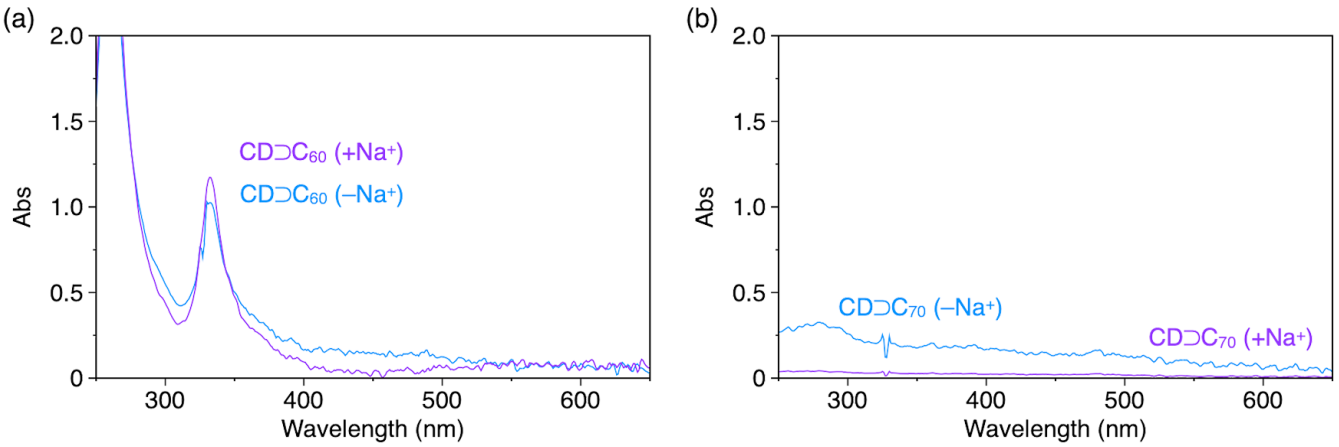


Fig. S11 Absorption spectra of the following aqueous solutions: (a) CD⊃C_{60} (10-fold dilution of the obtained solution) prepared in the absence (light blue) and presence of NaCl (purple), and (b) CD⊃C_{70} prepared in the absence (light blue) and presence of NaCl (purple).

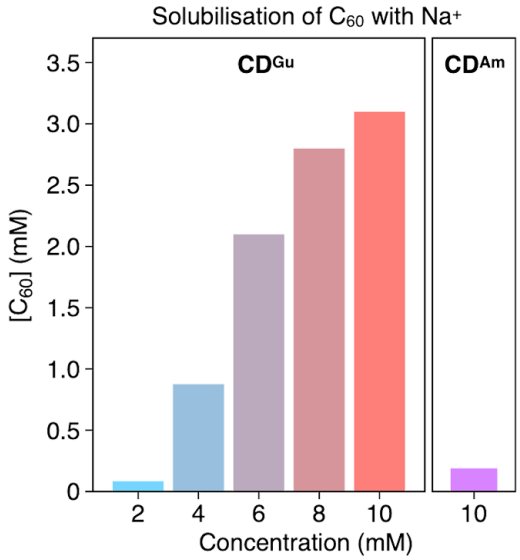


Fig. S12 Concentrations of C_{60} solubilised with CD^{Gu} (2–10 mM) or CD^{Am} (10 mM) in the presence of 1 M NaCl. Before the experiment, CD^{Am} was dissolved in aqueous HCl, reprecipitated with 1,4-dioxane, and dried under reduced pressure.

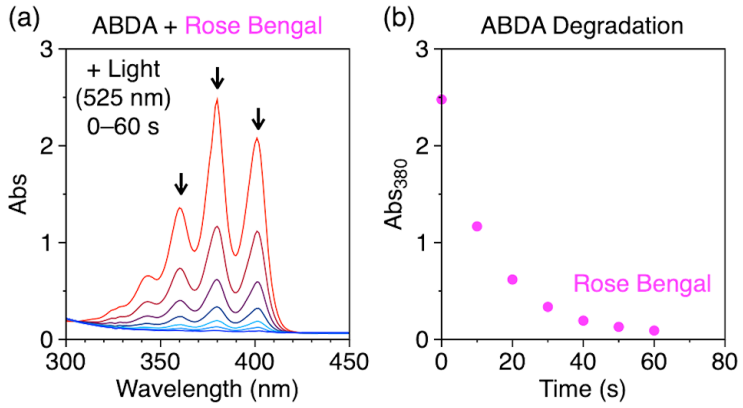


Fig. S13 (a) Absorption spectral changes upon exposure to 525 nm light (0–60 s) of an aqueous mixture of 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA; 0.2 mM) and Rose Bengal (10 μM). (b) Absorbance changes at 380 nm upon exposure to 525 nm light of an aqueous mixture of ABDA (0.2 mM) and Rose Bengal (10 μM).

4. Dynamic Light Scattering (DLS)

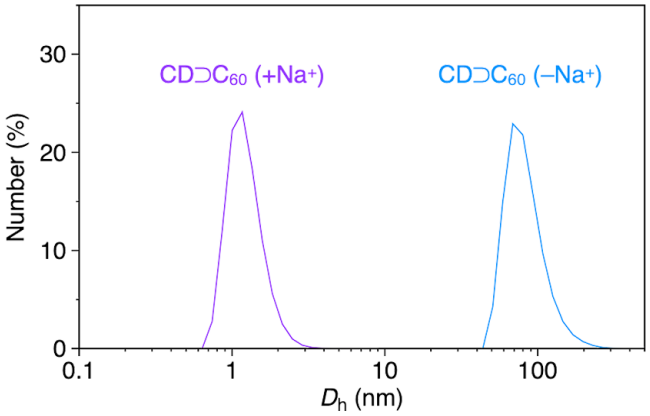


Fig. S14 Dynamic light scattering (DLS) profiles of aqueous solutions of CD⊃C_{60} prepared in the absence (light blue) and presence of NaCl (purple).

5. Zeta-potential Measurement

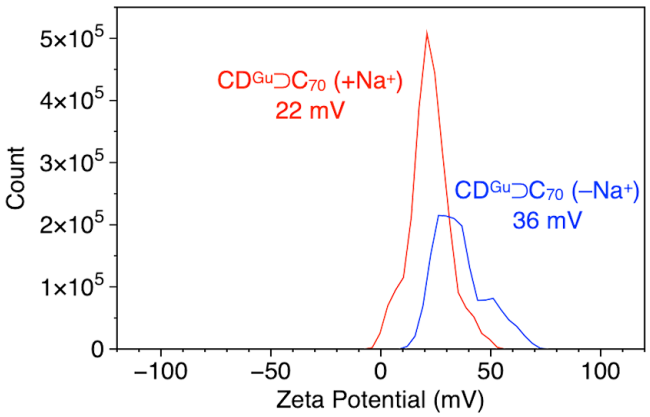


Fig. S15 Zeta-potential profiles of aqueous solutions of $CD^{Gu}⊃C_{70}$ prepared in the absence (blue) and presence of NaCl (red).

=> The difference in zeta-potential values can be attributed to the distinct morphologies of $CD^{Gu}⊃C_{70}$: $C_{70}⊃CD^{Gu}$ forms large aggregates when prepared in the absence of Na^+, with CD^{Gu} molecules thoroughly covering their surface and exposing Gu^+ moieties isotropically. The lower zeta potential observed for $CD^{Gu}⊃C_{70}$ prepared in the presence of Na^+ supports the formation of molecularly dispersed anisotropic complexes, where two CD^{Gu} molecules encapsulate one C_{70} molecule, resulting in less complete coverage of the complex surface by Gu^+ moieties.

6. Fluorescence Spectroscopy

HeLa cells were seeded on a 96-well culture plate (5.0×10^3 cells well^{-1}; 0.32 cm^2 well^{-1}) and incubated in DMEM (100 μL) containing 10% FBS at 37 °C under 5% CO_2 for 24 h. The cells were rinsed with D-PBS (100 μL × 3) and incubated in DMEM (10% FBS, 100 μL) containing 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI, 5 μM) at 37 °C under 5% CO_2 for 20 min, then in DMEM (10% FBS, 100 μL) at 37 °C under 5% CO_2 for 10 min. The cells were rinsed with D-PBS (100 μL × 3) and incubated in DMEM (100 μL) containing CD⊃C_{60} ([C_{60}] = 1–7 μM), CD^{Gu}⊃C_{60} ([C_{60}] = 1–7 μM) or CD^{Gu}⊃C_{70} ([C_{70}] = 1–7 μM) at 37 °C under 5% CO_2 for 4 h. The resulting cell samples were subjected to fluorescence spectroscopy (λ_{ex} = 530 nm and λ_{em} = 580 nm). Reference cell samples using CD^{Gu} (40–280 μM) without fullerenes and these using aggregated CD^{Gu}⊃C_{70} ([C_{70}] = 1–7 μM), prepared in the absence of NaCl, were prepared under otherwise identical conditions.

Analogous cell samples were prepared by incubation at 37 °C under 5% CO_2 in Hanks' Balanced Salt Solution containing LysoTracker Green (67 nM) for 45 min, in the same buffer containing MitoTracker Red CMX Ros (67 nM) for 30 min or Hoechst 33342 Staining Solution for 20 min. All samples were incubated for 4 h at 37 °C in DMEM without and with CD^{Gu}⊃C_{70} ([C_{70}] = 7 μM), and then subjected to fluorescence spectroscopy (LysoTracker Green: λ_{ex} = 471 nm and λ_{em} = 511 nm; MitoTracker Red: λ_{ex} = 569 nm and λ_{em} = 609 nm; Hoechst 33342: λ_{ex} = 350 nm and λ_{em} = 461 nm).

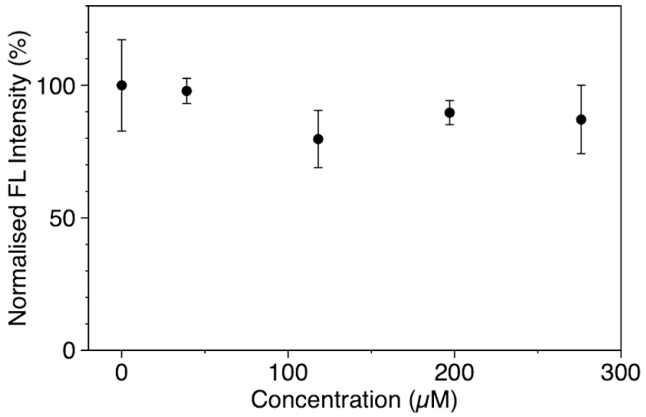


Fig. S16 Normalised fluorescence intensities (λ_{ex} = 530 nm and λ_{em} = 580 nm) of HeLa cells stained with 1,1'-dioctadecyl-3,3,3'3'-tetramethylindocarbocyanine perchlorate (DiI, 5 μM), followed by incubation for 4 h at 37 °C in DMEM containing CD^{Gu} (0–280 μM).

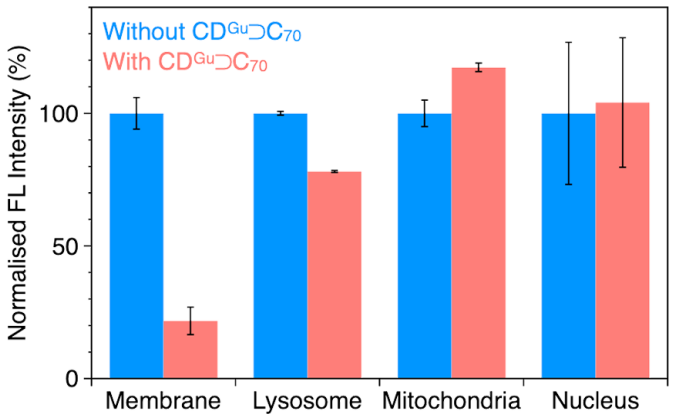


Fig. S17 Normalised fluorescence intensities of HeLa cells treated with DiI ($\lambda_{ex}/\lambda_{em}$ = 530/580 nm), LysoTracker Green ($\lambda_{ex}/\lambda_{em}$ = 471/511 nm), MitoTracker Red CMX Ros ($\lambda_{ex}/\lambda_{em}$ = 569/609 nm) and Hoechst 33342 ($\lambda_{ex}/\lambda_{em}$ = 350/461 nm) for staining cell membrane, lysosomes, mitochondria, and cell nucleus, respectively. The intensities were measured after incubation for 4 h at 37 °C in DMEM without (blue) and with $CD^{Gu}\supset C_{70}$ ([C_{70}] = 7 μM, red).

=> After incubating with $CD^{Gu}\supset C_{70}$, there was a notable decrease in the fluorescence of DiI, a dye marking the cell membrane, while the fluorescence emissions from MitoTracker Red CMX Ros and Hoechst 33342, dyes for mitochondria and cell nuclei, remained substantially unchanged. These results indicate that C_{70} was mainly transferred to the cell membrane. The small decrease in lysosomal fluorescence is likely due to C_{70} in the lysosomal membrane, presumably derived from the cell membrane.

7. Cell Viability Assay

HeLa cells were seeded on a 96-well culture plate (5.0×10^3 cells well^{-1}; 0.32 cm^2 well^{-1}) and incubated in DMEM (100 μL) containing 10% FBS at 37 °C under 5% CO_2 for 24 h. After rinsing with D-PBS (100 μL × 3), the cells were incubated in DMEM (10% FBS, 100 μL) containing CD⊃C_{60} ([C_{60}] = 0.5–5 μM), CD^{Gu}⊃C_{60} ([C_{60}] = 0.5–5 μM) or CD^{Gu}⊃C_{70} ([C_{70}] = 0.5–5 μM) at 37 °C under 5% CO_2 for 4 h. The cells were rinsed with D-PBS (100 μL × 3) and supplied with DMEM (10% FBS, 100 μL), followed by exposure to UV light (370 nm, 54 mW cm^{-2}), visible light (630 nm, 5.3 mW cm^{-2}), or white light (color temperature: 8300 K; 3 W) for 5–30 min. After incubation at 37 °C under 5% CO_2 for 24 h, the cell samples were supplied with Cell Counting Kit-8 reagent (10 μL) and incubated at 37 °C under 5% CO_2 for 1 h before absorption spectroscopy (λ = 450 nm). Analogous cell sample using CD^{Gu} (1–300 μM) withou exposure to light were prepared under otherwise identical conditions.

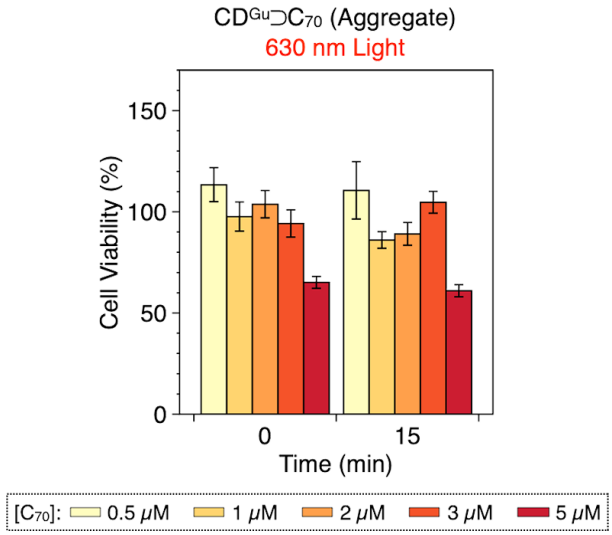


Fig. S18 Viabilities of HeLa cells incubated with DMEM containing aggregated CD^{Gu}⊃C_{70} ([C_{70}] = 0.5–5 μM) at 37 °C for 4 h, then exposed to 630 nm light (0–15 min), followed by incubation in DMEM (10% FBS) at 37 °C for 24 h.

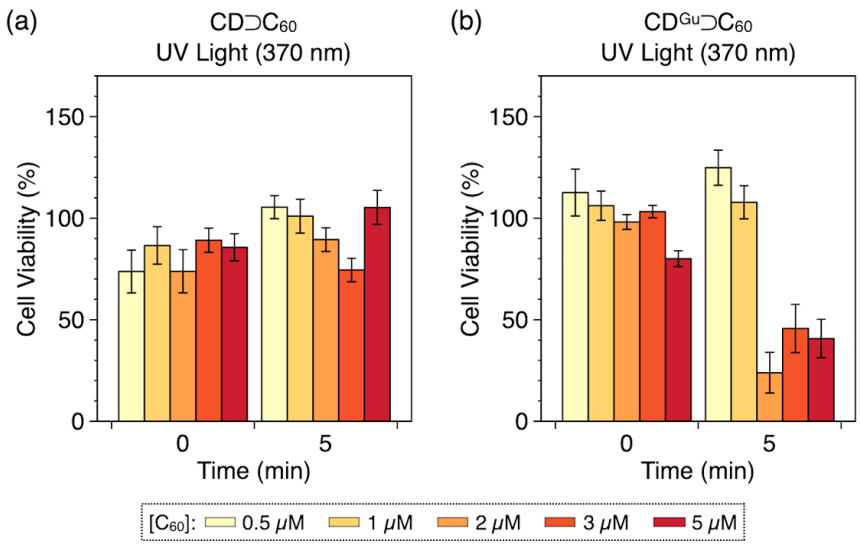


Fig. S19 Viabilities of HeLa cells incubated with DMEM containing (a) CD⊃C_{60} ([C_{60}] = 0.5–5 μM) or (b) CD^{Gu}⊃C_{60} ([C_{60}] = 0.5–5 μM) at 37 °C for 4 h, then exposed to 370 nm light (0–5 min), followed by incubation in DMEM (10% FBS) at 37 °C for 24 h.

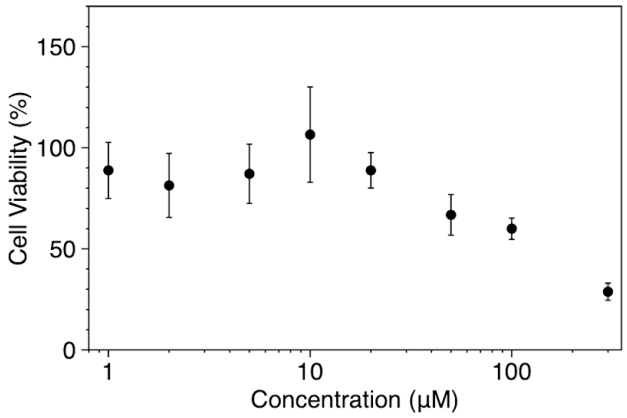


Fig. S20 Viabilities of HeLa cells incubated with DMEM containing CD^{Gu} (1–300 μM) at 37 °C for 4 h, followed by incubation in DMEM (10% FBS) at 37 °C for 24 h.

8. References

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