Supporting Online Material for

Self-assembled subphthalocyanine-based nanophotosensitiser for photodynamic therapy

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Experimental details

1. Materials

Chemicals were purchased from commercial suppliers (SIGMA Aldrich and Alfa Aesar) and used without further purification unless stated otherwise. All solvents were of AR quality and purchased from either Scharlab or Carlo Erba. Dry THF was degassed and obtained after passing through an activated alumina column in a solvent purification system. Water was purified using an EMD Milipore Mili-Q integral water purification system. Column chromatography was carried out on silica gel (Merk, kieselgel 60, 230-400 mesh, 60 Å). Size exclusion chromatography was carried out on Biorad Biobeads SX-1 (200-400 mesh). Reactions were followed by thin-layer chromatography on aluminum sheets precoated with 0.25 mm 60-F254 silica gel from Merck. All reactions were performed under an atmosphere of dry argon unless stated otherwise.

2. Methods

^{1}H-NMR and ^{13}C-NMR spectra were recorded on a Bruker AC-300 or a Bruker AC-500 spectrometer. Chemicals shifts are given in ppm (δ) values relative to tetramethylsilane (TMS). Splitting patterns are labelled as s, singlet; d, doublet; dd, doublet of doublet; t, triplet; q, quartet; quin, quintet; m, multiplet and b stands for broad. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker Reflex III instrument that was equipped with a nitrogen laser operated at 337 nm and recorded in the positive-ion mode. High-resolution mass spectra were acquired using a 9.4 T IonSpec QFT-MS FT-ICR mass spectrometer. The samples were analysed with a hybrid analyser QTOF model MAXIS II of Bruker. An ACQUITY UPLC system from the commercial house Waters was used as an entryway in the flow injection analysis mode.

Ultraviolet-visible (UV-Vis) absorption spectra and fluorescence spectra were recorded on a Jasco V-660-spectrophotometer and a Jasco FP-8600 spectrofluorometer respectively, both with a Jasco Peltier ETCS-761 temperature controller incorporated.

UV-Vis and fluorescence spectroscopic measurements were performed using quartz cuvettes (1 cm). Solutions were prepared by weighting the necessary amount of compound for a given concentration. Water solutions were prepared by injecting a concentrated DMSO solution (1 x 10^{-2} M) into water milli-Q to obtain the desired final concentration. In all cases, the solutions were optically transparent.

For transmission electron microscopy (TEM) study, 5 µL of sample was deposited on carbon film-coated copper grids (200 mesh; Beijing Zhongjingkeyi Technology Co. Ltd) and air-dried

before the TEM images were taken using a FEI Tecnai G2 Spirit transmission electron microscope operated at 120 kV acceleration voltage.

3. Singlet oxygen quantum yields (Φ_Δ)

Singlet oxygen quantum yields of **SubPc(PEG)₃** were measured in DMSO and D_2O following the well-known *relative method*,[1] based on the photoinduced consumption of a chemical trap (1,3-diphenylisobenzofuran (DPBF) in DMSO or 9,10-anthracenediyl-bis(methylene)dimalonic acid (ABDA) in D_2O) that easily reacts with $^1O_2$. Zinc phthalocyanine (ZnPc) (Φ_Δ (DMSO) = 0.56) was used as the reference compound in DMSO.[2] In D_2O, the reference compound employed was Eosin Y (Φ_Δ (D_2O) = 0.60).[3]

The procedure was as follows: 2.5 mL of a stock solution of DPBF or ABDA (with an absorbance of approximately 1) in DMSO or D_2O was transferred to a 10 x 10 mm quartz optical cell and bubbled with $^3O_2$ for 1 min. Next, a concentrated stock solution of **SubPc(PEG)₃** in the same solvent was added in a defined amount to achieve a final absorbance value of the Q band of approximately 0.1. The solution was irradiated with stirring for defined time intervals, using a halogen lamp (300 W). The duration of these intervals was adjusted in each experiment in order to obtain a decrease in the absorption of DPBF or ABDA of about 3-4%. Incident light was filtered through a water filter (6 cm) and an additional filter to remove light below 530 nm (Newport filter FSQ-OG530). Additional neutral density filters (FBS-ND03 or FB-ND10) were used when necessary. The decrease in absorbance of DPBF (at 414 nm) or ABDA (at 399 nm). The irradiation time was recorded and Φ_Δ was calculated using the following equation:

$$\phi_\Delta^S = \phi_\Delta^R \frac{K^S I_{aT}^R}{K^R I_{aT}^S}$$

where R and S indicate the reference and sample respectively. K is the slope obtained from the representation of ln (A_0/A_t) versus the irradiation time t, where A_0 and A_t are the absorbance of the chemical trap at the monitoring wavelength before and after the irradiation time t, respectively. I_{aT} is the total amount of light absorbed by the chromophore and is calculated as the sum of the intensities of the absorbed light I_a from wavelength 530 to 800 nm. I_a at the given wavelength is calculated using Beer's law:

$$I_a = I_o \left(1 - e^{-2.3A}\right)$$

where A is the absorbance of the photosensitiser at the determined wavelength, and I_0 is the transmittance of the filter at the same wavelength.

4. Fluorescence quantum yields (Φ_F)

The fluorescence quantum yields (Φ_F) were determined by comparing the properly integrated fluorescence intensity signal between solutions of the compounds to be studied and a solution of a reference compound by the method of Williams and co-workers.[4] For **SubPc(PEG)₃**, the reference compound was ZnPc in DMSO ($\Phi_F = 0.20 \pm 0.03$).[5] When the reference is presented in another solvent than the sample, the fluorescence intensity is corrected using the refractive index of the solvents used by applying the following equation:

$$\phi_F = \frac{A_S \cdot n_S^2}{A_R \cdot n_R^2}$$

where A_S and A_R are the areas corresponding to the sum of the intensities of the fluorescence band of the sample and the reference respectively, and n is the refractive index of the solvent used. The excitation wavelength was set at 529 nm.

5. Cell lines and culture conditions

HeLa (ATCC, no. CCL-2) and HepG2 (ATCC, no. HB-8065) cells were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with fetal bovine serum (10%) and penicillin-streptomycin (100 units mL^{-1} and 100 μg mL^{-1} respectively). All the cells were grown in a humidified incubator with 5% CO_2 at 37 °C.

6. Confocal laser scanning microscopy

Approximately 4×10^5 cells in DMEM (2 mL) were seeded on a confocal dish and incubated overnight at 37 °C with 5% CO_2. The cells were treated with the **SubPc(PEG)₃** nanoparticles. After that, the medium was removed, and the cells were rinsed with phosphate-buffered saline (PBS) and examined with a Leica TCS SP8 high speed confocal microscope. The excitation and emission wavelengths for the SubPc were 552 and 600–700 nm respectively.

7. Flow cytometry

Approximately 2×10^5 cells were inoculated into each of the wells in a 12-well plate and incubated in DMEM overnight at 37 °C and 5% CO_2. The cells were treated with the **SubPc(PEG)₃** nanoparticles. After that, the cells were rinsed with PBS and harvested by adding 0.5 mL of 0.25% trypsin–ethylenediaminetetraacetic acid (Invitrogen). After adding 0.5 mL of the medium to quench the activity of the trypsin, the cell mixture was centrifuged at 1500 rpm for

3 min at room temperature. The cell pellet was washed with 1 mL of PBS and subject to centrifugation for three times. After resuspending the cells in 1 mL of PBS, their intracellular fluorescence intensities were measured by using a BD FACSVerse flow cytometer (Becton Dickinson) with 10^4 cells counted in each sample. Data collected were analysed by using the BD FAC-Suite. All experiments were performed in triplicate.

8. Subcellular localisation studies

Approximately 2×10^5 cells were seeded on a confocal dish and incubated overnight at 37 °C in a humidified 5% CO_2 atmosphere. The cells, after being rinsed with PBS, were incubated with the **SubPc(PEG)$_3$** nanoparticles (with 5 μM SubPc) for 24 h. After being washed twice with PBS, the cells were stained with LysoTracker Green DND-26 (Thermo Fisher Scientific Inc., L7526) (2 μM), MitoTracker Green FM (Thermo Fisher Scientific Inc., M7514) (0.2 μM) or ER-Tracker Green (Thermo Fisher Scientific Inc., E34251) (1 μM) in a serum-free medium at 37 °C for 30, 15, and 15 min respectively. The solutions were then removed, and the cells were rinsed with PBS twice before being examined with a Leica TCS SP8 high-speed confocal microscope equipped with a 488 nm laser and a 552 nm laser. All the trackers were excited at 488 nm, and their fluorescence was monitored at 500−570 nm, while SubPc was excited at 552 nm and its fluorescence was monitored at 600−700 nm. The images were digitised and analysed using the Leica Application Suite X software.

9. *In vitro* cytotoxicity

Approximately 2×10^4 cells were inoculated into each of the wells in a 96-well plate and incubated in DMEM overnight at 37 °C and 5% CO_2. The cells were incubated with different concentrations of **SubPc(PEG)$_3$** nanoparticles for 24 h, and then were rinsed with 100 μL of PBS twice and replenished with 100 μL of fresh medium. For the dark cytotoxicity assay, the plate was directly incubated at 37 °C overnight. For the photocytotoxicity test, the cells were illuminated at room temperature for 20 min and then incubated overnight. The light source consisted of a 300 W halogen lamp, a water tank for cooling and a colour glass filter (Newport) cut on at $\lambda = 515$ nm. The fluence rate ($\lambda \geq 515$ nm) was 25.5 mW cm^{-2}. Illumination for 20 min led to a total fluence of 30.6 J cm^{-2}. A solution of MTT (Sigma) in PBS (3 mg mL^{-1}, 50 μL) was added to each well. After incubation for 4 h under the same condition, 100 μL of DMSO was added to each well and the plates were placed on a Bio-Rad microplate reader to detect the absorbance at 490 nm. The average absorbance of the blank wells (not seeded with cells) was subtracted from the measured absorbance values of wells of various treatment groups. Cell viability was determined by the equation %viability = $[\sum(A_i/A_{control} \times 100)]/n$, where A_i is the
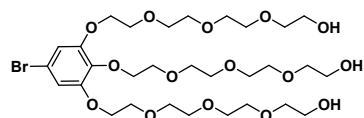
absorbance of the ith datum (i = 1, 2, ..., n), $A_{control}$ is the average absorbance of control wells in which the nanosystem was absent. The size of treatment group (n) is 4.

10. Synthetic procedures
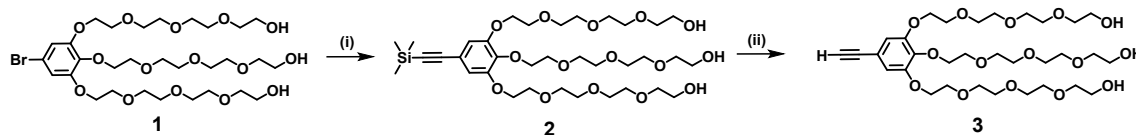
Scheme S1: Synthetic route for compound **3**. (i) Pd(PPh$_3$)$_4$, CuI, TMSA, Et$_3$N, 80 °C, 16 h (68%); (ii) K$_2$CO$_3$ MeOH, room temp., 3 h (89%).

Compound 1

Compound **1** was synthesised according to the procedure previously described by Deming and co-workers.[6] 1-Bromo-3,4,5-trihydroxylbenzene (0.15 g, 0.73 mmol), tetraethylene glycol *p*-toluenesulfonate (1.22 g, 3.50 mmol) and K$_2$CO$_3$ (0.60 g, 4.35 mmol) were mixed in DMF (8 mL). The mixture was heated at 90 °C under an argon atmosphere for 24 h. After cooling the mixture was poured into water and extracted by dichloromethane. The solvent in the organic layer was removed under vacuum and the residue was purified by column chromatography on a silica gel column using a mixture of CHCl$_3$: MeOH (95/5 v/v) as eluent, affording the product as a brown oil (0.41 g, 76%).

^{1}H-NMR (300 MHz, CDCl$_3$) δ: 6.72 (s, 2H, Ar), 4.12 (m, 6H, CH$_2$-OAr), 3.5-3.9 (m, 42H, TEG). **^{13}C-NMR** (101 MHz, CDCl$_3$) δ: 153.40, 137.88, 115.83, 111.48, 77.16, 72.73, 72.66, 70.94, 70.77, 70.75, 70.73, 70.63, 70.59, 70.47, 70.45, 69.72, 69.22, 61.79. **FT-IR** (ATR) ν (cm^{-1}): 3399 (O-H st), 2868, 1743, 1640, 1585, 1491, 1451, 1420, 1349, 1240, 1095, 940, 834. **HR-MS** (ESI$^+$; MeOH+NaI): *m/z* calc C$_{30}$H$_{53}$O$_{15}$BrNa: 755.2460 [M+Na]$^+$; *m/z* found 755.2472 (100).
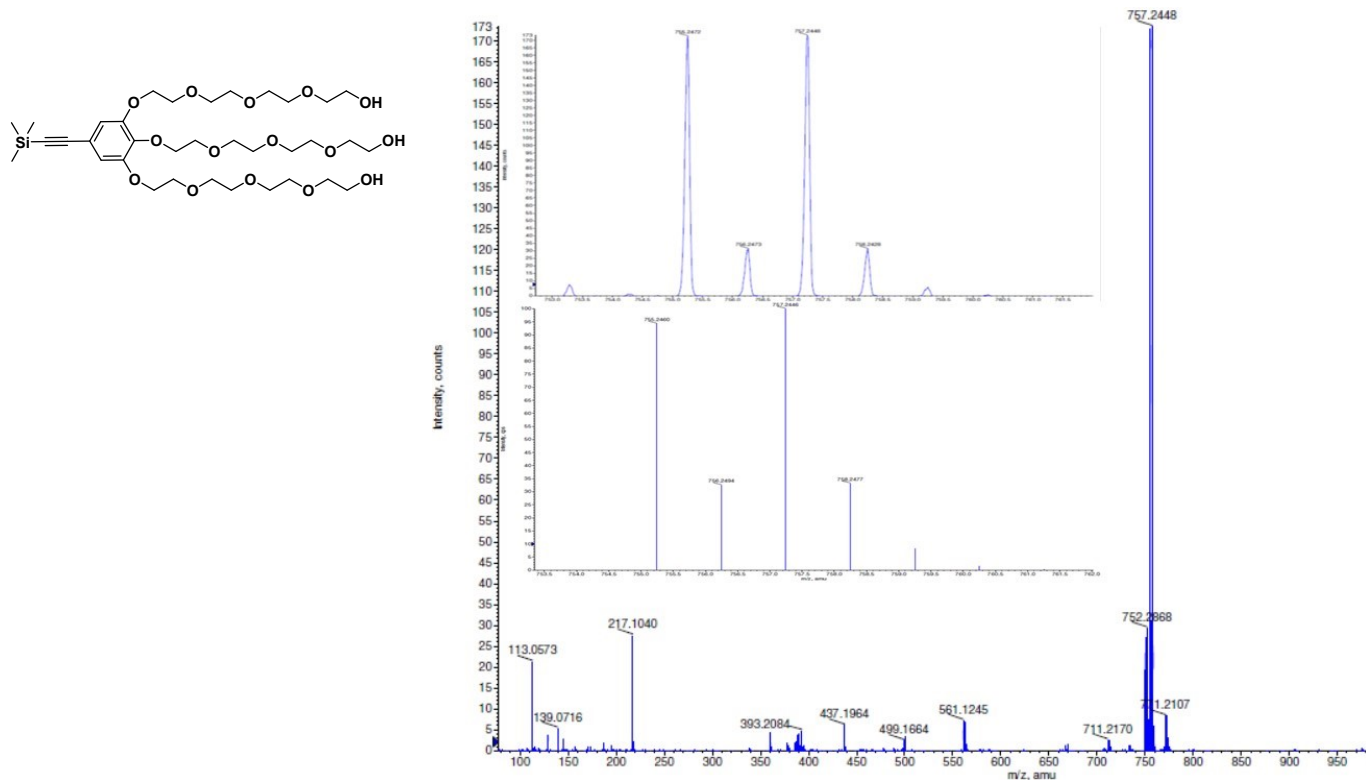
¹H NMR spectrum of 1 in CDCl₃
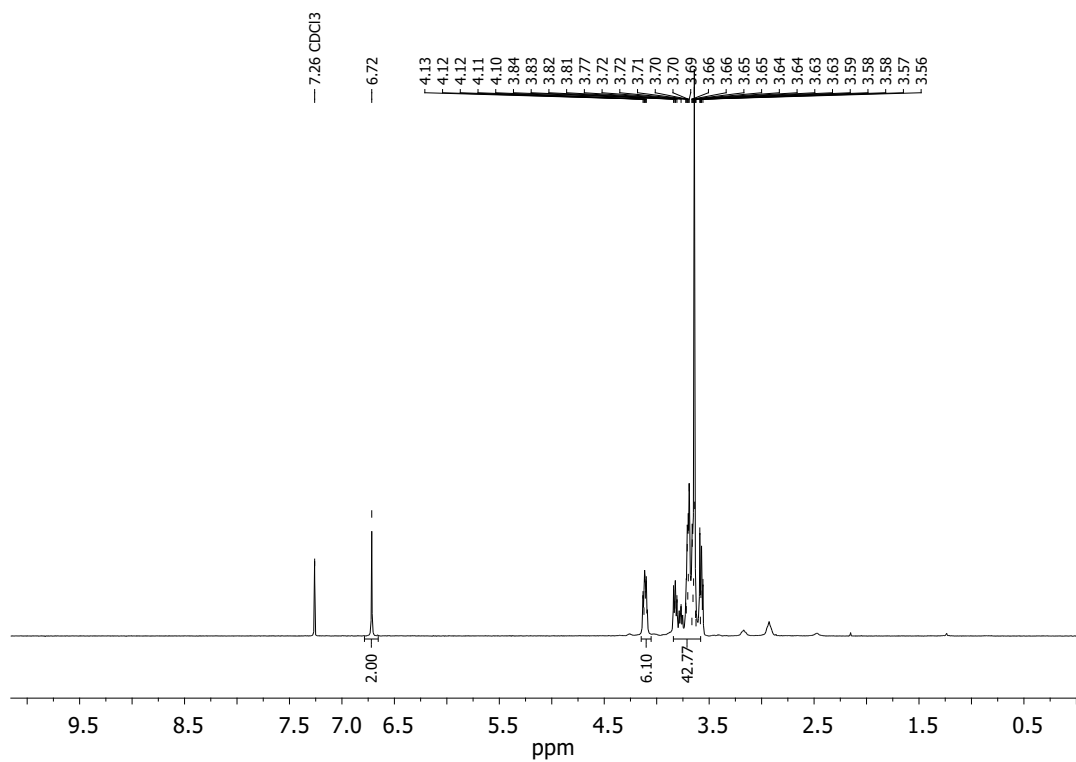
¹³C NMR spectrum of 1 in CDCl₃
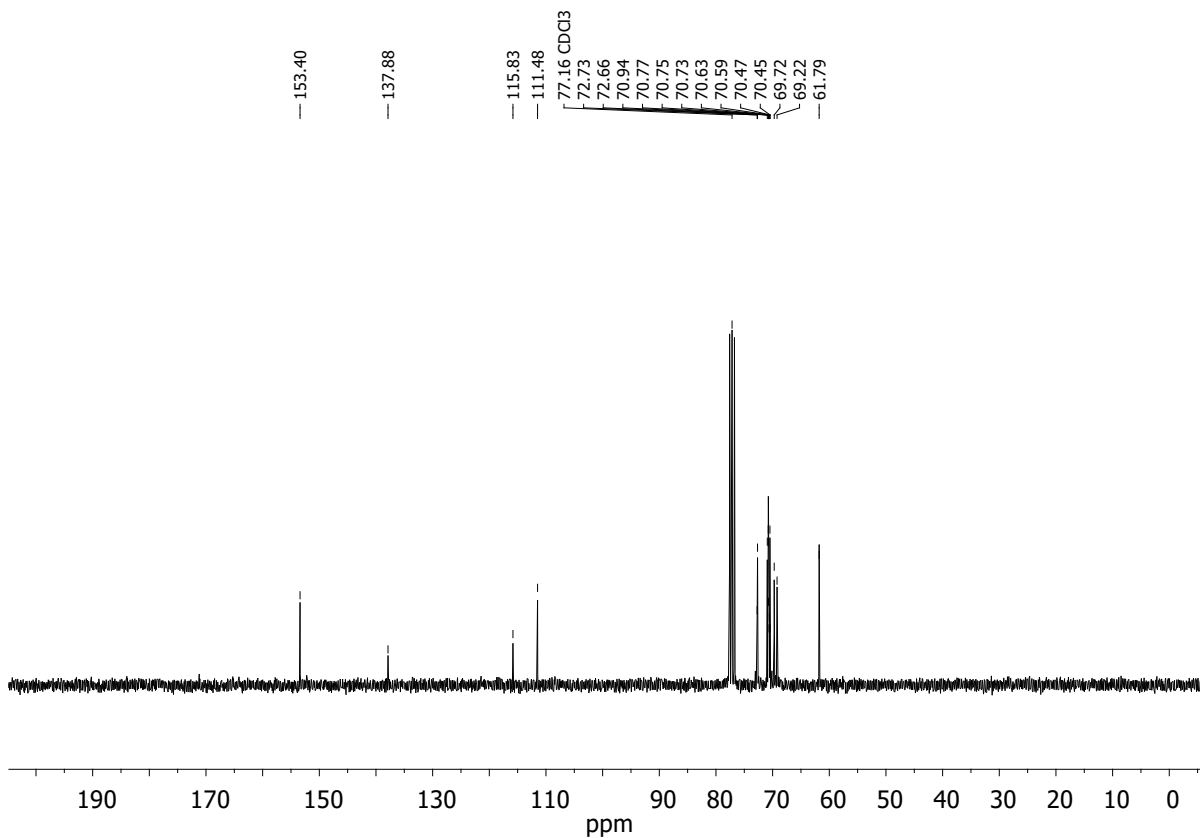
ESI mass spectrum of 1. The inset shows the experimental (top) and simulated (bottom) isotopic pattern of the molecular ion signal.
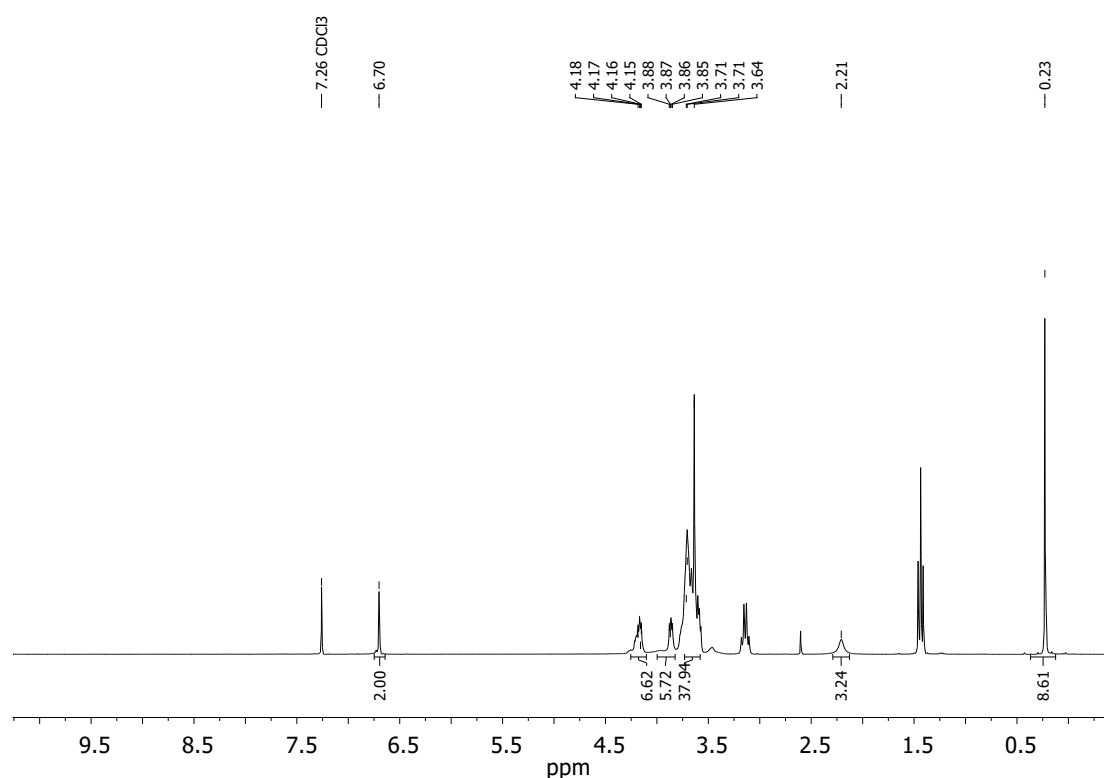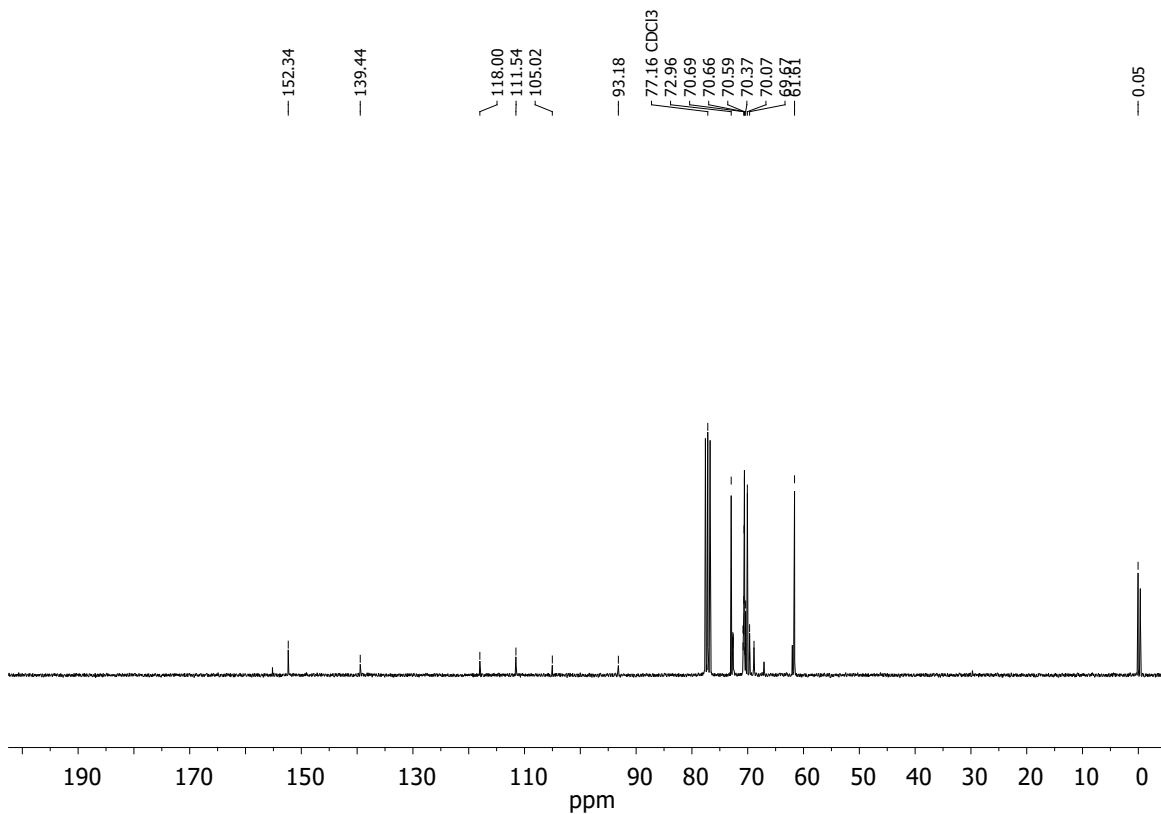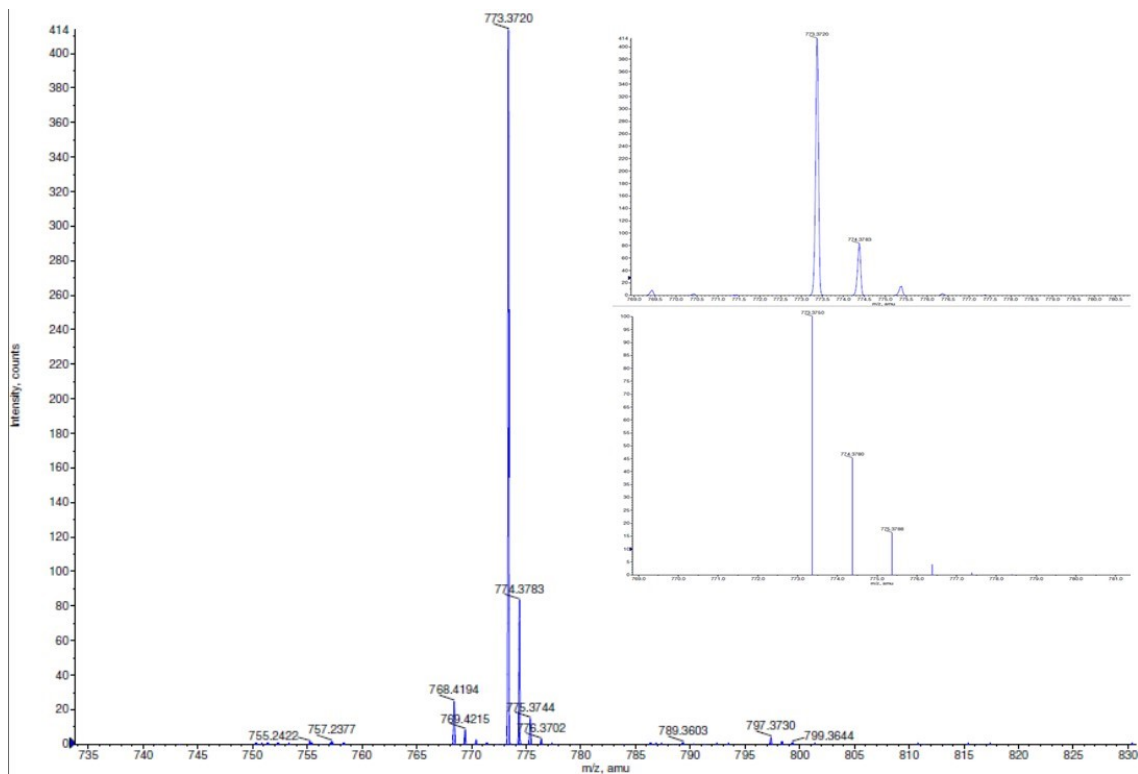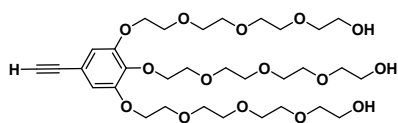

Compound 2

Compound **1** (0.25 g, 0.34 mmol), Pd(PPh$_3$)$_4$ (12 mg, 3 mol%), CuI (4.0 mg) and triethylamine (4 mL) were mixed and degassed by two "froze-pump-thaw" cycles. Then, trimethylsilylacetylene (TMSA) (0.50 g, 5.1 equiv) was added into the mixture. The mixture was heated at 80 °C under an argon atmosphere for 16 h. The solvent was removed under vacuum and the residue was purified by column chromatography on a silica gel column using a mixture of CHCl$_3$: MeOH (90/10 v/v) as eluent to afford the product as a colourless oil (0.17 g, 68%).

¹H-NMR (300 MHz, CDCl$_3$) δ: 6.70 (s, 2H, Ar) 4.16 (m, 6H, CH$_2$-OAr), 3.5-3.9 (m, 42H, TEG) 2.20 (br s, 3H, OH), 0.23 (s, 9H, TMSA). **¹³C-NMR** (101 MHz, CDCl$_3$) δ: 152.34, 139.44, 118.00, 105.02, 93.18 (C≡C), 77.16, 72.96, 72.71, 72.67, 72.63, 72.60, 70.84, 70.83, 70.71, 70.69, 70.66, 70.59, 70.51, 70.37, 70.07, 69.69, 69.67, 68.88, 61.61, 0.05. **FT-IR** (ATR) ν (cm^{-1}): 3429 (O-H st), 2870, 1743, 1571, 1496, 1453, 1420, 1329, 1243, 1104, 943, 886, 839. **HR-MS** (ESI$^+$; MeOH + NaI): *m/z* calc C$_{35}$H$_{62}$O$_{15}$SiNa: 773.3750 [M+Na]$^+$; *m/z* found 773.3720 [M+Na]$^+$ (100).

¹H NMR spectrum of 2 in CDCl$_3$

¹³C NMR spectrum of 2 in CDCl$_3$

ESI mass spectrum of 2. The inset shows the experimental (top) and simulated (bottom) isotopic pattern of the molecular ion signal.

Compound 3

 Compound **2** (0.17 g, 0.23 mmol) was dissolved in methanol (3 mL) and then K_2CO_3 (0.09 g) was added. The mixture was stirred at room temperature for 3 h and then filtrated. The filtrate was evaporated under vacuum and the residue was purified by column chromatography on a silica gel column using a mixture of $CHCl_3$:MeOH (90/10 v/v) as eluent to afford compound **3** (0.14 g, 89%).

¹H-NMR (300 MHz, $CDCl_3$) δ: 6.66 (s, 2H, Ar) 4.09 (m, 6H, CH_2-OAr), 3.5-3.9 (m, 42H, TEG) 3.00 (s, 1H, C≡CH). **¹³C-NMR** (101 MHz, $CDCl_3$) δ: 152.07, 138.28, 117.38, 111.01, 83.25 (C≡C), 77.16, 72.75, 72.49, 72.40, 72.28, 70.38, 70.27, 70.18, 70.00, 69.95, 69.91, 69.87, 69.54, 69.14, 68.16, 61.29, 61.08, 60.89. **FT-IR** (ATR) ν (cm^{-1}): 3396 (O-H st), 3224 (≡C-H st) 2919, 2871, 2094 (C≡C st) 1695, 1574, 1496, 1101, 941, 885, 838. **HR-MS** (ESI⁺; MeOH+NaI): *m/z* calc $C_{35}H_{54}O_{15}Na$: 701.3354, [M+Na],⁺ $C_{35}H_{54}O_{15}K$: 717.3094 [M+K]⁺; *m/z* found 701.3370 (49) [M+Na]⁺, 717.3100 (100) [M+K]⁺.
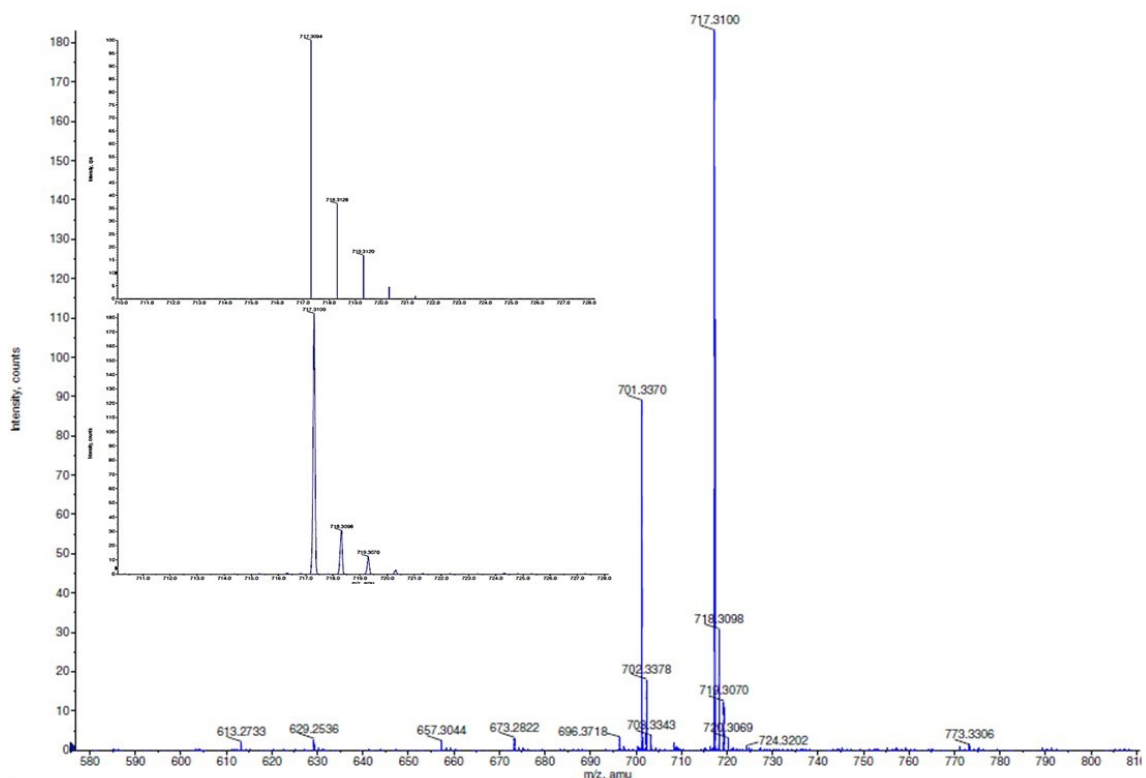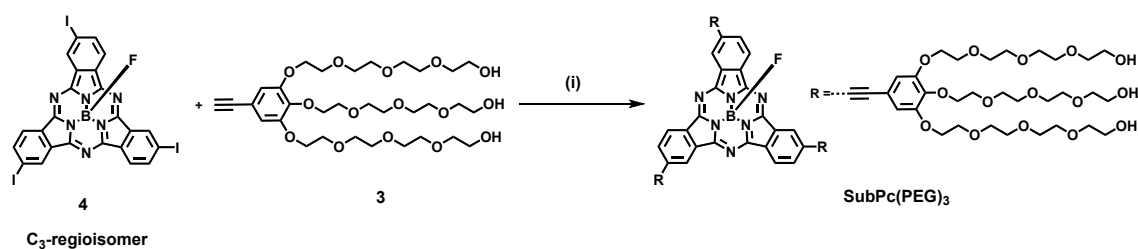
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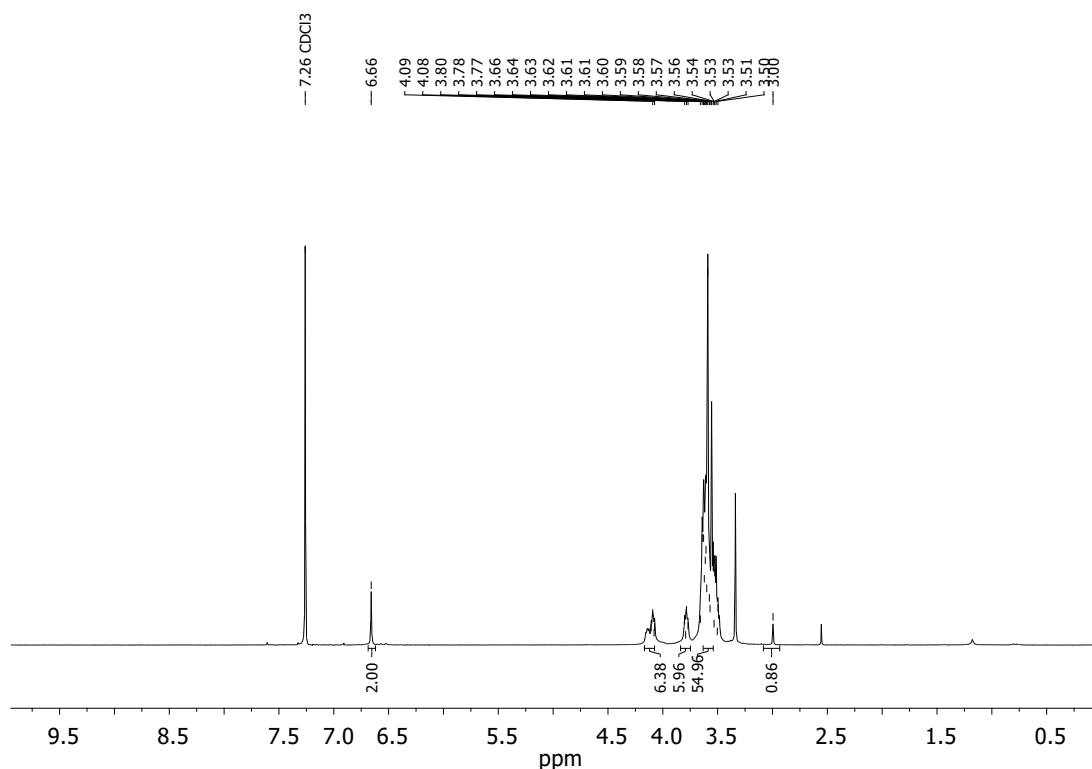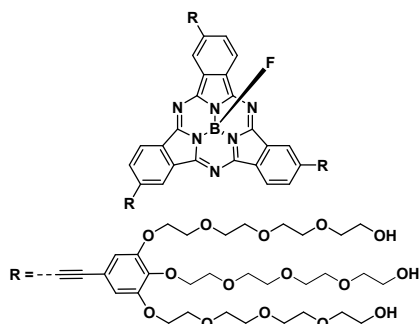

¹³C NMR spectrum of 3 in CDCl₃
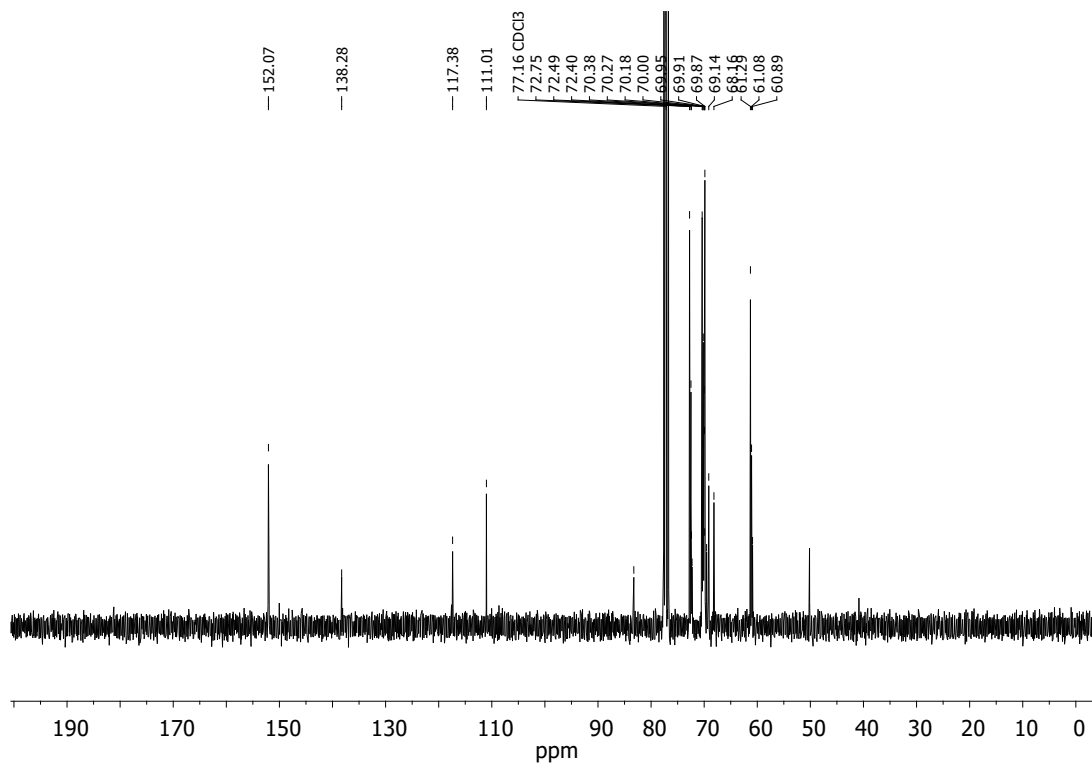
ESI mass spectrum of 3. The inset shows the experimental (top) and simulated (bottom) isotopic pattern of the molecular ion signal.

Scheme S2: Synthetic scheme of **SubPc(PEG)₃**. (i) Pd₂dba₃, CuI, AsPh₃, Et₃N/DMF, 80 °C, 12 h, 48%.
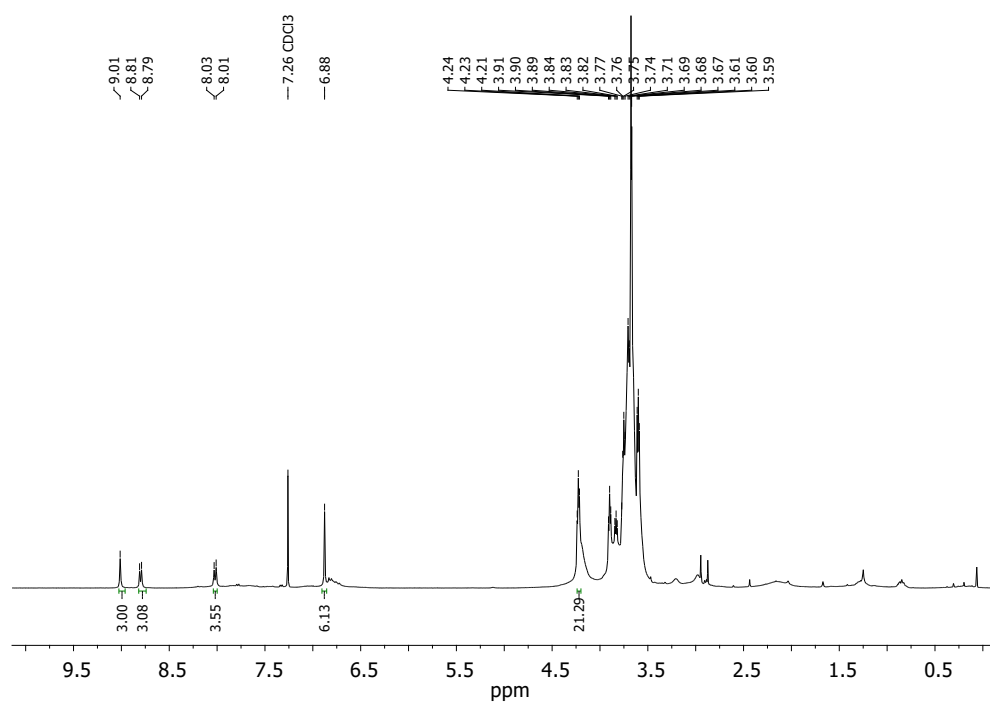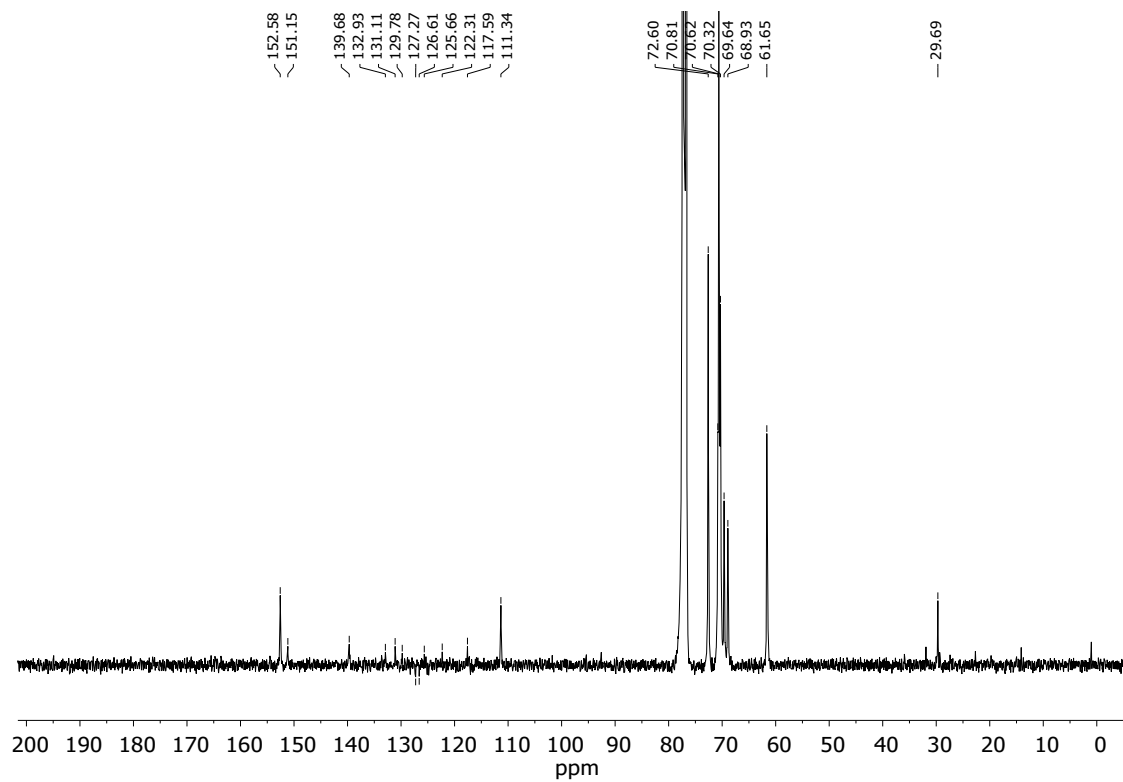
SubPc(PEG)₃

A degassed mixture of Et$_3$N/DMF (1:4 v/v) (1.5 mL) was added to an argon-purged reaction vessel charged with **3** (76 mg, 0.10 mmol), C$_3$-**4**[7] (20 mg, 0.025 mmol), Pd$_2$dba$_3$ (3.0 mg, 0.006 mmol), AsPh$_3$ (16 mg, 0.05 mmol) and CuI (13 mg, 0.006 mmol). The resulting mixture was stirred in the dark for 4 h. Then the reaction mixture was filtered over a celita plug. The liquid phase was dried under vacuum. The residue was further purified by size exclusion chromatography using CHCl$_3$ as eluent to afford the product as a purple oil (0.03 g, 48%).

¹H-NMR (300 MHz, CDCl₃) δ: 9.01 (s, 3H, SubPc), 8.79 (d, J = 8.2 Hz, 3H SubPc), 8.02 (d, J = 8.3, 3H, SubPc), 6.88 (s, 6H), 4.22 (m, 18H, ArO-CH₂), 3.6-3.8 (m, 126H, TEG) **¹³C-NMR** (101 MHz, CDCl₃) δ: 151.28, 151.15, 140.20, 139.68, 131.11, 127.27, 118.97, 117.59, 117.03, 116.82, 116.58, 115.65, 111.34, 72.60, 70.81, 70.62, 70.32, 69.64, 68.93, 61.65, 29.69. **¹⁹F NMR** (300 MHz, CDCl₃) δ: -156.52. **MS** MALDI-TOF (DCTB): *m/z* calc $C_{120}H_{168}BFN_6O_{45}$: 2467.1 (100) [M+Na]⁺; *m/z* found $C_{120}H_{168}BFN_6O_{45}Na$: 2444.1 (17) [M⁺], 2467.1 (100) [M+Na]⁺, 2488.2 (10) [M+K]⁺. **HR-MS** MALDI-TOF (DCTB + PPGNa2000+ PPGNa2700): *m/z* calc $C_{120}H_{168}BFN_6O_{45}Na$: 2467.1085 (100) [M+Na]⁺; m/z found $C_{120}H_{168}BFN_6O_{45}Na$: 2467.1055 (100) [M+Na]⁺.

¹H NMR spectrum of SubPc(PEG)₃ in CDCl₃

¹³C NMR spectrum of SubPc(PEG)₃ in CDCl₃

152.58
151.15
139.68
132.93
131.11
129.78
127.27
126.61
125.66
122.31
117.59
111.34
72.60
70.81
70.62
70.32
69.64
68.93
61.65
29.69

200 190 180 170 160 150 140 130 120 110 100 90 80 70 60 50 40 30 20 10 0
ppm

¹⁹F NMR spectrum of SubPc(PEG)₃ in CDCl₃

−156.52

-130 -135 -140 -145 -150 -155 -160 -165 -170 -175 -180 -185 -190 -195 -200
ppm
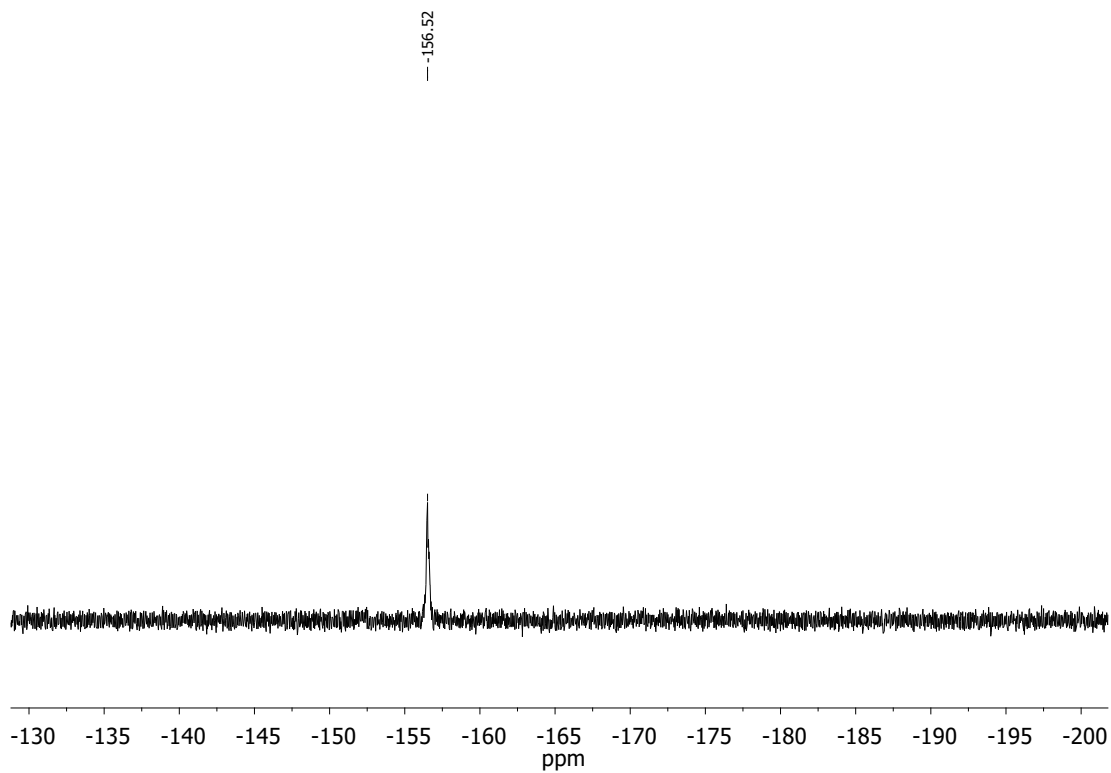
MALDI-TOF mass spectrum of SubPc(PEG)₃. The inset shows the experimental (top) and simulated (bottom) isotopic pattern of the molecular ion signal.
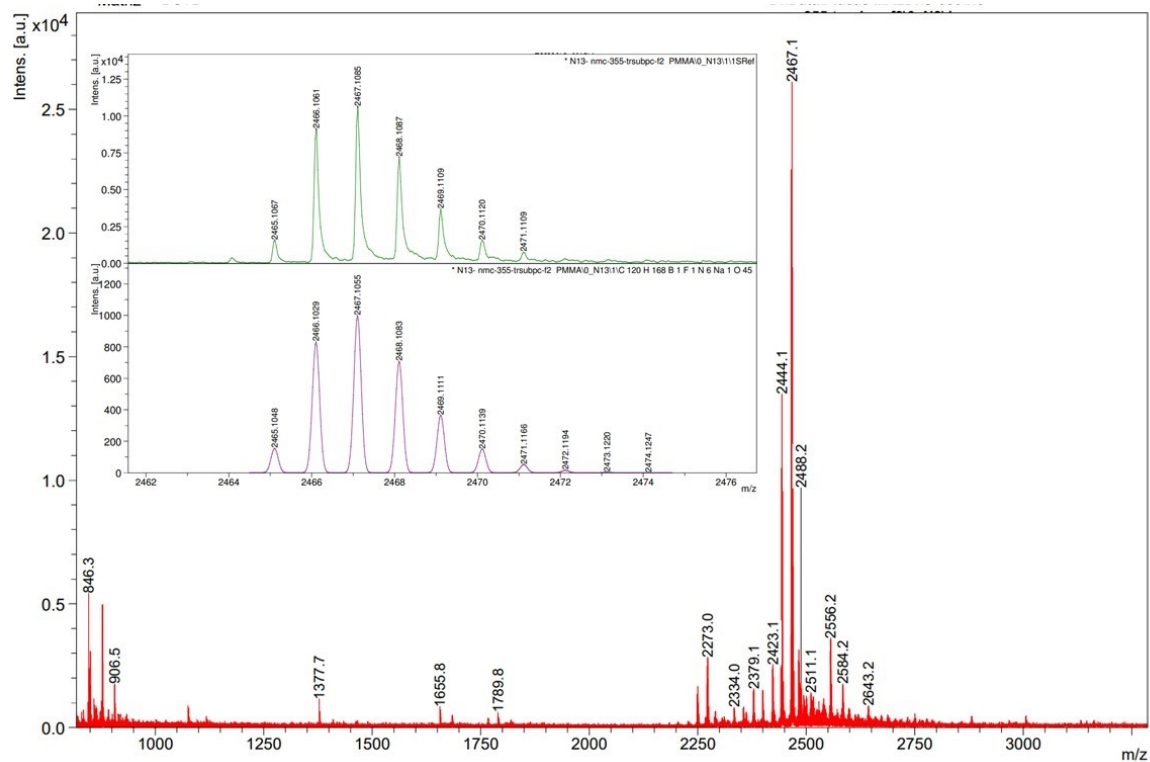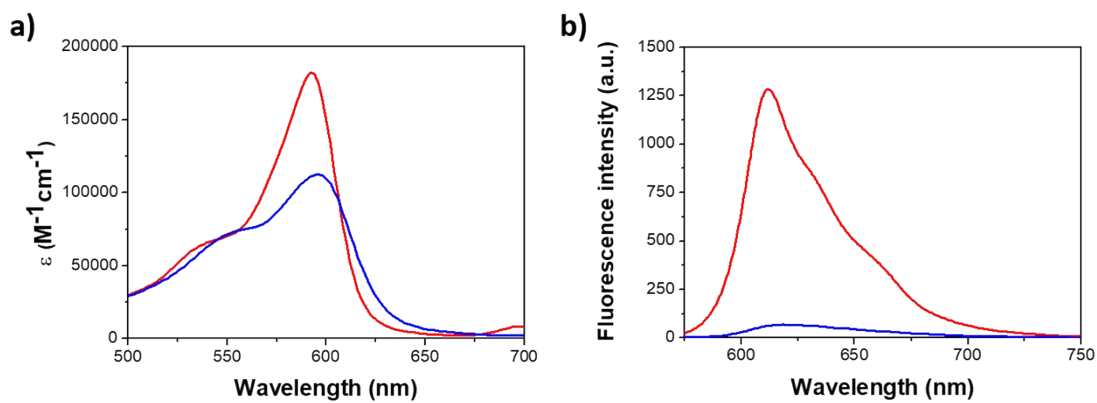

Supporting data

Fig. S1 (a) UV-Vis and (b) fluorescence (λ_{ex} = 550 nm) spectra of **SubPc(PEG)₃** in water (blue) and in DMSO (red) (c = 5 x 10^{-5} M) at room temperature.

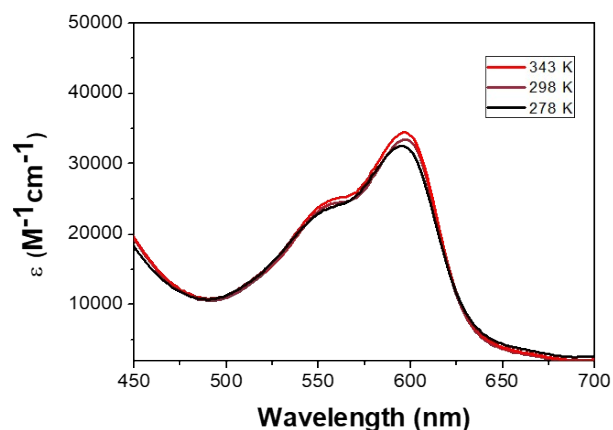**Fig. S2** UV-Vis spectra of **SubPc(PEG)₃** in water (c = 2.5 x 10⁻⁵ M) at different temperatures between 278 K (black) and 343 K (red).

The Gibbs free energy of monomer association (ΔG), the parameter *m*, that relates the ability of the good solvent to interact with the monomer, and the cooperativity degree (σ) have been derived by using the SD model[8] (Fig. 1d,e in the main text). The application of eqn (1) – (3) allows the derivation of the complete set of thermodynamic parameters associated with the supramolecular polymerisation mechanism of **SubPc(PEG)₃**.

$$\Delta G^{'} = \Delta G + mf \quad (1)$$

$$\Delta G^{'} = - RTlnK_e \quad (2)$$

$$\sigma = K_n/K_e \quad (3)$$

Table S1. Thermodynamic parameters obtained from global fitting of the temperature-dependent UV (λ = 419 nm) and fluorescence (λ$_{ex}$ = 520 nm, λ = 594 nm) data for **SubPc(PEG)3** in water at different concentrations (Fig. 1d,e in the main text) on the basis of the SD model.[8]

ΔG [kJ mol^{-1}]	m	σ*	*ΔG´ [kJ mol^{-1}]	K* [M^{-1}]
-43.0	48.1	1.0	-33.3	6.9 X 10^{-4}

*ΔG´, K$_e$, K$_n$ and σ were calculated at 298 K for f = 0.2.
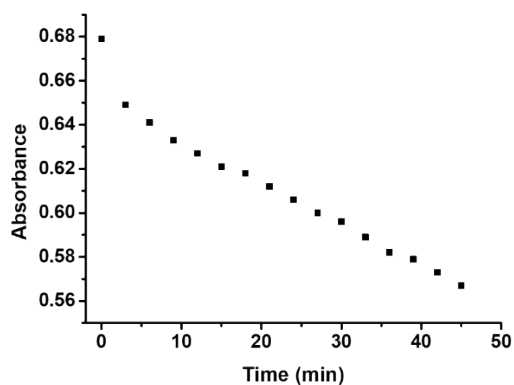
a)

b)

c)
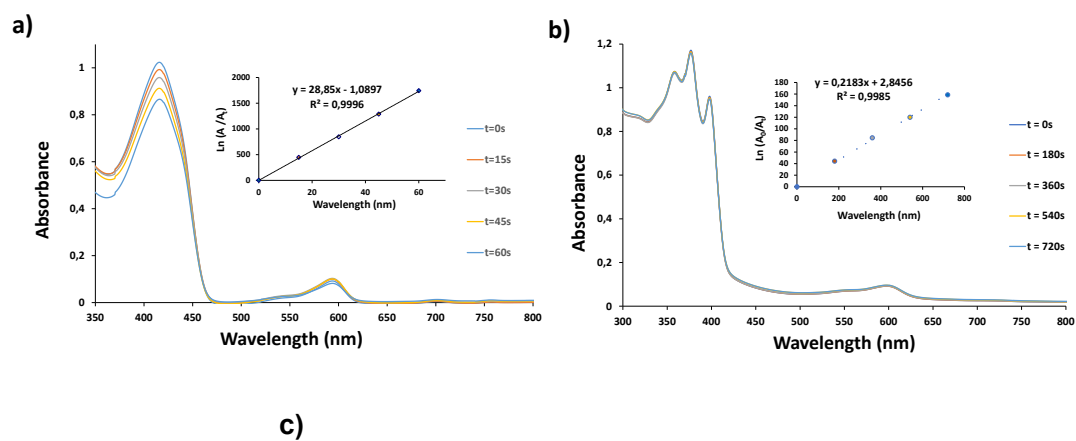
Fig. S3 Time-dependent photobleaching of (a) DBPF in DMSO and (b) ABDA in D$_2$O in the presence of **SubPc(PEG)$_3$**, which is directly related to the generation of $^1O_2$ induced by the PS upon irradiation. (c) Photo-decay of ABDA (30 μM) sensitised by **SubPc(PEG)$_3$** (20 μM) in DMSO as monitored by the decrease in absorbance at 399 nm along with the irradiation time ($\lambda > 515$ nm).

Table S2. Photophysical data for **SubPc(PEG)$_3$**.

PS	Φ_Δ (DMSO)[I]	Φ_Δ (D$_2$O)[II]	λ_F[III]	Φ_F [IV] (DMSO)
SubPc(PEG)$_3$	0.54	0.11	618 nm	0.26

[I] Using ZnPc in DMSO as the reference ($\phi_\Delta = 0.56$). [II] Using Eosin Y in D$_2$O as the reference ($\phi_\Delta = 0.60$). [III] Excited at 550 nm. [IV] Using ZnPc in DMSO as the reference ($\phi_F = 0.20$).
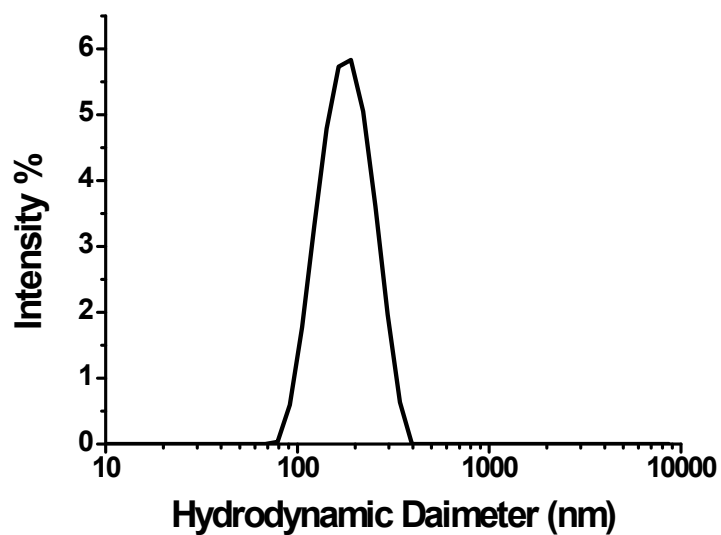
Fig. S4 Hydrodynamic diameter distribution of **SubPc(PEG)₃** nanoparticles (with 5 µM SubPc) in DMEM measured by DLS.
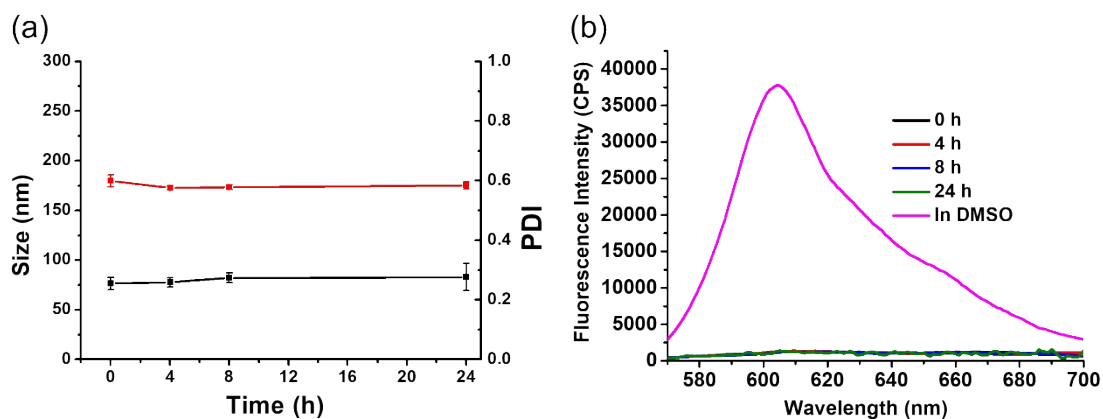

Fig. S5 (a) Hydrodynamic diameter (red) and PDI (black) of **SubPc(PEG)₃** nanoparticles in DMEM over a period of 24 h. (b) Fluorescence spectra (λ_{ex} = 552 nm) of **SubPc(PEG)₃** nanoparticles in DMSO and in DMEM over a period of 24 h.
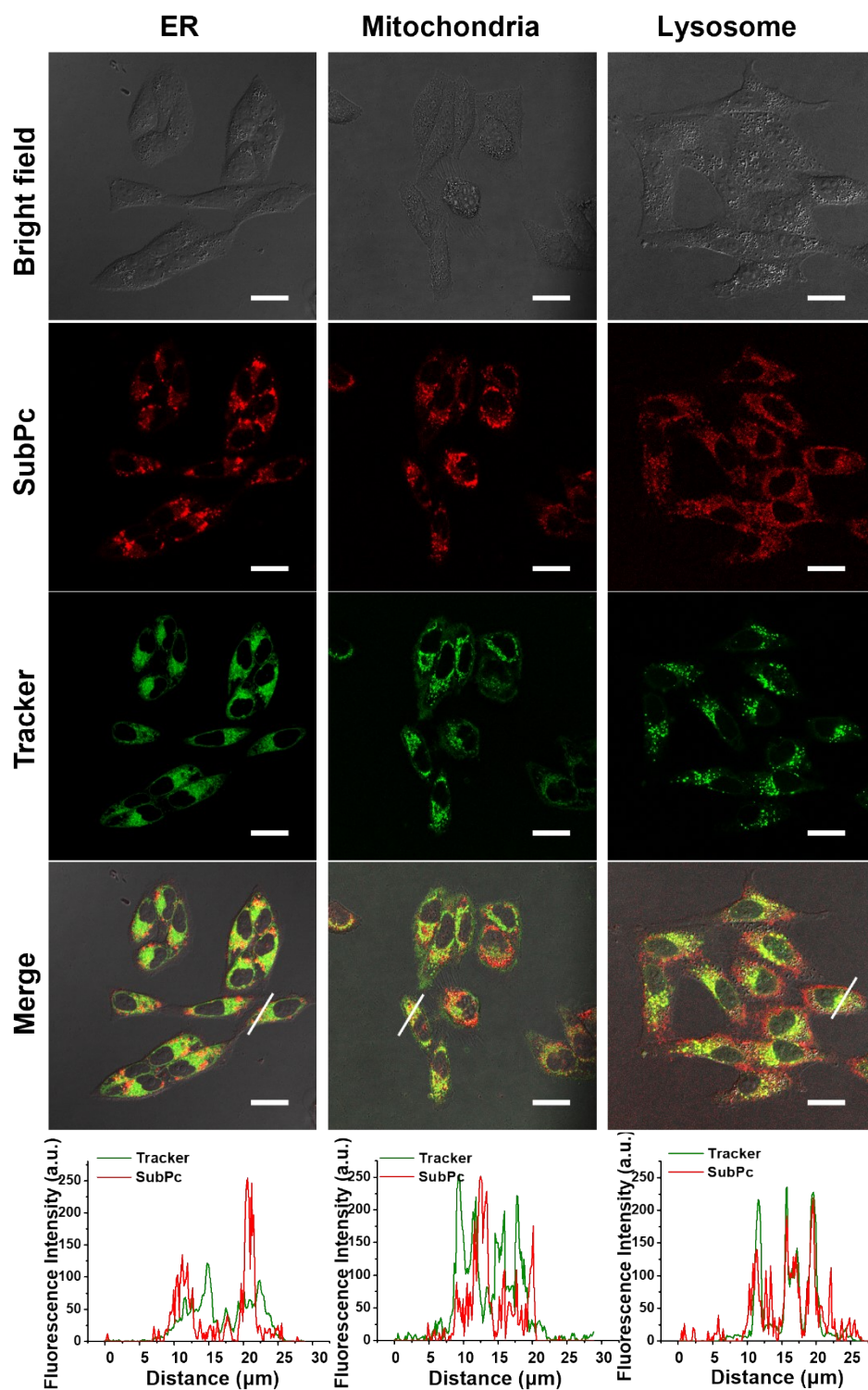
Fig. S6 Visualisation of the intracellular fluorescence of **SubPc(PEG)₃** nanoparticles (after incubation for 24 h) and various subcellular trackers in HepG2 cells. The corresponding bright-field and merged images are given in row 1 and 4 respectively. The figures on the bottom show the fluorescence intensity profiles of **SubPc(PEG)₃** nanoparticles and the different trackers traced along the lines in the corresponding images on row 4. Scale bar: 25 µm.
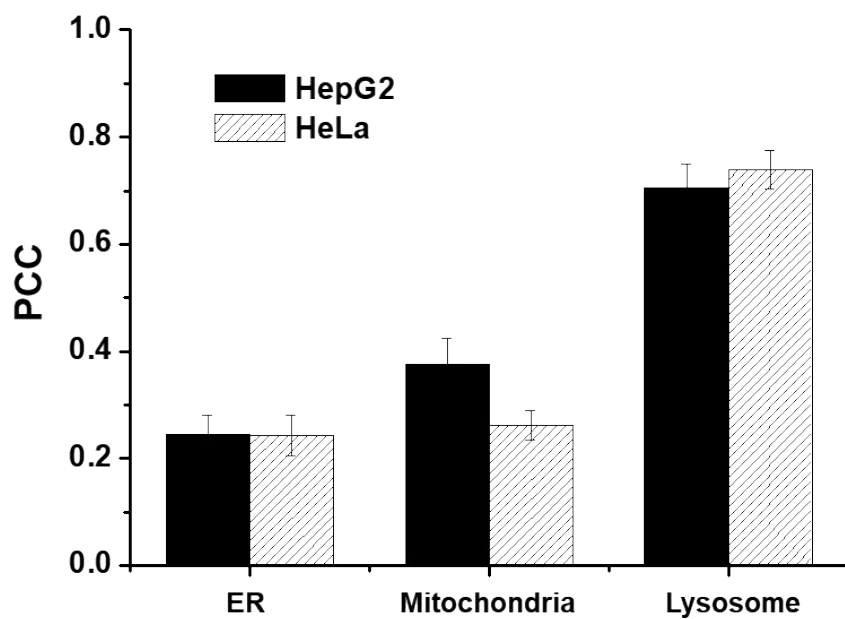
Fig. S7 Pearson's correlation coefficients between the intracellular fluorescence of **SubPc(PEG)₃** nanoparticles (after incubation for 24 h) and that of various subcellular trackers in HeLa and HepG2 cells. Data were obtained by analysing 50 cells and are presented as the mean ± SD of three independent experiments.
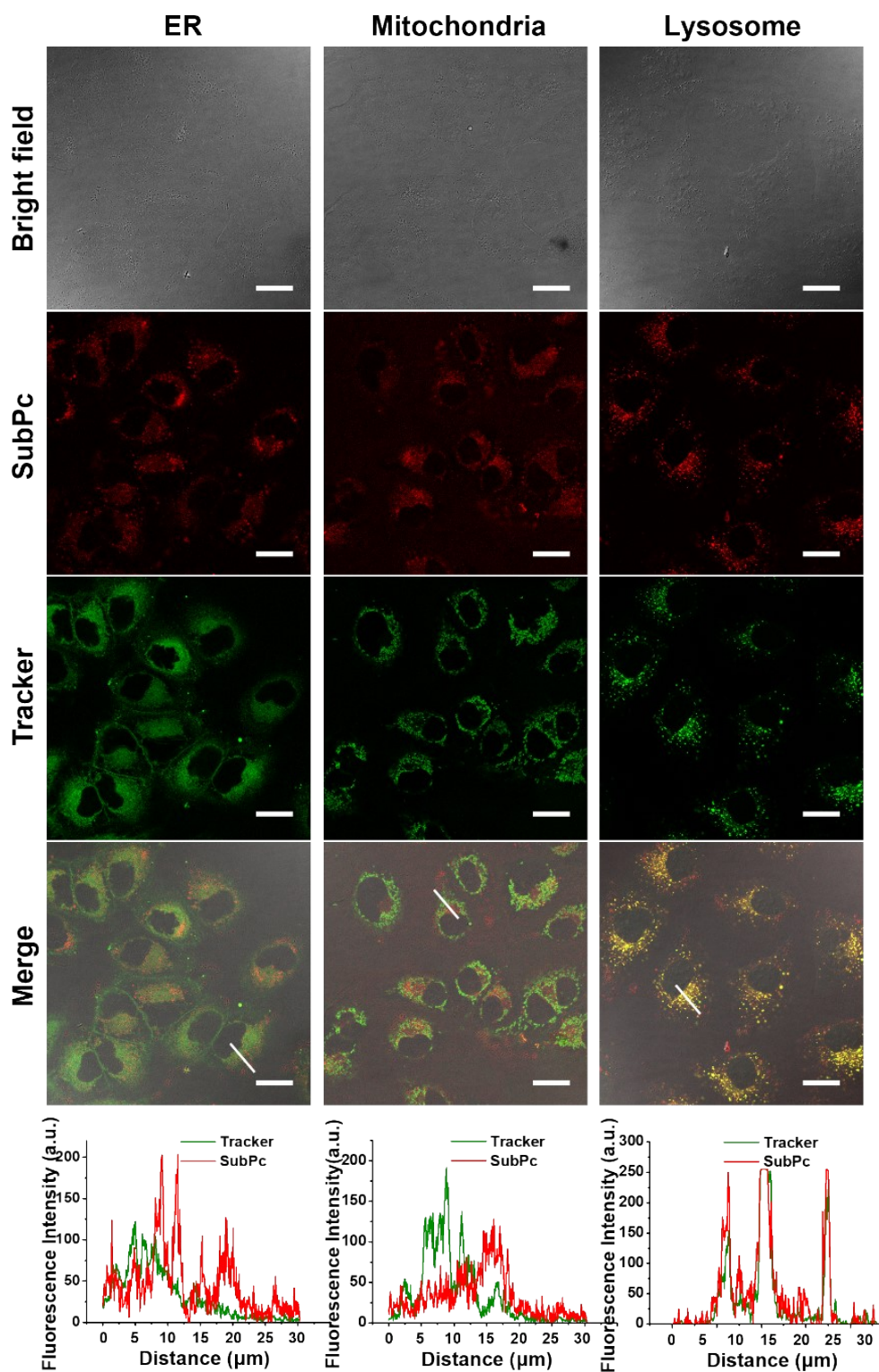
Fig. S8 Visualisation of the intracellular fluorescence of **SubPc(PEG)₃** nanoparticles (after incubation for 4 h) and various subcellular trackers in HeLa cells. The corresponding bright-field and merged images are given in row 1 and 4 respectively. The figures on the bottom show the fluorescence intensity profiles of **SubPc(PEG)₃** nanoparticles and the different trackers traced along the lines in the corresponding images on row 4. Scale bar: 25 μm.
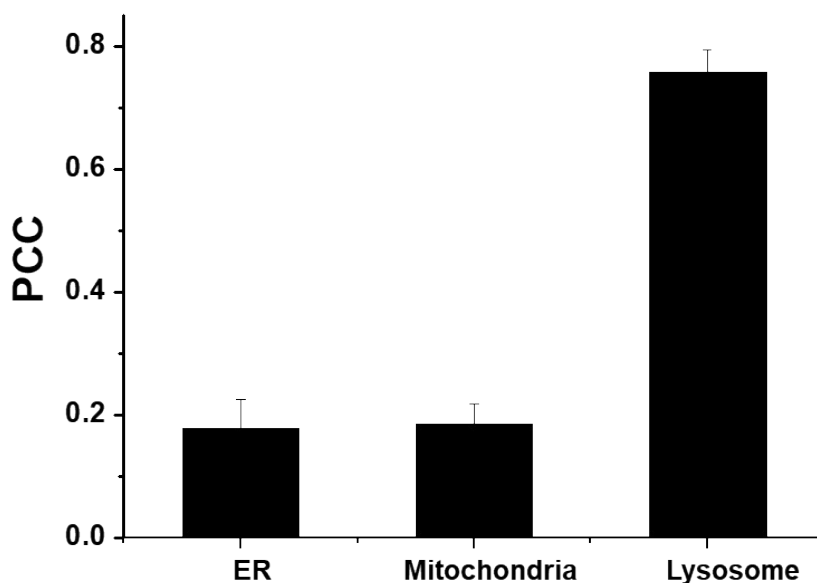
Fig. S9 Pearson's correlation coefficients between the intracellular fluorescence of **SubPc(PEG)₃** nanoparticles (after incubation for 4 h) and that of various subcellular trackers in HeLa cells. Data were obtained by analysing 50 cells and are presented as the mean ± SD of three independent experiments.

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