Electronic Supplementary Information

An acid-cleavable phthalocyanine tetramer as an activatable photosensitiser for photodynamic therapy

Sun Y. S. Chow, Pui-Chi Lo* and Dennis K. P. Ng*

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Experimental Section

General

All the reactions were performed under an atmosphere of nitrogen. \(N,N\)-dimethylformamide (DMF) was dried over barium oxide and distilled under reduced pressure. Tetrahydrofuran (THF) and toluene were distilled from sodium benzophenone ketyl and sodium, respectively, under nitrogen prior to use. All other solvents and reagents were of reagent grade and used without further purification. Chromatographic purification was performed on silica gel (Macherey-Nagel, 230–400 mesh) with the indicated eluents. Size-exclusion chromatography was carried out on Bio-Rad Bio-Beads S-X1 beads (200-400 mesh) using THF as the eluent. Compounds 1\(^{R1}\) and 3\(^{R2}\) were prepared as described.

\(^1\)H and \(^{13}\)C\(^{\{1\}H}\) NMR spectra were recorded on a Bruker AVANCE III 400 spectrometer (\(^1\)H, 400 MHz; \(^{13}\)C, 100.6 MHz) in deuterated solvents. Spectra were referenced internally by using the residual solvent \([\^1\)H: \(\delta = 7.26\) (for CDCl\(_3\)) and 7.18 (for the most upfield signal of pyridine-\(d_5\))] or solvent \([^{13}\)C: \(\delta = 77.2\) (for CDCl\(_3\)) and 123.3 (for the most upfield signal of pyridine-\(d_5\))] resonances relative to SiMe\(_4\). Electrospray ionisation (ESI) mass spectra were recorded on a Thermo Finnigan MAT 95 XL mass spectrometer. Matrix-assisted laser desorption/ionisation time-of-flight (MALDI-TOF) mass spectra were recorded on a Bruker Autoflex MALDI-TOF mass spectrometer.

UV-Vis and steady-state fluorescence spectra were taken on a Cary 5G UV-Vis-NIR
spectrophotometer and a Hitachi F-7000 spectrofluorometer respectively. The fluorescence quantum yields ($\Phi_F$) of the samples in DMF were determined by the equation: 

$$\Phi_{F(\text{sample})} = \left( \frac{F_{\text{sample}}}{F_{\text{ref}}} \right) \left( \frac{A_{\text{ref}}}{A_{\text{sample}}} \right) \left( \frac{n^2_{\text{sample}}}{n^2_{\text{ref}}} \right) \Phi_{F(\text{ref})},$$

where $F$, $A$ and $n$ are the measured fluorescence (area under the emission peak), the absorbance at the excitation position (610 nm) and the refractive index of the solvent respectively. Unsubstituted zinc(II) phthalocyanine (ZnPc) in DMF was used as the reference [$\Phi_{F(\text{ref})} = 0.28$]. To minimise reabsorption of radiation by the ground-state species, the emission spectra were obtained in very dilute solutions (absorbance $< 0.05$ at 610 nm). The singlet oxygen quantum yields ($\Phi_\Delta$) were measured in DMF by the method of chemical quenching of 1,3-diphenylisobenzofuran (DPBF) by using ZnPc as the reference ($\Phi_\Delta = 0.56$).

**Preparation of tetraazido diacetal linker 2**

A mixture of terephthalaldehyde (0.26 g, 1.94 mmol), 3-azidopropan-1-ol (2.00 g, 19.8 mmol) and a catalytic amount of $p$-toluenesulfonic acid (TsOH) in toluene (50 mL) was heated under reflux for 12 h and a Dean-Stark apparatus was used to continuously remove the water formed. After cooling, triethylamine (3 drops) was added to quench the TsOH, and then the solvent was removed under reduce pressure. The residue was purified by column chromatography using CH$_2$Cl$_2$ as the eluent to give a colourless liquid (0.53 g, 54%). $^1$H NMR (CDCl$_3$): $\delta$ 7.45 (s, 4 H, Ar-\text{H}), 5.52 (s, 2 H, CH), 3.59-3.63 (m, 4 H, OCH$_2$), 3.52-3.56 (m, 4 H, OCH$_2$), 3.43 (t, $J =$
6.8 Hz, 8 H, CH₂N₃), 1.87 (quintet, J = 6.8 Hz, 8 H, CH₂). ¹³C{¹H} NMR (CDCl₃): δ 138.7, 126.7, 101.6, 62.2, 48.6, 29.2. MS (ESI): an isotopic cluster peaking at m/z 525 (100%, [M+Na]⁺). HRMS (ESI): m/z calcd for C₂₀H₃₀N₁₂NaO₄ [M+Na]⁺: 525.2405; found: 525.2412.

**Preparation of cPC4**

A solution of CuSO₄·5H₂O (15 mg, 60 μmol) and sodium ascorbate (12 mg, 61 μmol) in a 1:1 mixture of water and pyridine (2 mL) was added to a mixture of alkynyl phthalocyanine 1 (2.5 g, 2.7 mmol) and the linker 2 (10 mg, 20 μmol) in CHCl₃ (12 mL). The mixture was stirred at room temperature for 8 h. After mixing with water (10 mL), the mixture was extracted with CHCl₃ (10 mL x 2). The combined organic layer was dried over anhydrous Na₂SO₄ and evaporated in vacuo. The residue was purified by size-exclusion chromatography using THF as the eluent, followed by silica gel column chromatography using CHCl₃/MeOH (30:1, v/v) as the eluent to give a dark green solid (45 mg, 54%). ¹H NMR (CDCl₃ with a trace amount of pyridine-d₅): δ 9.14 (d, J = 7.2 Hz, 8 H, Pc-Hα), 9.08-9.10 (m, 8 H, Pc-Hα), 8.87-8.89 (m, 8 H, Pc-Hβ), 7.39 (s, 4 H, Ar-H), 7.11 (s, 4 H, triazole-H), 7.02 (s, 8 H, Pc-Hβ), 5.08 (s, 2 H, CH), 4.72-4.75 (m, 16 H, OCH₂), 4.46 (s, 8 H, OCH₂-triazole), 4.35-4.38 (m, 16 H, OCH₂), 4.04-4.14 (m, 24 H, OCH₂), 3.77-3.84 (m, 16 H, OCH₂), 3.59-3.69 (m, 24 H, OCH₂), 3.52-3.54 (m, 8 H, OCH₂), 3.34 (s, 12 H, NCH₂), 3.16-3.19 (m, 4 H, NCH₂), 3.05-3.08 (m, 4 H, NCH₂), 1.79-1.82 (m, 8 H, CH₂). ¹³C{¹H} NMR (pyridine-d₅): δ 153.8, 153.6,
153.5, 152.9, 150.7, 149.3, 145.4, 139.4, 139.0, 138.9, 136.1, 135.1, 129.5, 129.3, 127.3, 127.0, 124.1, 123.0, 115.5, 101.9, 72.3, 71.5, 71.2, 71.1, 70.8, 70.2, 69.6, 65.0, 62.3, 58.6, 47.3, 41.1, 30.7 (some of the signals were overlapped). MS (MALDI-TOF): m/z calcd for C_{212}H_{207}N_{44}O_{36}Zn_{4} [M+H]^+: 4208.2884; found, m/z 4208.6524.

**Preparation of ncPC4**

According to the above procedure, phthalocyanine 1 (1.2 g, 1.3 mmol) was treated with the tetraazido linker 3 (15 mg, 64 μmol), CuSO_{4}·5H_{2}O (48 mg, 192 μmol) and sodium ascorbate (38 mg, 192 μmol) in a mixture of CHCl_{3} (12 mL), water (1 mL) and pyridine (1 mL) to give the product as a dark green solid (140 mg, 56%). ^1H NMR (pyridine-d$_5$): δ 9.40 (br. s, 24 H, Pc-H$_a$), 8.32 (s, 4 H, triazole-H), 8.10 (br. s, 16 H, Pc-H$_b$), 8.02 (br. s, 8 H, Pc-H$_a$), 7.27 (s, 8 H, Pc-H$_b$), 4.85 (br. s, 16 H, OCH$_2$), 4.71 (s, 8 H, OCH$_2$-triazole), 4.61 (s, 8 H, NCH$_2$), 4.43 (br. s, 16 H, OCH$_2$), 4.07 (br. s, 16 H, OCH$_2$), 3.79 (br. s, 16 H, OCH$_2$), 3.67 (br. s, 16 H, OCH$_2$), 3.60 (t, $J = 4.8$ Hz, 8 H, OCH$_2$), 3.41 (t, $J = 4.8$ Hz, 8 H, OCH$_2$), 3.14 (s, 12 H, OCH$_3$). ^13C{^1H} NMR (pyridine-d$_5$): δ 153.5, 153.4, 153.3, 153.2, 153.1, 151.9, 150.9, 150.0, 147.6, 143.7, 138.7, 138.4, 128.8, 128.7, 122.4, 113.7, 105.7, 72.1, 71.2, 71.1, 70.8, 70.7, 70.6, 70.5, 70.4, 69.8, 68.6, 59.2 (some of the signals were overlapped).
**pH-responsive fluorescence and singlet oxygen measurements**

**cPC4** and **ncPC4** were dissolved in DMF to give 100 μM solutions, which were diluted to 0.5 μM with phosphate buffered saline (PBS) at different pH (7.4, 6.5, 6.0, 5.5 or 5.0) with 1% (v/v) Cremophor EL with continuous stirring. The fluorescence spectra ($\lambda_{ex} = 610$ nm, $\lambda_{em} = 630-800$ nm) of these solutions were recorded at different time intervals. For the study of singlet oxygen generation efficiency, **cPC4** and **ncPC4** were dissolved in DMF to give 100 μM solutions, which were diluted to 0.5 μM with PBS at pH 7.4 or 5.0 with 1% (v/v) Cremophor EL. The mixtures were stirred continuously for 24 h. An aliquot of these solutions (3 mL) was then mixed with a solution of DPBF in DMF (24 mM, 10 μL) followed by illumination with red light coming from a 300 W halogen lamp after passing through a water tank for cooling and a colour glass filter (Newport) cut-on 610 nm. The decay of DBPF at 417 nm was monitored with time.

**Cell lines and culture conditions**

The HT29 human colorectal adenocarcinoma cells (from ATCC, no. HTB-38) were maintained in Dulbecco's Modified Eagle's medium (DMEM) (Invitrogen, no. 10313-021) supplemented with fetal calf serum (10%), penicillin-streptomycin (100 units mL$^{-1}$ and 100 μg mL$^{-1}$ respectively), L-glutamine (2 mM) and transferrin (10 μg mL$^{-1}$).
**Photocytotoxicity assay**

Approximately $3 \times 10^4$ cells per well in the medium were inoculated in 96-multiwell plates overnight at 37 °C in a humidified 5% CO$_2$ atmosphere. cPC4 and ncPC4 were dissolved in DMF to give 1.6 mM solutions, which were diluted to 80 μM with DMEM in the presence of 0.5% (v/v) Cremophor EL. These solutions were further diluted with DMEM to various concentrations. The cells, after being rinsed with PBS twice, were incubated with 100 μL of these phthalocyanine solutions for 2 h at 37 °C under 5% CO$_2$. The cells were then rinsed again with PBS and refilled with 100 μL DMEM before being illuminated at ambient temperature. The light source consisted of a 300 W halogen lamp, a water tank for cooling and a colour glass filter (Newport) cut-on 610 nm. The fluence rate ($\lambda > 610$ nm) was 40 mW cm$^{-2}$. Illumination of 20 min led to a total fluence of 48 J cm$^{-2}$. Cell viability was determined by means of the colourimetric MTT assay.$^R_6$ After illumination, the cells were incubated at 37 °C under 5% CO$_2$ for 24 h. An MTT (Sigma) solution in PBS (3 mg mL$^{-1}$, 50 μL) was added to each well followed by incubation for 2 h under the same environment. A solution of sodium dodecyl sulfate (Sigma, 10% by weight, 50 μL) was then added to each well. The plate was incubated at 37 °C under 5% CO$_2$ for 30 min, and then 80 μL of iso-propanol was added to each well. The plate was agitated on a Bio-Rad microplate reader at ambient temperature for 10 sec before the absorbance at 540 nm for each well was taken. The average absorbance of the blank wells, which did not contain the cells, was subtracted from the readings of the other wells. The cell
viability was then determined by the following equation: 

\[ \% \text{ viability} = \frac{\left[ \sum (A_i / A_{\text{control}} \times 100) \right]}{n}, \]

where \( A_i \) is the absorbance of the \( i \) th data \( (i = 1, 2, \ldots, n) \), \( A_{\text{control}} \) is the average absorbance of the control wells in which the phthalocyanine was absent and \( n (= 5) \) is the number of the data points.

**Intracellular ROS measurements**

Approximately \( 3 \times 10^4 \) HT29 cells per well in DMEM were inoculated in 96-multiwell plates and incubated overnight at 37 °C in a humidified 5% CO\(_2\) atmosphere. \text{cPC4} and \text{ncPC4} were dissolved in DMF to give 1.6 mM solutions, which were diluted to 80 μM with DMEM in the presence of 0.5% (v/v) Cremophor EL. These solutions were further diluted with DMEM to various concentrations. The cells, after being rinsed with PBS twice, were incubated with 100 μL of these phthalocyanine solutions for 2 h at 37 °C under 5% CO\(_2\). After being rinsed with PBS twice, the cells were incubated with 100 μL of 2',7'-dichlorodihydrofluorescein diacetate (DCFDA) (Molecular Probes) in PBS (10 μM) at 37 °C for 30 min in the dark. The cells were then rinsed again with PBS and refilled with 100 μL of PBS in the dark before the photodynamic treatment. Fluorescence measurements were made in a fluorescence plate reader (TECAN Polarion) with a 485 nm excitation filter and a 535 nm emission filter set at a gain of 60.
Intracellular fluorescence imaging

Approximately $3 \times 10^4$ HT29 cells in DMEM (2 mL) were seeded on a coverslip and incubated overnight at $37 \, ^\circ\text{C}$ under 5% CO$_2$. The medium was then removed. The cells were incubated with a solution of cPC4 or ncPC4 in the medium [1 $\mu$M with 0.6% (v/v) DMF and 0.06% (v/v) Cremophor EL, 2 mL] for 2 h under the same conditions. The cells were rinsed with PBS and then viewed with an Olympus FV1000 IX81 confocal microscope equipped with a 633 nm helium neon laser. Emission signals in 650-750 nm were collected and the images digitised and analysed using Fluoview. The average intracellular fluorescence intensities (for a total of 50 cells in each sample) were also determined.

References


Fig. S1 Electronic absorption spectra of (a) cPC4 and (b) ncPC4 in DMF at different concentrations. The insets plot the Q-band absorbance versus the concentration of cPC4 and ncPC4. Figure (c) shows the absorption spectra of 1, cPC4 and ncPC4 in DMF at the same concentration of the phthalocyanine unit.
Fig. S2 Fluorescence spectra of 1, cPC4 and ncPC4 in DMF (excited at 610 nm).
Fig. S3 Change in fluorescence intensity of ncPC4 (0.5 μM) in PBS (with 1% Cremophor EL) at different pH with time.
Fig. S4 Comparison of the rate of decay of DPBF (initial concentration = 80 μM) sensitised by cPC4 and ncPC4 (both at 0.5 μM) after incubation in PBS (with 1% Cremophor EL) at pH 7.4 and 5.0 for 24 h.
Fig. S5 $^1$H NMR spectrum of 2 in CDCl$_3$. 
Fig. S6 $^{13}$C$\{^1$H$\}$ NMR spectrum of 2 in CDCl$_3$. 

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Fig. S7 $^1$H NMR spectrum of cPC4 in CDCl$_3$ with a trace amount of pyridine-$d_5$. 
Fig. S8 $^{13}$C-$^1$H NMR spectrum of cPC4 in pyridine-$d_5$. 
Fig. S9 $^1$H NMR spectrum of ncPC4 in pyridine-$d_5$. 
Fig. S10 $^{13}\text{C}^{1\text{H}}$ NMR spectrum of ncPC4 in pyridine-$d_5$. 