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Supporting information for

A charge-switchable, four-armed polymeric photosensitizer for photodynamic cancer therapy

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Experimental details

Materials. All reagents and solvents were obtained commercially and used without further purification. β-Benzyl-L-aspartate (BLA), bis-(trichloromethyl)-carbonate (triphosgene), 1-(3-aminopropyl)imidazole (API), N,N-dimethylformamide (DMF), tetrahydrofuran (THF), dimethyl sulfoxide (DMSO) and deuterium oxide (D_2O, 99% D) were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). *Meso*-tetra(4-aminophenyl) porphyrin (TAPP) was purchased from Frontier Scientific, Inc. (Salt Lake City, UT, USA). Dimethyl sulfoxide-d_6 (DMSO-d_6, 99% D) was purchased from Cambridge Isotope Laboratories. Inc. (Andover, MA, USA). The dialysis membranes were obtained from Spectrum Laboratories Inc. (Rancho Dominguez, CA, USA). RPMI-1640 medium, fetal bovine serum (FBS), antibiotics (penicillin/streptomycin), and Dulbecco's phosphate buffered saline (DPBS) were obtained from Gibco BRL (Invitrogen Corp., Carlsbad, CA, USA). Singlet Oxygen Sensor Green (SOSG) and the LIVE/DEAD® Viability/Cytotoxicity Kit were purchased from Molecular Probes, Inc. (Eugene, OR, USA). Cell counting kit-8 (CCK-8) was purchased from Enzo Life Science, Inc. (NY, USA).

The synthesis of β-Benzyl-L-aspartic acid *N*-carboxyanhydride (BLA-NCA). BLA-NCA was synthesized by the Fuchs-Farthing method using triphosgene.[1, 2] Briefly, L-aspartate-β-benzyl ester (3 g, 13.44 mM) was suspended in THF (50 mL) containing triphosgene (3 g, 10.11 mM) and stirred at 60 ℃ for 2 h. The crude mixture was filtered twice. The product was precipitated by the addition of hexane (900 mL) and collected by filtration and drying under vacuum to obtain BLA-NCA as a white powder.

The synthesis of *meso*-tetra(4-aminophenyl) porphyrin-poly(β-benzyl-L-aspartate) (TAPP-pBLA). TAPP-pBLA was synthesized via the ring-opening polymerization of BLA-NCA initiated by the four terminal amino groups of TAPP. The TAPP (0.16 g, 0.23 mM) was dissolved in DMF (20 mL). A

solution of BLA-NCA (2.87 g, 11.52 mM) in DMF (20 mL) was added to the solution of TAPP and the reaction mixture was stirred for 2 days at 25 °C. The resulting solution was dialyzed against distilled water using a dialysis membrane (Spectra/Por; molecular weight cut off (MWCO) = 3,500 Da) for 2 days. Lyophilization afforded TAPP-pBLA as a powder. The degree of polymerization (DP) of the BLA units was calculated to be 26 (M_n = 6,400; M_w = 9,000, M_w was determined by gel permeation chromatography) from ^{1}H-NMR measurements. ^{1}H-NMR spectra were recorded in DMSO-d_6 at 25 °C using a Bruker 300 MHz NMR Spectrometer (Bruker, Germany).
^{1}H-NMR (DMSO-d_6): δ -2.74 (2H, inner NH), δ2.82 (52H, -COCHC$\underline{H}_2$COOCH$_2$Ph), δ4.63 (26H, -COC$\underline{H}$NH-), δ5.12 (52H, -COOC$\underline{H}_2$-), δ7.02 (4H, -Ph-N$\underline{H}$-), δ7.34 (130H, -COOCH$_2\underline{Ph}$), δ8.10 (16H, ArH), δ8.45 (26H, -COCHN$\underline{H}$-), δ8.89 (8H, pyrrole βCH).

Synthesis of the charge-switchable, four-armed polymeric photosensitizer (C4P-PS, *meso*-tetra(4-aminophenyl) porphyrin-poly(aminopropyl imidazole-grafted-L-aspartic acid), TAPP-p(API-*g*-L-ASP)). TAPP-pBLA (200 mg, 28.17 μM) was dissolved in DMSO (10 mL). API (3.20 mM) was added to the solution and the reaction mixture was stirred for 12 h. The resulting solution was dialyzed against 0.01M hydrochloric acid solution (replaced three times) and distilled water (replaced three times). The hydrochloride salt of TAPP-pBLA was obtained as a white powder after lyophilization. ^{1}H-NMR spectra were recorded in DMSO-d_6 at 25 °C using a Bruker 300 MHz NMR Spectrometer (Bruker, Germany).
^{1}H-NMR (DMSO-d_6): δ2.74 (2H, inner NH), δ1.85 (26H, -CONHCH$_2$C$\underline{H}_2$-), δ2.69 (26H, -CONHC$\underline{H}_2$CH$_2$-), δ2.97 (52H, -COCHC$\underline{H}_2$CONH-), δ3.87 (26H, -COC$\underline{H}$NH-), δ4.04 (26H, -CONHCH$_2$CH$_2$C$\underline{H}_2$N-), δ5.06 (13H, -CON$\underline{H}$CH$_2$CH$_2$CH$_2$N-), δ 6.90, δ7.16, δ7.65 (39H, Imidazole), δ7.02 (4H, -Ph-N$\underline{H}$-), δ8.10 (16H, ArH), δ8.89 (8H, pyrrole βCH).

Synthesis of the noncharge-switchable, four-armed polymeric photosensitizer (NC4P-PS, *meso*-tetra(4-aminophenyl) porphyrin-poly(L-aspartic acid), TAPP-(L-ASP)). To remove the β-benzyl group, TAPP-pBLA (200 mg; 28.17 μM) was dissolved in 0.5 M NaOH solution and stirred for 10 min at 25 °C. The resulting clear solution was neutralized with acetic acid, followed by dialysis against deionized water using a dialysis membrane (Spectra/Por; MWCO = 1,000 Da), and then lyophilized. ^{1}H-NMR spectra were recorded in D_2O at 25 °C using a Bruker 300 MHz NMR Spectrometer (Bruker, Germany).
^{1}H-NMR (D_2O): δ2.69 (52H, -COCHC$\underline{H}_2$COOH), δ4.35 (26H, -COC$\underline{H}$NH-), δ7.08 (4H, -Ph-N$\underline{H}$-), δ7.85 (16H, ArH), δ8.75 (8H, pyrrole βCH).

Gel permeation chromatography (GPC). The samples were analyzed using an HPLC system (Waters) equipped with a 515 HPLC pump, a pump control module Ⅱ, a 717plus autosampler, a column oven, and a GPC KF-804 or GPC KF-805 column (Shodex, Tokyo, Japan) at a flow rate of 1.0 mL/min with *N,N*-dimethylformamide as the mobile phase at room temperature. Detection was performed using a 410 differential refractometer. The average molecular weight of the sample was calibrated against narrow molecular weight pullulan standards. The M_w obtained from GPC showed a difference from the M_n obtained from NMR, because the retention time of GPC is affected not only by the molecular weight but also by the branched structure. Generally the calibration curve from GPC is based on the linear polymers and has a limitation providing the actual molecular weights of branched polymers.[3,4]

Zeta-potential analysis. The zeta-potential was determined using dynamic light scattering (DLS; Zetasizer Nano ZS, Malvern Instruments Ltd., UK). DLS was performed at 25 °C in aqueous solution (sample concentration, ~ 1 mg/mL) with the sampling time and analysis set to automatic.

Optical photography and fluorescence analysis. Free TAPP and C4P-PS (1.0×10^{-5} M TAPP) were dissolved in distilled water and DMSO, respectively. Optical photographs were taken using a digital

camera (nx1000, Samsung Electronics Co., Korea) at 25 °C. Fluorescence intensity was measured in 96-well plates using a plate reader (Tecan Genios, Durham, NC, USA) with an excitation wavelength of 650 nm and emission wavelength of 675 nm at 25 °C. Fluorescence images were captured using a 12-bit CCD camera (Image Station 4000 MM; Kodak, New Haven, CT, USA) with a special C-mount lens and a long-wave emission filter (600~700 nm; Omega Optical, Brattleboro, VT, USA).

Measurement of singlet oxygen generation (SOG). The SOG by TAPP derivatives was detected chemically using SOSG as a probe. The SOSG probe works via an intramolecular electron transfer, which quenches the fluorescence from the light-emitting chromophore prior to reaction with singlet oxygen. Reaction with singlet oxygen results in the formation of the endoperoxide, prohibiting electron transfer and thus leading to the recovery of fluorescence.[5, 6] TAPP derivatives were dissolved in distilled water (1.0×10^{-5} M TAPP) and mixed with a SOSG solution (2 mM). The mixture was then irradiated with 10 mW/cm^2 of 670 nm laser source (fiber coupled laser system, LaserLab®, Korea) for 100 seconds. The fluorescence intensity of SOSG (λ_{ex} 494 nm, λ_{em} 534 nm) was recorded using fluorescence spectroscopy (RF-5301; Shimadzu, Japan).

In vitro cell culture and incubation conditions. HCT-116 (human colon cancer) and CT-26 (murine colon cancer) cells were obtained from the Korean Cell Line Bank (HCT-116; KCLB No. 10247, CT-26; KCLB No. 80009) and were cultured in RPMI-1640 (HCT-116) and DMEM (CT-26) supplemented with 10% heat inactivated FBS and penicillin/streptomycin (100 U/mL and 100 μg/mL, respectively), which is called complement medium (CM medium) in this study. The cells were cultured at 37 °C in a humidified atmosphere containing 5% CO_2 and subcultured in new medium every 2-3 days. C4P-PS or NC4P-PS was dissolved in serum-free (SF) medium.

In vitro cellular uptake test. To verify the cellular uptake of TAPP derivatives at two different pHs (pH 7.4 and 6.5), HCT-116 cells were seeded in 12-well cell culture plate at a density of 5×10^5 cells per well and incubated for 12 h at 37 °C in 5% CO_2. The medium was then removed and the cells were incubated in serum-free (SF) medium containing C4P-PS or NC4P-PS (1.0×10^{-5} M TAPP) for 1 h at pH 7.4 or 6.5. The cells were rinsed, harvested and re-suspended with DPBS. Cellular uptake was quantitatively analyzed using flow cytometry (Beckman, San Jose, CA, USA). For each sample 10,000 cells (gated events) were counted, and TAPP fluorescence was detected with logarithmic settings (FL4; Em = 670 nm). Each experiment was analyzed statistically using the CXP analysis program (Beckman, San Jose, CA, USA).

To observe the cellular localization of TAPP derivatives at two different pHs (pH 7.4 and 6.5), HCT-116 cells (1×10^5 cells per well in a 12-well plate) were treated with C4P-PS or NC4P-PS (1.0×10^{-5} M TAPP) for 2 h at pH 7.4 or 6.5. The cells were then washed twice with DPBS, fixed with 4% paraformaldehyde and stained with DAPI. The cells were mounted in mounting medium (Dako, Glostrup, Denmark) and visualized using a confocal laser scanning microscope (LSM 710 Meta; Carl Zeiss, Germany). A laser line with a wavelength of 633 nm was used for excitation. A long-pass filter (LP 650 nm) was used at the emission end for detection. Fluorescence images were analyzed using the LSM Image Browser software (Carl Zeiss, Germany).

In vitro cell viability study (Cell Counting Kit-8, CCK-8 assay). HCT-116 cells were seeded in black 96-well cell culture plates at a density of 2×10^4 cells per well and incubated for 12 h. C4P-PS or NC4P-PS (1×10^5 M TAPP) was added to each well in SF medium (100 μL), and the plates were returned to the incubator for 4 h. After incubation, the wells were rinsed twice with DPBS to remove material that had not been internalized by the cells. One hundred microliters of complement medium were added to the wells, and each well was irradiated using the pre-determined 670 nm laser source (0, 3, 6, 12, 24 J/cm^2, fiber coupled laser system, LaserLab®, Korea). The cells were incubated for a further 24 h. Cell viability was assessed using the CCK-8 assay according to the manufacturer`s protocol. In brief, ten microliter of CCK-8 solution (2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium,

monosodium salt) was added to 100 μL of each well containing complement medium in a 96-well plate for 2 h. And then, 50 μL of medium was transferred to new 96-well plate. Absorbance intensity was measured at 450 nm using a microplate reader (Bio-Tek, VT, USA).

Live/dead assay. The Live/dead viability/cytotoxicity assay kit (Molecular Probes, USA) was used as a two-color fluorescence cell viability assay. The optimal dyes used were calcein AM (2 μM) and EthD-1 (4 μM); the former stains live cells green, whereas the latter stains dead cells red. HCT-116 cells were seeded in 35-mm cell culture dishes at a density of 5×10^5 per well. The cells were incubated for 12 h at 37 °C in 5% CO_2. After incubation, the medium was removed and the cells were incubated in SF medium containing C4P-PS or NC4P-PS (1×10^5 M TAPP) at pH 7.4 and 6.5 for 4 h. The medium was removed and the cells were rinsed twice with DPBS. Then, 670 nm laser irradiation (0 and 24 J/cm^2) was performed with a 670 nm laser source (fiber coupled laser system, LaserLab®, Korea). The cells were then incubated in complement medium for 1 h. The cell viability was observed using fluorescence microscopy (Carl Zeiss, Germany). Fluorescence images were analyzed using LSM Image Browser software (Carl Zeiss, Germany).

Animal model. CT-26 cells were implanted into 6-week-old BALB/c nude mice. Briefly, the cells (5×10^4) in 100 μL of SF DMEM medium were injected subcutaneously. Tumor volume was calculated using the following equation: Volume = $0.5 \times L \times W^2$, where "W" and "L" are the width and length of the tumor, respectively. All procedures were approved by the Institutional Animal Care and Use Committee (IACUC) of College of Pharmacy, the Catholic University of Korea and Use committee in accordance with the "Principles of Laboratory Animal care", NIH publication no. 85–23, revised in 1985.

In vivo tumor growth inhibition effect. The tumor model was established as described above. When the tumor volume was around 100-150 mm^3 at 15 days after cell inoculation, the mice were randomly divided into five groups (n=4). Solutions of PBS, C4P-PS and NC4P-PS in 100 μL of commercially used NaCl solution were injected via tail vein at a dose of 10 mg TAPP per kg body weight per injection at day 0. Tumor regions were irradiated with a 670 nm laser (fiber coupled laser system, LaserLab®, Korea) at power densities of 150 J/cm^2 (0.5 W/cm^2, 5 min) at 12 h post-injection. Tumor sizes and body weights were measured at each time point. Optical images were captured by digital camera.

Statistical analysis. Data are expressed as the mean ± standard deviation (SD). Differences between the values were assessed using Student's t-test.

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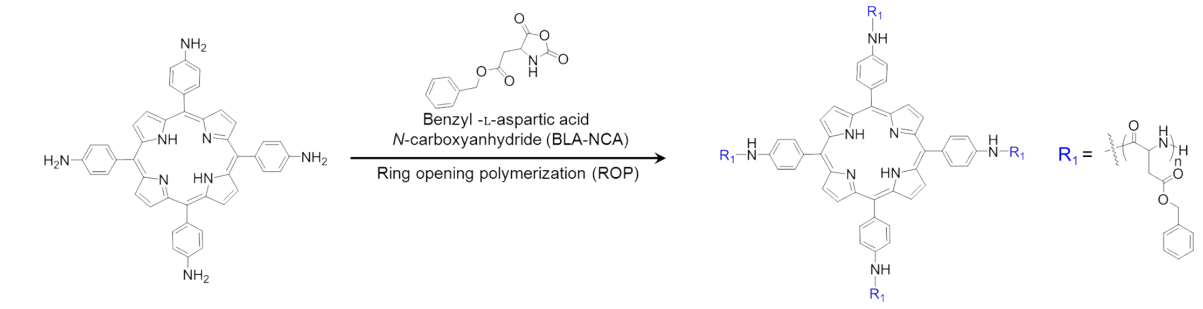
Table S1. Characterization of C4P-PSs.

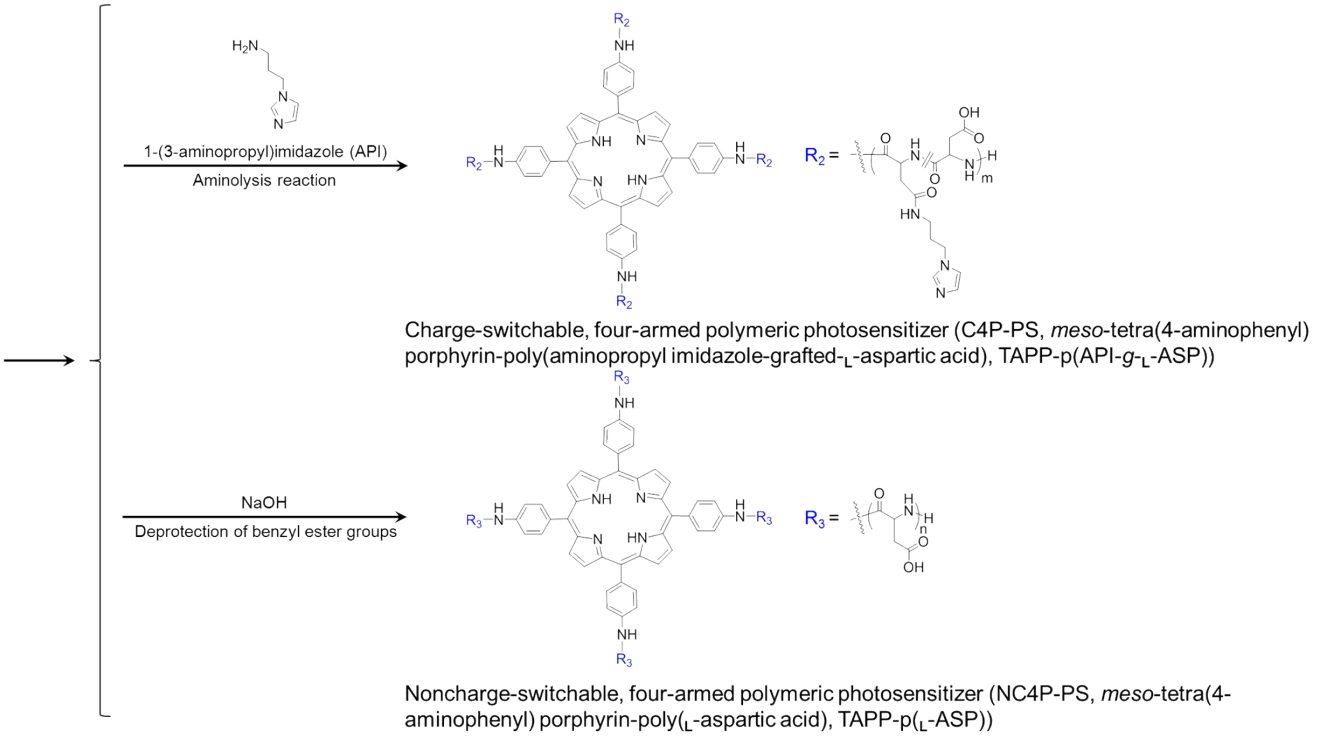
Code	Molar feed ratio (API)[a]	Actual content (mole, wt %)[b]						M_n[b]	M_w[c]	PZC (pH)[g]	ZP at pH 7.4[h]	ZP at pH 6.5[i]
		TAPP[d]		DP-L-ASP[e]		API-*g*-L-ASP[f]						
Compound **1**	1	1	14	18	50	7	36	4,800	8,600	4.9	-28 ± 2	-22 ± 1
Compound **2**	4.5	1	13	12	30	13	57	5,400	8,800	6.7	-29 ± 2	+8 ± 1
Compound **3**	10	1	12	6	14	17	74	5,600	9,400	N.D.	+29 ± 8	+30 ± 1
Compound **2** after four weeks[j]	4.5	1	13	12	30	13	57	5,400	-.	6.7	-24 ± 2	14 ± 1

[a]Feed ratio of API to benzyl ester groups in TAPP-pBLA.
[b] Number average molecular weight (M_n) as determined by ^{1}H-NMR.
[c] Weight average molecular weight (M_w) as determined by GPC.
[d]Actual content of *meso*-tetra(4-aminophenyl) porphyrin (TAPP) in synthesized compound.
[e]Actual content of deprotected-L-aspartic acid (DP-L-ASP) in synthesized compound.
[f]Actual content of 1-(3-aminopropyl)imidazole-grafted-L-aspartic acid (API-*g*-L-ASP) in synthesized compound.
[g] Point of zero charge (PZC, pH value) of synthesized compound determined by DLS.
[h]Zeta potential (ZP) of synthesized compound at pH 7.4 determined by DLS.
[i]Zeta potential (ZP) of synthesized compound at pH 6.5 determined by DLS.
[j]Ccompound **2** was characterized after incubation for 4 weeks in PBS buffer (50 mM, pH 7.4).



meso-tetra(4-aminophenyl)porphyrin (TAPP)

TAPP-poly(benzyl-L-aspartic acid) (TAPP-pBLA)



Charge-switchable, four-armed polymeric photosensitizer (C4P-PS, *meso*-tetra(4-aminophenyl) porphyrin-poly(aminopropyl imidazole-grafted-$_L$-aspartic acid), TAPP-p(API-*g*-$_L$-ASP))

Noncharge-switchable, four-armed polymeric photosensitizer (NC4P-PS, *meso*-tetra(4-aminophenyl) porphyrin-poly($_L$-aspartic acid), TAPP-p($_L$-ASP))

Scheme S1. Synthetic route of charge-switchable, four-armed polymeric photosensitizer (C4P-PS), and noncharge-switchable, four-armed polymeric photosensitizer (NC4P-PS).

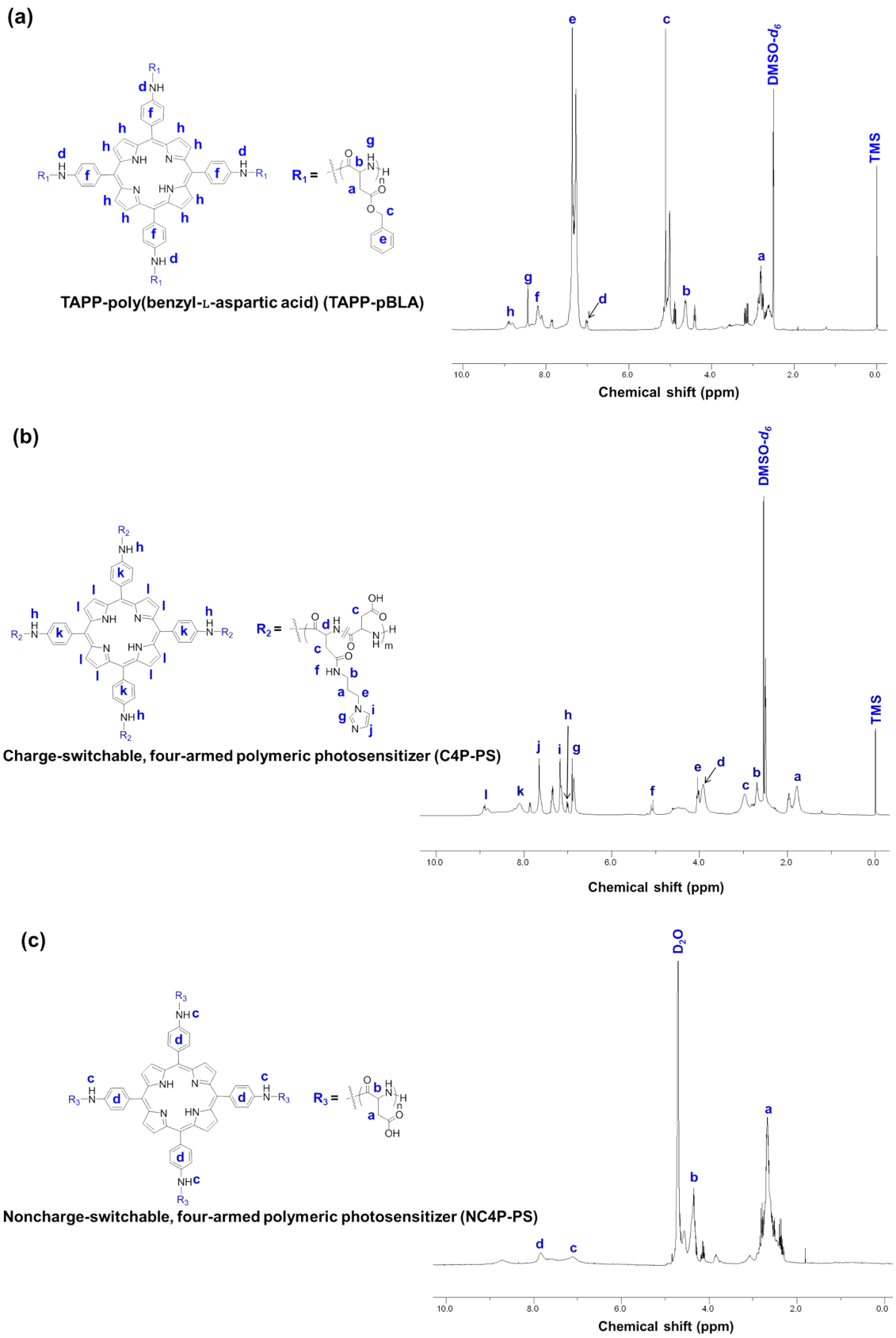


Figure S1. ^{1}H NMR analysis of (a) TAPP-pBLA in DMSO-d_6, (b) C4P-PS in DMSO-d_6, and (c) NC4P-PS in D_2O.

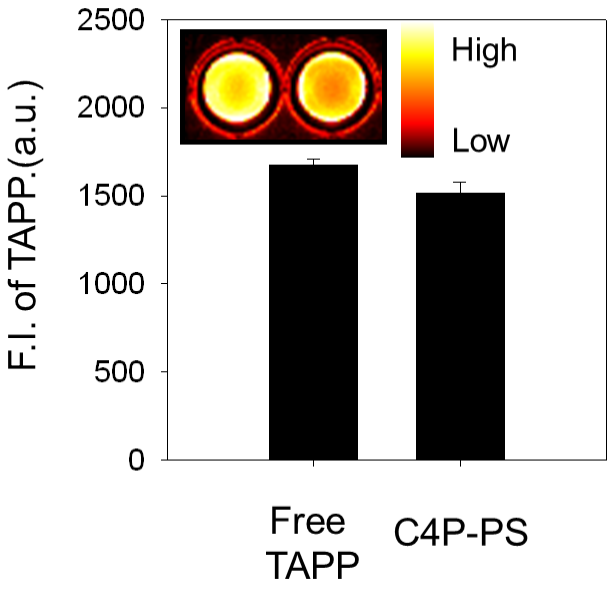


Figure S2. Fluorescence intensity of free TAPP and C4P-PS in organic solvent (DMSO). Inset: fluorescence image from wells containing free TAPP and C4P-PS. TAPP concentration was 1.0×10^{-5} M.

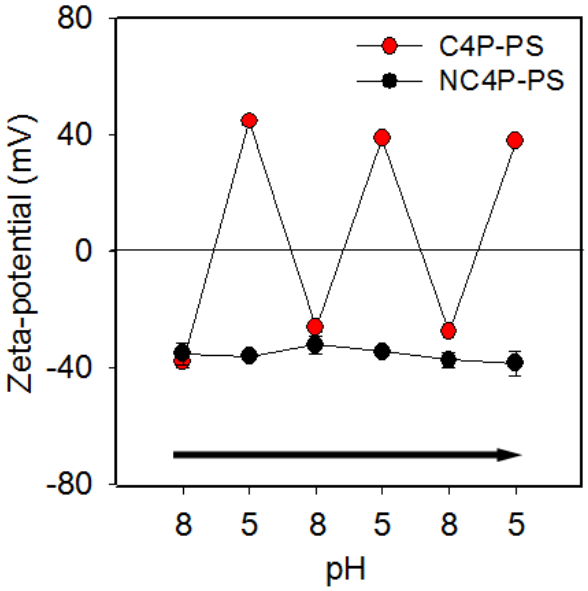


Figure S3. Reversible variation of the zeta potential of C4P-PS and NC4P-PS at two representative pHs after incubation for 4 weeks in PBS buffer (50 mM, pH 7.4).

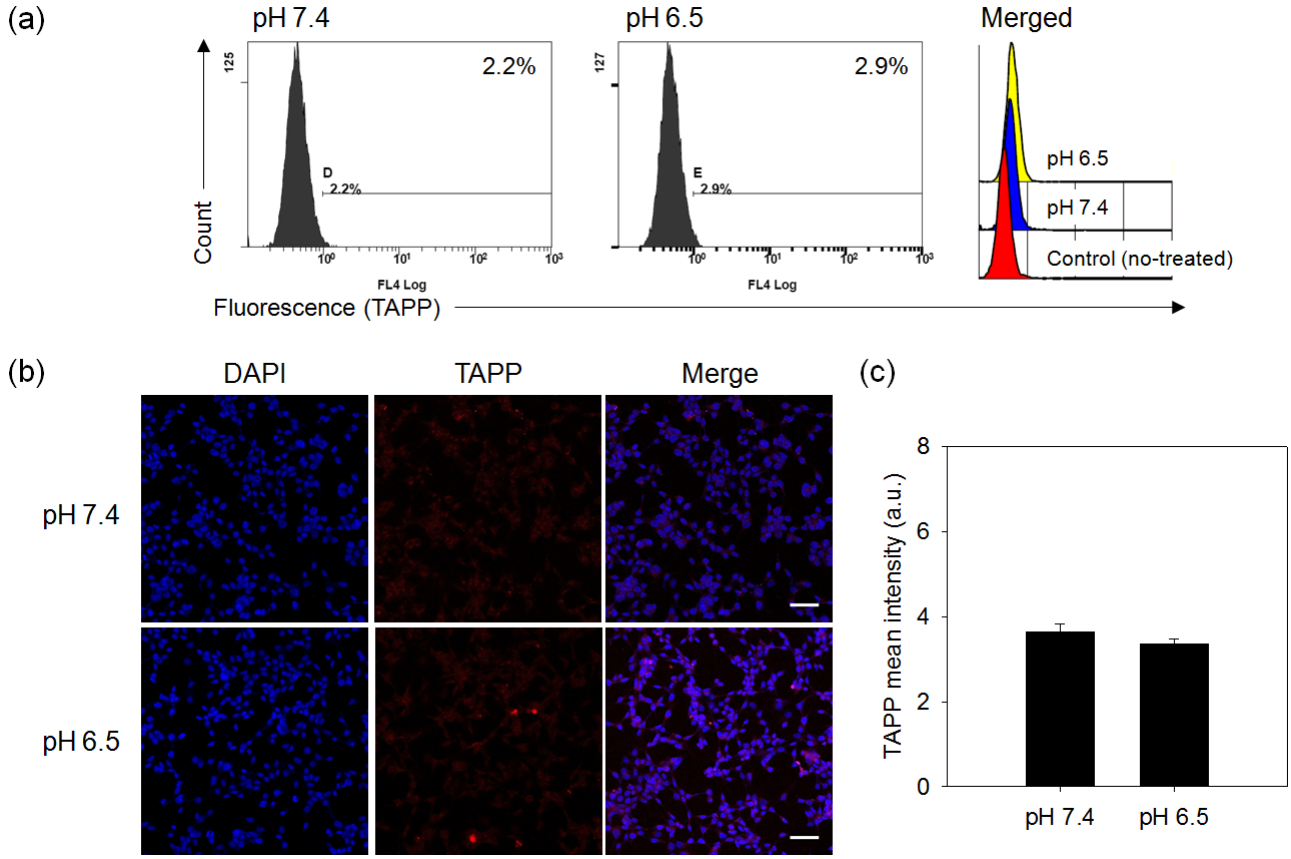


Figure S4. (a) Flow cytometry quantification of cellular internalization of NC4P-PS at pH 7.4 and 6.5. (b) CLSM image of HCT-116 cells treated with NC4P-PS at pH 7.4 and 6.5 (scale bar = 50 μm). (c) The TAPP mean fluorescence value of HCT-116 cells treated with NC4P-PS at pH 7.4 and 6.5 (n=3).

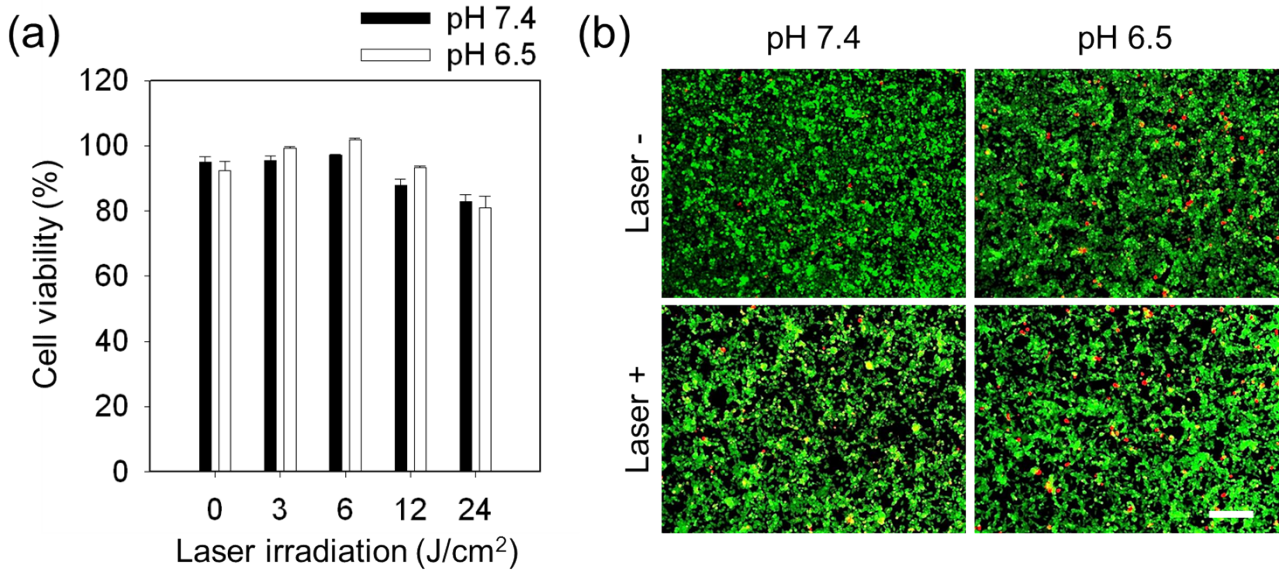


Figure S5. (a) Cell viability of HCT-116 cells treated with NC4P-PS with or without laser irradiation (3 to 24 J/cm^2) at pH 7.4 and 6.5, and (b) Live/dead assay of HCT-116 cells treated with NC4P-PS with or without laser irradiation (24 J/cm^2) at pH 7.4 and 6.5 (scale bar = 200 μm).

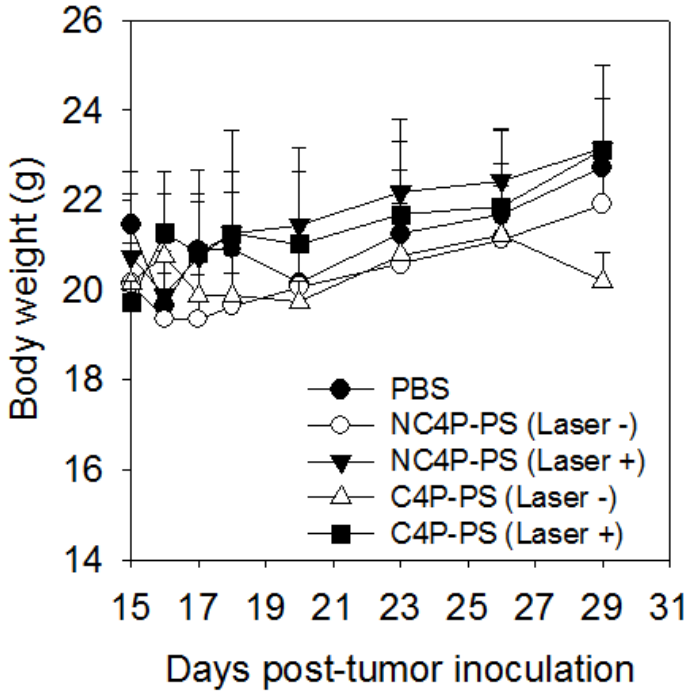


Figure S6. Body weight measurements of CT-26 tumor-bearing mice after tail-vein injection of PBS, NC4P-PS and C4P-PS (dose: 10 mg/kg TAPP, *n*=4) with or without irradiation (150 J/cm^2). Values are mean ± SD.