Electronic Supplementary Information

Photosenstizier-complexed polypyrrole nanoparticles for activatable fluorescence imaging and photodynamic therapy

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Materials and methods

Materials

Pyrrole (98 %), polyvinylpyrrolidone (PVP, MW ~29,000), and iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), trypsin from porcine pancreas (13,100 U/mg), and hyaluronidase (1,228 U/mg) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Hyaluronic acid (HA, Mw 39,000 Da) was obtained from Lifecore Biomedical (Chaska, MN, USA) and used as received. Chlorin e4 (Ce4) was purchased from Frontier Scientific, Inc. (Logan, UT, USA). Singlet oxygen sensor green (SOSG), which is an agent for detecting singlet oxygens, was obtained from Invitrogen Corp. (Carlsbad, CA, USA).

Methods

Synthesis and characterization of positively charged-Ce4 (PCe4)

Positively charged photosensitizer (PCe4) was prepared by conjugating Ce4 with RRK peptide. Briefly, the peptide (RRK) was synthesized using a standard solid-phase peptide synthesis protocol. Coupling was carried out using Fmoc amino acid (8 eq), *N*-hydroxybenzotriazole (HOBt, 8 eq), *N*,*N*-diisopropylethylamine (DIPEA,16 eq) in DMF for 2 h. After each step, resin washing was performed using DMF and methanol 2 times each. The Fmoc protecting group was removed by rocking in 20% piperidine in DMF for 10 min (twice). Ce4 and HOBt/DIPEA in DMF were added to the peptide-attached resin. The mixture was reacted for 12 h and suctioned, and then the resin was washed with DMF, MeOH, and DMF. When the reaction was completed, crude peptide was cleaved from the resin using a mixture of TFA/EDT/thioanisole/TIS/deionized water (90/2.5/2.5/2.5/2.5 volume) for 2 h. The solution was precipitated with cold ether and pelleted using a centrifuge. The solid was collected and air-dried. Crude peptide was dissolved in deionized water and purified by prep-LC, lyophilized, and preserved in a refrigerator before use.

The molecular weights of PCe4 were measured using LC/MSD (Agilent Hewlett Packard 1100 series, Santa Clara, CA, USA).

Synthesis of HA-coated polypyrrole nanoparticles (HANPs)

HA-coated polypyrrole nanoparticles (HANPs) were synthesized as described by Park et al.[1] Briefly, HA (400 mg) was dissolved in 20 mL of deionized water. PVP (0.5 g) was dissolved in 12.5 mL of deionized water and stirred for 30 min at 25°C, followed by addition of 65 μL of pyrrole. After 10 min, 0.5 mL of iron(III) chloride hexahydrate (0.75 g/mL) was poured into the reaction mixture, followed by the addition of pre-dissolved HA solution (20 mL). After 3 h of incubation, the solution was purified using membrane dialysis (MWCO: 100,000) for 2 days. The precipitate was removed by centrifugation at 1200 rpm for 2 min. Black powder was obtained by freeze-drying.

Fluorescence quenching of PCe4 by HANPs

Fluorescence quenching of PCe4 during formation of the charge complex with HANPs was analyzed as follows: 100 μL of PCe4 solution (100 μg/mL) was mixed with 200 μL of various concentrations of HANP and the solution was vortexed. After incubation for 1 h, the fluorescence spectra of PCe4 were acquired using a multifunctional microplate reader (Safire 2; Tecan, Männedorf, Switzerland) (λ_{ex} = 405 nm) (Fig. S4).

Preparation and characterization of PCe4-complexed HANPs (PCe4@HANPs)

PCe4 solution (0.8 mg PCe4 in 100 μL deionized water) was slowly added to the HANP solution (4 mg HANP in 400 μL deionized water), and the mixture was incubated for 24 h to form a charge complex. Unbound free PCe4 was removed by passing the reaction mixture through a PD Minitrap G-25 column (GE Healthcare, Little Chalfont, UK) according to the manufacturer's protocol. The volume of the obtained solution was adjusted to 1 mL and the product was filtered through a 0.45-μm pore size membrane to remove aggregated nanoparticles and obtain PCe4@HANPs.

The hydrodynamic size and zeta-potential of PCe4@HANPs were measured using a zeta potential/particle-sizer (Malvern Instrument, Malvern, UK). Scanning electron microscope (SEM) and transmission electron microscope (TEM) images of PCe4@HANP were acquired using a JEOL-7001F and JEM-2010 (JEOL Ltd., Tokyo, Japan), respectively. A UV/Vis absorption spectrophotometer (DU730, Beckman Coulter, Brea, CA, USA) and multifunctional microplate reader (Safire 2; Tecan) were used to measure the UV/Vis absorption and fluorescence spectra, respectively.

To measure the amount of PCe4 in PCe4@HANPs, PCe4@HANPs were dissolved in deionized water containing 20% wt/v SDS to detach PCe4s from HANPs, and then UV/Vis absorption spectra of the solution were measured and compared with the standard curve of PCe4s in deionized water containing 20% wt/v SDS.

Analysis of singlet oxygen generation

To measure singlet oxygen generation (SOG), SOSG was used as a singlet-oxygen-detecting reagent. PCe4 and PCe4@HANP (2 μg/mL PCe4 equivalent) was dissolved in oxygen-saturated water solution and SOSG was then added. The concentration of SOSG reagent in the test solution was fixed at 1 μM. Increases in SOSG fluorescence (λ_{ex} = 504 nm, λ_{em} = 525 nm) were measured periodically during light illumination with a 670 nm CW laser (50 mW/cm^2). To normalize the fluorescence intensity of SOSG, initial fluorescence intensity of SOSG control was deducted from the fluorescence intensity of the sample solutions. All experiments were carried out in quadruplicate. Data are expressed as the mean ± standard deviation (SD).

***In vitro* release profile of PCe4 from PCe4@HANPs**

The *in vitro* release profile of PCe4 was examined in 5 mL of phosphate-buffered saline (PBS; 160 mM, pH 7.4, NaCl 150 mM) at 25°C. Briefly, PCe4@HANPs (150 μL, 80 μg/mL PCe4 equivalent) were dialyzed using D-Tube Dialyzer Minis (MWCO 12–14 kDa) with gentle shaking. At each selected time point, the samples inside the D-tubes were dissolved in deionized water containing 20% wt/v SDS to detach PCe4s from HANPs, and then the UV/Vis absorption spectrum of the solution was measured and compared with a standard curve of PCe4s in deionized water containing 20% wt/v SDS. The amount of PCe4 released at each time point was calculated by deducting the remaining PCe4 inside the D-Tubes. All experiments were performed in triplicate.

To evaluate whether low pH and enzymes stimulate PCe4 release from the nanoparticles, PCe4@HANPs (100 μL, 40 μg/mL PCe4 equivalent) were incubated in 900 μL of acetate buffer (200 mM, pH 5.0) or acetate buffer (200 mM, pH 5.0) containing hyaluronidase (500 U/mL) or trypsin (940 μg/mL). After 5 h incubation, the samples were applied to an Amicon® ultra-0.5 centrifugal filter (30k, Merck Millipore, Billerica, MA, USA) and centrifuged twice to remove the released PCe4. The remaining PCe4@HANP in the upper part of the Amicon tubes was dissolved in deionized water containing 20% wt/v SDS, and then the UV/Vis absorption spectra were analyzed. The amount of PCe4 released at each time point was calculated by deducting the remaining PCe4 in the upper part of the Amicon tubes. All experiments were performed in triplicate.

Real-time near-infrared (NIR) fluorescence imaging of cancer cells incubated with free PCe4 or PCe4@HANP

To evaluate fluorescence quenching in the extracelluar region and subsequent activation in the NIR region of PCe4@HANP inside cancer cells, real-time NIR fluoresce imaging was performed. Squamous cell carcinoma (SCC7) cells obtained from the American

Type Culture Collection (Manassas, VA, USA) were maintained in Dulbecco's modified essential medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 1% antibiotic and antimycotic (Thermo Fisher Scientific) and 10% fetal bovine serum (FBS) at 37°C, in a 5% CO_2 atmosphere. SCC7 cells were seeded into a 12-well (Thermo Scientific, Waltham, MA, USA) at a density of 1×10^5 cells per well and incubated for 24 h for cell attachment. The cells were then replaced with phenol red-free medium containing PCe4@HANP or PCe4, equivalent of 5 μM of PCe4. Without washing the cells, NIR fluorescence images (λ_{ex} = 640 ± 15 nm, λ_{em} = 690 ± 25 nm) of the SCC7 cells were acquired every 30 min for 5 h using a Live Cell Imaging System (Axio observer Z1, 10x, NA 0.55, Carl Zeiss, Jena, Germany). All images were acquired using the same microscope settings and analyzed using Carl Zeiss software (ZEN lite 2012).

Intracellular localization of PCe4@HANPs

To analyze the intracellular localization of PCe4@HANPs, SCC7 cells were seeded into 8-well lab-tek chamber slide at a density of 2×10^4 cells per well and then incubated overnight for cell attachment. PCe4@HANP was dispersed and diluted with cell culture medium to obtain 2 μM PCe4 equivalent. The cell culture medium of SCC7 cells was replaced with fresh medium containing PCe4@HANP and the cells were incubated for 5 h. Next, the cells were stained with LysoTracker (Blue DND-22, Thermo Scientific) for 30 min to stain the lysosomes of the cancer cells. After washing the cells twice with cell culture medium, the cells were incubated in phenol red-free cell culture medium. Finally, fluorescence images of PCe4@HANP (λ_{ex} = 405 nm, λ_{em} = 625–754 nm) and LysoTracker (λ_{ex-}= 405 nm, λ_{em-}= 411–497 nm) in the cells were obtained using confocal laser scanning microscopy (CLSM, ZEISS LSM 510 META, Carl Zeiss).

***In vitro* cytotoxicity of PCe4@HANPs**

The cytotoxicity of PCe4@HANP was evaluated by treating cells for 16 h in the absence of light illumination. SCC7 cells were seeded into a 96-well plate at a density of 1×10^4 cells per well and incubated overnight for cell attachment. The cell culture medium was replaced with fresh media containing various concentrations of PCe4@HANP and incubated for 16 h. After washing the cells twice with cell culture medium, the cells were further incubated for 24 h. Cell viability was evaluated using a CCK-8 assay kit (Dojindo Laboratories, Kumamoto, Japan). Untreated control cells were used as a reference for 100% viable cells, and their medium served as the background. Data are expressed as the mean ± SD of 4 samples.

***In vitro* phototoxicity test of PCe4@HANPs**

To evaluate selective photodynamic therapy using the nanoparticles, SCC7 cells were seeded into a 24-well plate at a density of 5×10^4 cells per well and incubated for 24 h for cell attachment. The cell culture medium was replaced with fresh medium containing various concentrations of PCe4@HANPs, and the cells were incubated for 16 h. After washing the cells twice with cell culture medium, the cells were irradiated with a 670-nm CW laser at a dose rate of 100 mW/cm^2 for 300 s. Five hours later, the cells were stained with a calcein AM staining kit (Invitrogen) for fluorescence microscopy of live and dead cells. Live cells were stained green with calcein (470/540 nm) and the nuclei of dead cells were stained red with EthD-1 (546/607 nm).

SCC7 cells were seeded on 96-well plates at a density of 1×10^4 cells per well and incubated for 24 h at 37ºC to allow for cell attachment. PCe4@HANP was dissolved and diluted with DMEM medium with 10% FBS to various concentrations (0.1, 0.2, 0.5, 1, 2, 5, and 10 μg PCe4 equiv./mL). The existing medium was replaced with 100 μL of fresh cell culture medium containing PCe4@HANP, and the cells were then incubated further for 16 h at 37ºC. Finally, all cells were

washed twice, and fresh culture medium was added. Thereafter, the cells were irradiated with a 670-nm CW laser at a dose rate of 100 mW/cm^2 for 300 s. After further incubating the cells overnight, cell viability was analyzed using a CCK-8 solution. Untreated control cells were used as a reference for 100% viable cells, while the cell culture medium served as the background. Results are represented as the mean (±SD) of four data samples.

Statistical analysis

Data are expressed as the mean ± SD. Student's *t*-test was performed for statistical analyses.

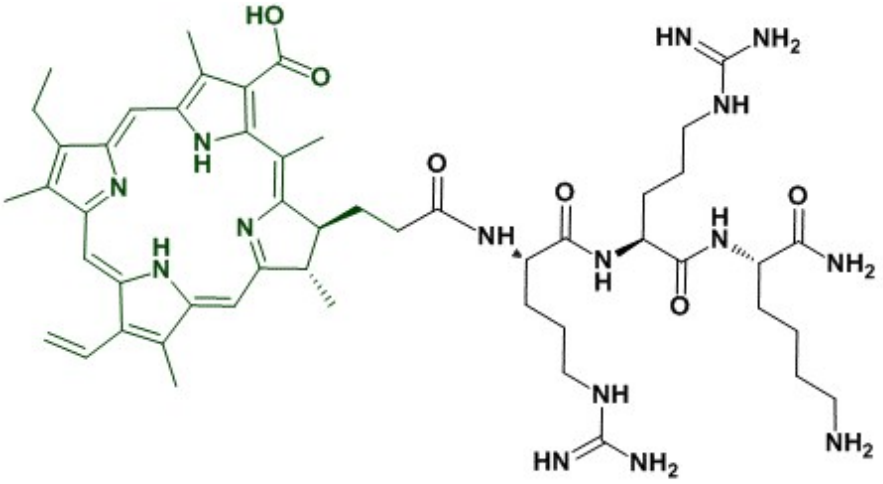

Fig. S1 Chemical structure of PCe4.

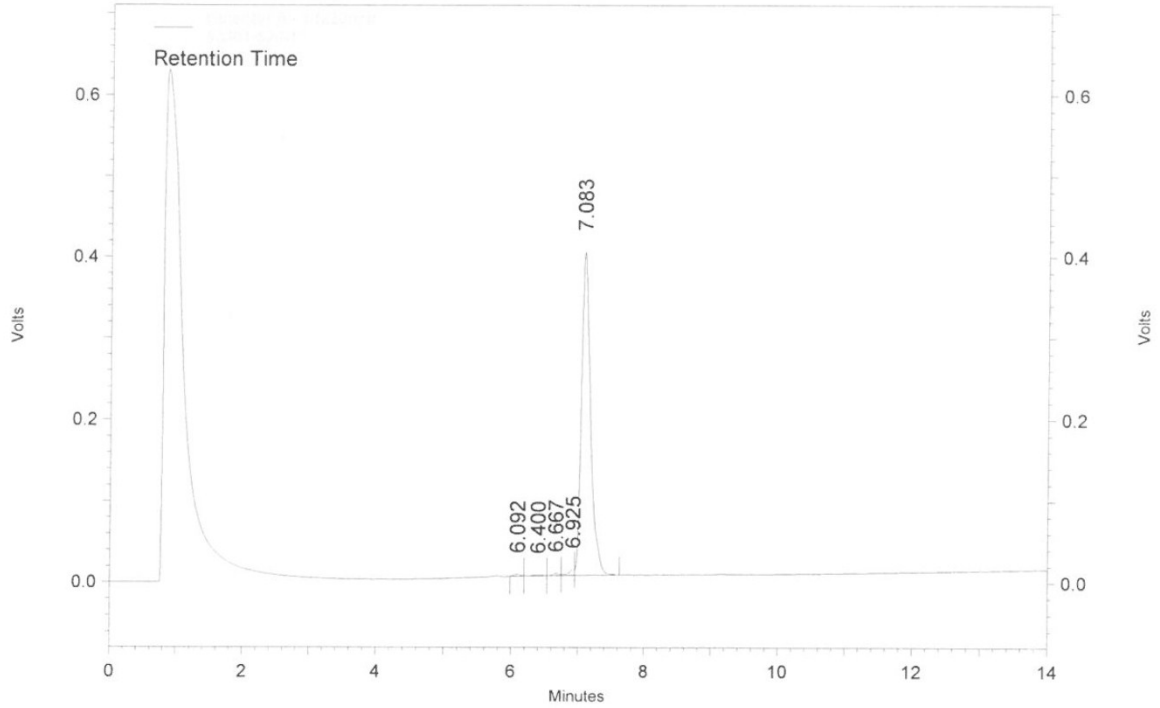

Fig. S2 HPLC chromatogram of PCe4. Purified PCe4 was analyzed by reverse-phase HPLC (Shimadzu prominence HPLC, Kyoto, Japan) using a Vydac Everest C18 column (250 mm × 22 mm, 10 μm). Elution was carried out with a water-acetonitrile linear gradient containing 0.1% (v/v) trifluoroacetic acid. The flow rate was 1 mL/min. The PCe4 was detected at 220 nm with a retention time of 7.083 min and a measured purity of 97.736%.

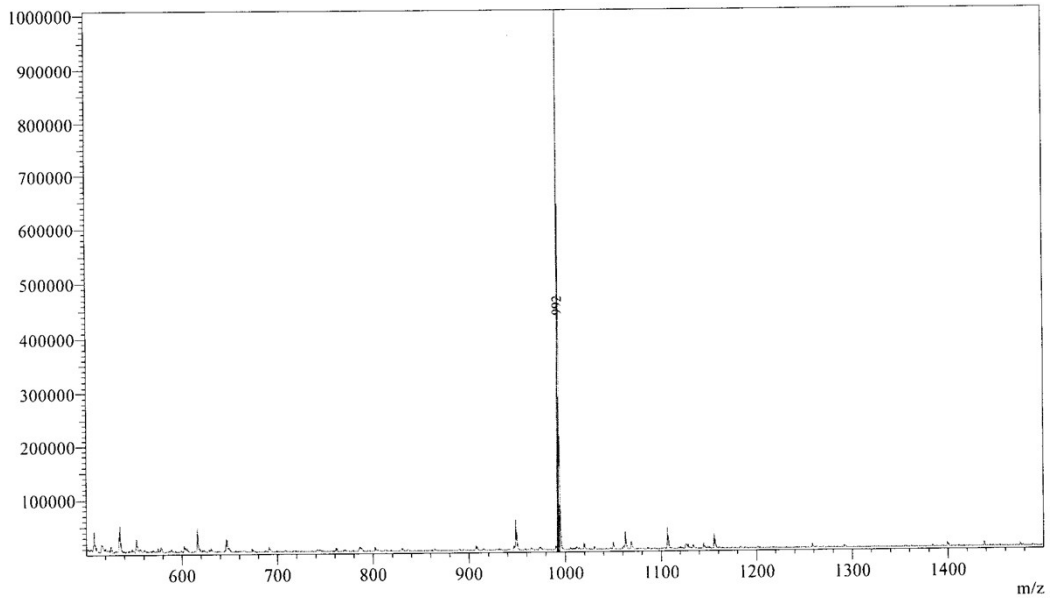

Fig. S3 Mass spectrum of the PCe4. The molar mass of PCe4 was 992 g/mol (the theoretical molar mass of PCe4 is 992.22 g/mol).

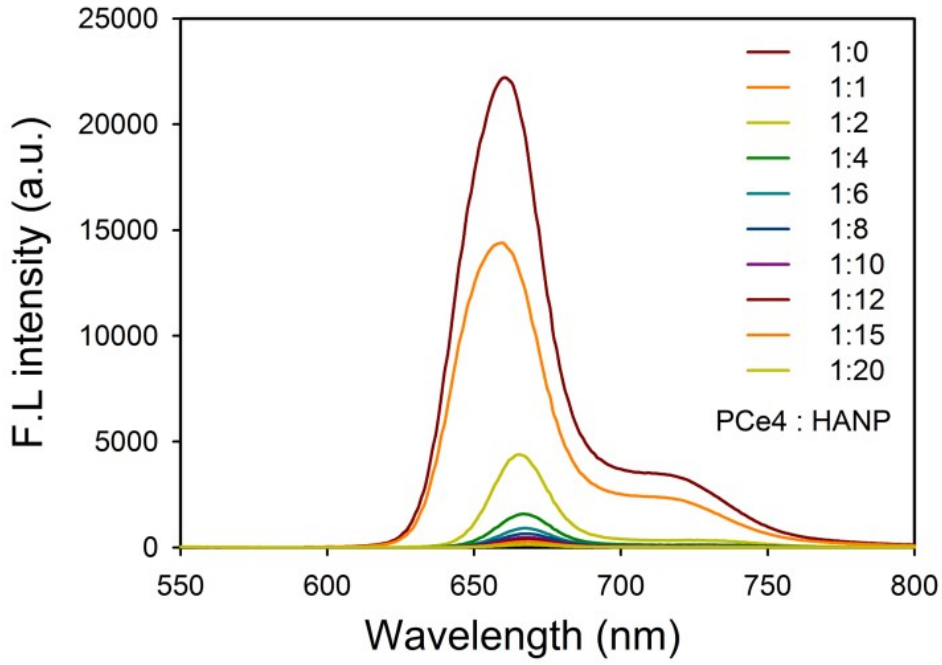

Fig. S4 Fluorescence quenching of PCe4 with various concentrations of HANPs. PCe4 solutions (10 μg in 100 μL) were mixed with 200 μL of HANPs (0, 10, 20, 40, 60, 80, 100, 120, 150, 200 μg) and incubated for 1 h. Next, the fluorescence spectra (λ_{ex} = 405 nm) of the solutions were obtained.

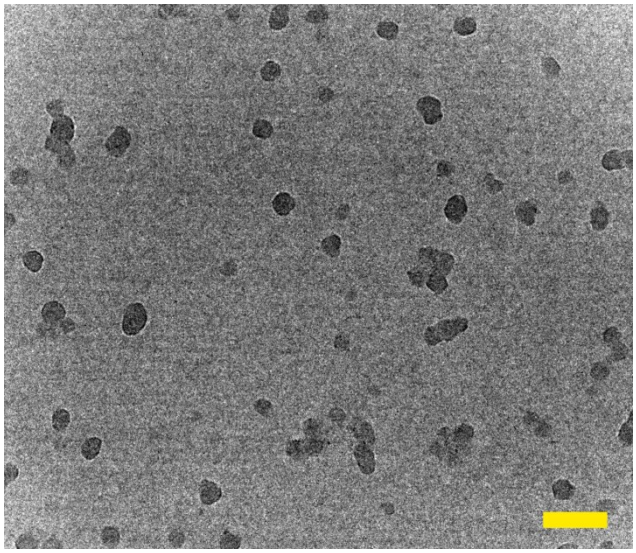

Fig. S5 TEM image of PCe4@HANPs. Scale bar (yellow) represent 100 nm.

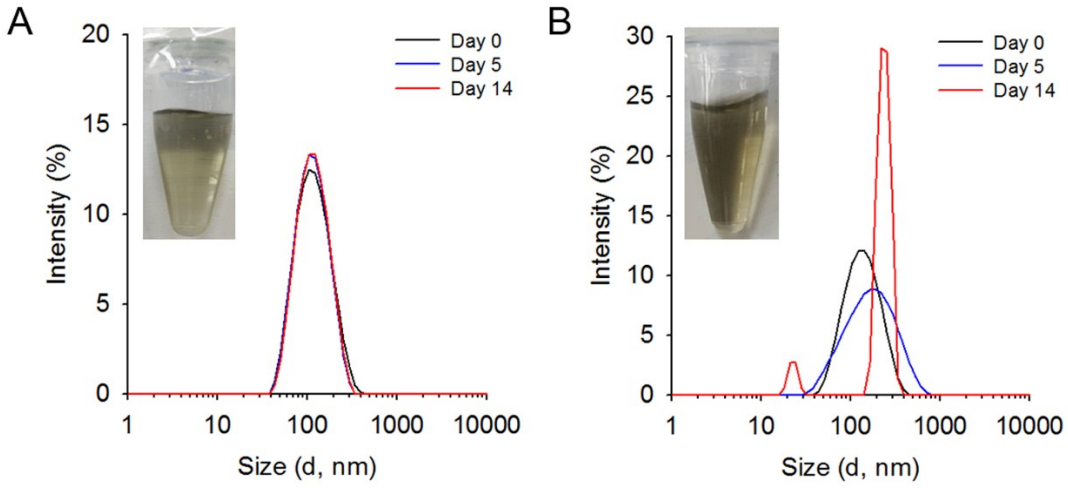

Fig. S6 Colloidal stability of PCe4@HANPs in different solutions. PCe4@HANPs were dispersed in (A) PBS solution (pH 7.4) and (B) DMEM culture medium (without phenol red) with 10% FBS. Hydrodynamic sizes of PCe4@HANPs were measured periodically for 14 days. Inset images: Photographs of sample solutions acquired at 14 days of incubation. As a result, no precipitates were observed in both sample solutions at 14 days of incubation, indicating good dispersion stability of the nanoparticles. Hydrodynamic size of PCe4@HANPs in PBS solution was uniform (i.e., 100 nm) for 14 days. However, hydrodynamic size of PCe4@HANPs increased from 116 nm at day 0 to 238 nm at day 14 in DMEM/FBS solution.

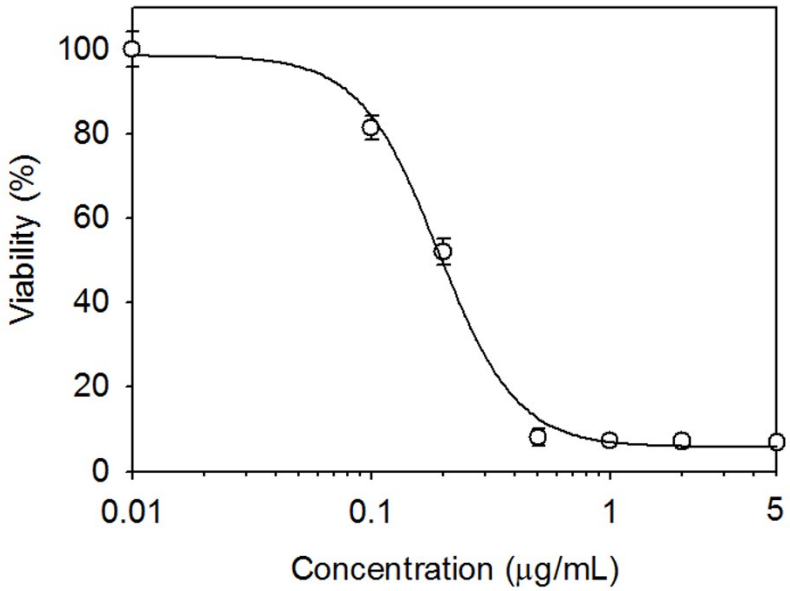

Fig. S7 *In vitro* phototoxicity test of PCe4@HANP. SCC7 cells were treated with PCe4@HANP for 16 h at various concentrations, washed, and irradiated with a 670 nm CW laser (100 mW/cm^2, 300 s). IC_{50} was 0.202 μg PCe4 equiv./mL.

References

1. D. Park, K.-O. Ahn, K.-C. Jeong and Y. Choi, *Nanotechnology*, 2016, **27**, 185102.