Supporting Information

Bioactivatable self-quenched nanogel for targeted photodynamic therapy

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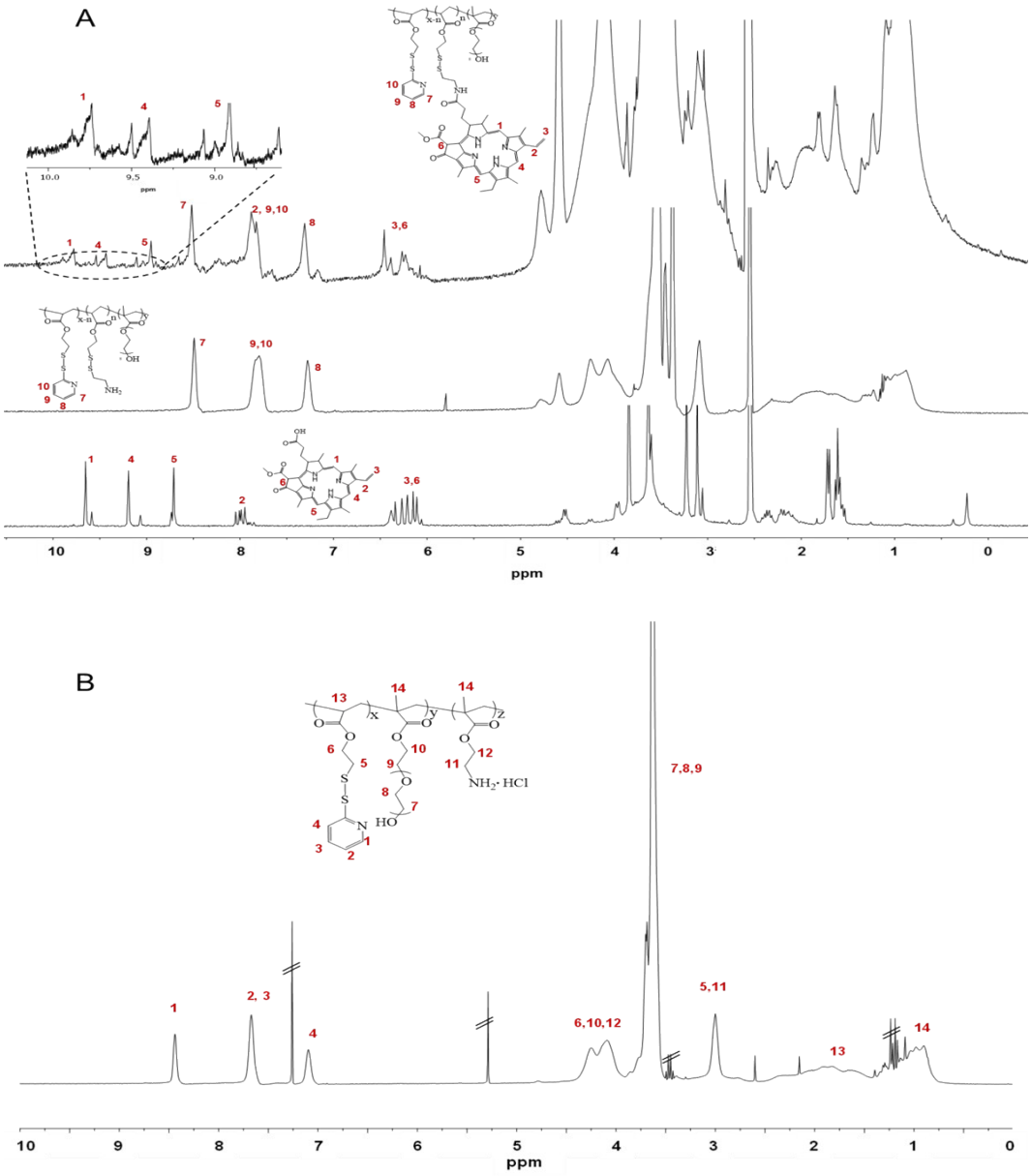

Figure S1. ^{1}H-NMR spectra of (A) PhA, PDA-PEG and PDA-PEG-PhA (from bottom to top) recorded in DMSO-d_6; and (B) PDA-PEG-AEME recorded in $CDCl_3$.

Table S1. Summary of size, PDI and zeta potential of PhA nanogels.

Nanogels	Size (nm)	PDI	Zeta potential (mV)
PhA-NG	129.3±1.1	0.28	0.22 ± 0.83
PhA-ENG	178.8 ± 4.8	0.22	-7.95 ± 4.04

2. Experimental Section

2.1. Chemicals

Aldrithiol-2 and Silica gel (Spherical, 100 mm) were purchased from Tokyo Chemical Industry Co., LTD (Harborgate Street, Portland, OR). 2-Mercaptoethenol, DL-dithiothreitol (DTT), 1-Ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC), N-hydroxysuccinimide (NHS), tris(2-carboxyethyl)phosphine (TCEP), 2, 2-Azobisisobutyronitrile (AIBN), Poly(ethylene glycol)methacrylate (Mn=360 Da, PEG360), 9,10-dimethylanthracene (DMA) and Thiazolyl Blue Tetrazolium Bromide (MTT) were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO). Pheophorbide A (PhA) was purchased from Frontier Scientific, Inc (Logan, UT). Penicillin (10,000 U/mL), streptomycin (10,000 mg/mL), 0.25% trypsin-EDTA, Dulbecco's Modified Eagle Medium (with L-glutamine) and fetal bovine serum (FBS) were obtained from American Type Culture Collection (ATCC, Manassas, VA). Anti-EGFR Affibody® Molecule was purchased from Abcam plc. (Cambridge, MA). All the other solvents used in this research were purchased from Sigma Aldrich Chemical Co. (St. Louis, MO) and used without further purification unless otherwise noted.

2.2. polymer synthesis and nanogel fabrication

PDA-PEG-PhA polymer synthesis

To provent the premature-release of PhA, Pha was covalently conjugated to PDA-PEG polymer. Briefly, PDA-PEG (20 mg in 250 μL DMSO) was first reacted with cysteamine hydrochloride (1.857 mg in 250 μL DMSO, 60% PDA function group) over night at room temperature. TEA (20 μL) and PhA (16.13 mg in 200 μL DMSO) activated by EDC (10.44 mg in 200 μL DMSO) and NHS (6.27 mg in 200 μL DMSO) were then added. The reaction

mixture was stirred over night at room temperature, followed by a thorough dialysis in DMSO (MW CO=6,000 Da) to remove unconjugated PhA. The resulted PDA-PEG-PhA DMSO solution was directly used for nanogel fabrication. The structure of PDA-PEG was confirmed by ^{1}H-NMR, and its molecular weight and polydispersity were evaluated by gel permeation chromatography (GPC).

PDA-PEG-AEME polymer synthesis

The synthesis of PDA-PEG-AEME polymer was similar to the synthesis of PDA-PEG polymer. Briefly, PDA (125 mg, 0.52 mmol) and PEG360 (187.5 mg, 0.52 mmol) were first dissolved in 5 mL degassed anisole, following the addition of 2-aminoethyl methacrylate hydrochloride (AEME, 8.28 mg, 0.05 mmol) in 1 mL CH_3OH. AIBN (7 mg, 0.0425 mmol) in 1 mL degassed anisole was then added, and the reaction mixture was stirred for 24 hours at 65 ^{o}C. The final product was precipitated (3×) in ice cold ether and dried for 48 h in vacuum.

PDA quantification in polymer: For the quantification of PDA in the polymer, PDA-PEG, PDA-PEG-PhA or PDA-PEG-AEME (50 μg/mL in DMSO) was incubated with tris(2-carboxyethyl)phosphine (TCEP, 10 mM and 20 mM) for 1 hour at room temperature, and then the amount of 2-pyridinethione released was quantified through UV-Vis spectrophotometer (DU® 650 Spectrophotometer, Beckman Coulter, Inc.) at λ = 375 nm (ε, molar absorption coefficient = 8080 $M^{-1}cm^{-1}$)

PDA-PEG-PhA nanogel fabrication (PhA-NG)

PDA-PEG-PhA (2.0 mg in 500 μL DMSO) and PDA-PEG-AEME (1 mg in 50 μL DMSO) was mixed well. TCEP (110.4 μg in 20 μL DMSO) was then added and the final mixture solution was immediately added dropwise into 5 mL ddH2O and stirred overnight in a glass vial with opened cap to form the nanogel. The nanogel was further purified by dialysis

in DMSO to remove free PhA and then PBS (pH 7.4, 10 mM) to change the solvent. The nanoparticle size, zeta potential, and morphology were measured by DLS (Zetasizer Nano ZS, Malvern Instruments Ltd) and TEM (Hitachi H8000, Hitachi High Technologies America, Inc.), respectively.

EGFR conjugation to the nanogel (PhA-ENG)

Mal-PEG-COOH (Mw=3,500 Da, 500 μg in 100 μL DMSO) was activated by EDC (100 μg in 100 μL DMSO) and NHS (60 μg in 100 μL DMSO) and added into the PhA-NG (0.6 mg polymer in 2 mL PBS). The mixture was stirred at 4 ^{o}C overnight and then dialyzed in PBS to remove unreacted Mal-PEG3500-COOH (MW CO=6,000 Da). The conjugation of anti-EFGR affibody to the nanogel was realized by the reaction between maleimide and thiol group. Briefly, Anti-EGFR Affibody® Molecule (20, 100 and 500 μg, respectively) was pretreated with 20 mM DTT to expose the reactive cysteine residue and then added to the nanogel (~0.3 mg in 1 mL PBS) and reacted overnight at 4 ^{o}C to obtain the PhA-ENG. The nanogel was purified by passing through Sephadex® G-25 column (Sigma-Aldrich Co. LLC) to remove unconjugated affibody and filtrated through 0.22 μm filter.

2.3. Nanogel characterization

UV-Vis spectra investigation

PhA and PhA-NG were dissolved in DMSO and TCEP was added to get a final concentration of 10 mM. UV-Vis spectra of PhA and PhA-NG were recorded by UV-Vis spectroscopy (DU® 650 Spectrophotometer, Beckman Coulter, Inc).

Serum Stability

The stability of the nanogel was evaluated in 10% serum condition. Briefly, PhA-NG was diluted in PBS supplemented with 10% FBS and 0.05% sodium azide. The final

concentration of PhA was 800 nM. The nanogel was incubated at 37 ^{o}C for up to 7 days. The size of the nanogel was monitored by DLS every day.

PhA quantification in the nanogel

PhA concentration in the nanogel was quantified by UV-Vis spectrophotometer. In brief, the nanogel was diluted in DMSO with 50 mM and 100 mM TCEP for 2 h to release PhA. The absorbance at 670 nm was then recorded and PhA concentration was calculated according to a pre-generated calibration curve.

PhA nanogel fluorescence kinetic profile study

The kinetic profile of the nanogel was investigated by measuring the PhA fluorescence at different GSH concentration. Briefly, PhA-NG was diluted in PBS supplemented with 1% Tween 80 and 10 mM EDTA to yield a final PhA concentration of 800 nM. To mimic the tumor and normal tissue reducing conditions, GSH (100 μM and 10 mM) was added into the nanogel and incubated at 37 ℃. At pre-determined time points, 1 mL samples were retrieved and frozen in liquid nitrogen immediately. At the end of the experiment, all samples were quickly thawed at 37 ℃ and their fluorescence were measured by a plate reader (Ex=405 nm, Em=680 nm, Molecular Devices SpectraMax M2, Molecular Devices, LLC).

Determination of singlet oxygen generation

Singlet oxygen generation was determined by monitoring the decrease of fluorescence intensity of 9,10-dimethylanthracene (DMA) as a singlet oxygen sensor. Briefly, PhA, PhA-NG, PhA-NG supplemented with 10 mM GSH were diluted in PBS with 1% Tween 80 and 10 mM EDTA to get a final PhA concentration of 800 nM. All samples were incubated at 37 ^{o}C for 1 h, 3 h and 5 h, respectively. DMA was then added (final concentration was 25 μM), following the irradiation under a 670 nm laser (15 mW/cm^2, Intense HPD 7404 diode laser,

Intense Ltd.). At every minute interval, 200 μL samples were retrieved and the fluorescence intensity of DMA was measured by a microplate reader (Ex=370 nm, Em=535 nm, Beckman Coulter DTX 880 Multimode Detector, Beckman Coulter, Inc). The singlet oxygen quantum (SOQ) yield was calculated as follows:

$$\emptyset_{\Delta} = \frac{K_S}{K_R} \times \frac{A_R}{A_S} \times ref.\emptyset_{\Delta} \quad (2\text{-}1)$$

where A is absorbance at 670 nm and K is the slope of the absorbance curve; S and R represent the sample and the reference, respectively. The SOQ ($\emptyset_{\Delta}$) of each sample was calculated from the reference $\emptyset_{\Delta}$value (PhA 0.52: $ref.\emptyset_{\Delta}$).

GSH-mediated photoactivity of PhA-NG

The fluorescence emission spectra of the PhA-NG was studied by fluorescence spectroscopy. In brief, PhA-NG equivalent to 800 nM PhA was suspended in PBS or PBS supplemented with 1% Tween 80 and were added with GSH at concentration of 10 μM, 100 μM, 1 mM, 10 mM, 25 mM and 50 mM. Then nanogel solution was incubated at 37 ^{o}C for 1 h and its fluorescence emission spectra was recorded by Varian Cary Eclipse Fluorescence Spectrophotometer (Agilent technologies, Inc.) with an excitation wavelength at 405 nm. The activatable photoactivity of PhA-NG depending on the gradient of GSH concentrations were investigated. In brief, PhA-NG with the equivalent PhA concentration to 800 nM in PBS supplemented with 1% Tween 80 was exposed to different concentrations of GSH (0.01-50 mM) and incubated at 37 ^{o}C for 1 h, 2 h, and 4 h. The nanogel was then loaded into a black 96-well plate and the fluorescence imaging was taken by IVIS spectrum fluorescence and bioluminescence imaging system (Ex=430 nm, Em=680 nm, PerkinElmer Inc.).

2.4. *In vitro* assay

Confocal microscopy

UMSCC 22A (200,000 cells/dish) were seeded on 35mm^2 Petri dishes (Mat Tek, MA, USA) for overnight. PhA, PhA-NG and PhA-ENG were then diluted in culture medium and added into the dishes with the final equivalent PhA concentration at 800 nM in each dish. After 3 h of incubation under a humidified atmosphere of 95/5% air/CO_2, cells were washed by PBS (3×), fixed with formaldehyde (4.5 % in PBS), and then cells were analyzed under a confocal microscope (LSM 700, Carl-Zeiss Inc.).

Flow cytometry

UMSCC 22A cells (300,000 cells/well) were seeded on a 6-well plate for overnight. PhA, PhA-NG and PhA-ENG were then diluted in culture medium and added into the plate with the final PhA concentration of 800 nM in each well. After 3 h incubation under a humidified atmosphere of 95/5% air/CO_2, cells were washed by PBS, trypsinized and resuspened in PBS. PhA positive cell population was quantified at λ_{ex}488 and λ_{em}670 nm using flow cytometry (BD Accuri C6, BD Biosciences).

Sub-cellular co-localization

UMSCC 22A cells (150,000 cells/dish) were seeded on 35mm^2 Petri dishes (Mat Tek, MA, USA) for overnight and incubated with PhA-ENG at indicated times. Before imaging, fresh FBS-free medium supplemented with Insulin-Transferrin-Selenium-X (ITX) reagent was added. Cells were loaded with MitoTracker Green (MTG, 500 nM) and LysoTracker Green (LTG, 500 nM) respectively to assess the co-localization of PhA-ENG with mitochondria and lysosomes. Cells were placed in an environmental chamber at 37 °C and

analyzed by laser scanning confocal microscope (LSM 510, Carl-Zeiss Inc). A 63× N.A. 1.4 oil immersion planapochromat objective was used for all experiments. For the mitochondria and NP colocalization experiment, NPs and MTG fluorescence was imaged using 2-photon 720 nm excitation/ 650-710 nm emission and 488 nm excitation/ 500-550 nm emission, respectively. For the lysosome and NP colocalization experiment, LTG and PhA fluorescence was imaged using 488 nm excitation/500–530-nm emission and 580 nm excitation/650-710 nm emission, respectively. All images were processed by ImageJ software to calculate the co-localization coefficients.

***In vitro* phototoxicity assay**

In vitro phototoxicity of PhA nanogels were tested in UMSCC 22A cell line. Cells were seeded on two 96-well plates (20,000 cells/well) for 24 h prior to the study. Subsequently, the culture medium was removed and replaced with fresh medium, including a serial of concentrations of PhA, PhA-NG and PhA-ENG. After 24 h incubation, cells in one plate were exposed to 670 nm laser for irradiation (800 mJ/cm^2), and followed by another 24 h of incubation in 95/5% air/CO_2 at 37 ^{o}C. For another plate, cells were kept incubating in the dark for a total 48 h. Finally, for both plates, MTT reagent (100 μL, 10%(w/w) in medium) was added and incubated for 4h, followed by the addition of MTT stop solution and the measurement of the optical density of the medium using a microplate reader (ELX808, Bio-Tech Instrument, Inc) at λ = 595 nm.

2.5. *In vivo* assay

Tumor model

All animal procedures were conducted in accordance with NIH regulations and approved by the Institutional Animal Care and Use Committee of the University of South

Carolina. UMSCC 22A cells suspended in DMEM medium at the density of 1.5×10^6/100 μL were inoculated subcutaneously in flanks of female Balb/c nude mice (8-10 week old, ~20 g, The Jackson Laboratory). The tumor volume was measured by a digital caliper and calculated according to the following formula: Tumor volume = (tumor length) × (tumor width)^2/2.

Biodistribution observation

When the tumor reached 50~150 mm^3, PhA, PhA-NG, and PhA-ENG were administered by retro-orbital injection (100 μL, PhA concentration equal to 1 mg/kg). For the control group, the same volume of PBS was injected. After injection, mice were imaged by IVIS imaging system (IVIS Lumina III In Vivo Imaging System, Perkin, Ex=620 nm, Em=670 nm) at predetermined time points. All the image analyses were performed using a Living Image Software (IVIS Imaging Systems).

In vivo antitumor efficacy evaluation

When the tumor reached to 50~150 mm^3, PhA, PhA-NG, and PhA-ENG were administered by retro-orbital injection (100 μL, PhA concentration equal to 1 mg/kg). At 24 h post-injection, tumors were irradiated with a 670 nm laser at a light density of 38.0 mW/cm^2 for 5 min. Then the tumor volume (V) and body weight (m) of the mice were measured every three days up to 21 days. The relative tumor volume expressed as V/V_0 (V_0 was the tumor volume when the treatment was initiated) was used to represent the tumor size change during the whole treatment process. After 21 days, the mice were sacrificed, and the blood, tumor, liver, heart, lung, kidneys, and spleen were collected for further analysis. All organs were fixed in 10% neutral buffered formalin for 48 h and then washed with PBS and finally kept in 70% ethanol at 4 ^{o}C.

Histological Examination

The formalin fixed organ tissues were embedded in OCT gel, sectioned into ~5 μm, stained with hematoxylin and eosin (H&E) and analyzed under light microscopy (Leica DM1000 LED, Leica Microsystems Inc.). The histology was performed in a blinded fashion by professional personnel at the University of South Carolina.

2.6. Statistical Analysis

The results were reported as the mean ± standard deviation of three different studies. Student's two-tailed t-test assuming equal variance was used to determine statistical significance ($p < 0.05$) of the experimental data.