Supplementary Information

In vivo tumor diagnosis and photodynamic therapy *via* tumoral pH-responsive polymeric micelles

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Experimental section

Materials. Analytical grade methyl ether poly(ethylene glycol) (MPEG, M_n=4,850, determined by GPC), acryloyl chloride, hexane- 1,6-diol diacrylate (HDD), 4,4′-trimethylene dipiperidine (TDP), triethylamine, anhydrous chloroform, anhydrous dichloromethane (DCM), and protophorphyrin IX (PpIX) were purchased from Sigma Chemical Co. (St. Louis, MO) and used without further purification.

Synthesis of MPEG-poly (β-amino ester) block copolymers. MPEG-poly (β-amino ester) block copolymer was synthesized as described previously.[1] Briefly, MPEG (1 equiv.), dissolved in anhydrous DCM and protected from moisture with $CaCl_2$, was mixed with triethylamine (2 equiv.) and cooled to 0 °C. Following the dropwise addition of acryloyl chloride (1.5 equiv.), the mixture was stirred at 0 °C for 2 h, allowed to warm to room

temperature, and stirred for 1 day. The product was extracted with dilute HCl and precipitated with hexane to obtain poly (ethylene glycol) methyl ether acrylate (monoacrylated MPEG). MPEG-poly (β-amino ester) block copolymer was synthesized via a Michael-type step polymerization using monoacrylated MPEG as the monocrylate, HDD as the diacrylate ester and TDP as the diamine. HDD (1 equiv.) and TDP (1.1 equiv.), each dissolved in chloroform, were mixed, and the resulting solution was added to monoacrylated MPEG (0.1 equiv.) and allowed to react for 2 days at 50 °C. The final product, designated MPEG-poly (β-amino ester) block copolymer, was precipitated in diethylether and vacuum dried.

The average molecular weight of the synthesized polymer was measured by gel permeation chromatography (GPC), using KF-803L and KF-802.5 (Shodex) columns in series, with tetrahydrofuran (THF) as the eluent at a flow rate of 1 ml/min. The chemical structure of MPEG-poly (β- amino ester) block copolymer was further characterized by ^{1}H NMR spectroscopy. The ^{1}H NMR spectra were recorded using a 500 MHz FT-NMR (Utility Inova 500 MB, Varian) spectrometer with $CDCl_3$ as the solvent. M_ns of of MPEG and poly (β-amino ester) blocks were determined to 4850 Da and 12,580 Da, respectively. MPEG-PLLA polymer (pH-independent control) was synthesized by traditional ring opening polymerization.[2]

Preparation of PpIX-loaded polymeric micelles. PpIX-loaded pH-responsive micelles(PpIX-pH-PMs) of MPEG-poly (β-amino ester) block copolymer were prepared by a solvent evaperation method.[3] Briefly, PpIX (10 mg) was dissolved in 1 ml of chloroform/methanol (1:1, v/v), and the solution was added dropwise to the stirred solution containing 100 mg of MPEG-poly (β-amino ester) block copolymer in chloroform/methanol (1:1, v/v). After evaporating the co-solvent using a rotary evaporator, the polymer/drug thin film was dispersed by adding 10 ml of PBS (pH 7.4) under gentle shaking for 10min,

followed by sonication using a probe-type sonifier (Sigma Ultrasonic Processor, GEX-600) at 90 W for 3 min, with the pulse turned off for 1 s after 5 s sonication to prevent heat build-up. The dispersed PpIX-loaded polymeric micelles were passed through syringe filters (0.8μm, Millipore) and free PpIX and by products were further removed by dialysis (molecular weight cutoff=8000, Spectrum®), and the PpIX loaded micelles were lyophilized to give a purple powder. The drug loading efficiency of PpIX-loaded polymeric micelles dissolved in DMSO was quantified by absorbance using UV–vis spectroscopy (Lambda Vis 7 spectrophotometer, Perkin-Elmer, CT) at 405nm.

The particle sizes of PpIX unloaded and loaded polymeric micelles were measured by dynamic light scattering (DLS) (127-35 laser, Spectra Physics, Mountain View, CA). PpIX unloaded and loaded polymeric micelles (1mg/ml in PBS) were measured by using a BI-9000AT digital autocorrelator (Brookhaven, NY, USA), which is operated at 633nm and 25°C. Also, the morphological shapes of PpIX-loaded and unloaded polymeric micelles were confirmed using transmission electron microscopy (TEM) (CM30 electron microscope, Philips, CA), operated at an acceleration voltage of 80kV. Each sample (1mg/ml in distilled water) was placed on a 300-mesh copper grid coated with carbon. After drying the sample, negative staining was performed using a droplet of 2wt% uranyl acetate.

Table. SI1 Characterization of pH-responsive polymeric micelles.

sample	feed amount (wt%)	loading efficiency (%)	size (nm) [a]	μ^2/I^2 [b]
pH-PM	-	-	42	0.013
PpIX-pH-PM	10	75.3 ± 3.7	122	0.023
MPEG-PLLA	-	-	47	0.026
PpIX-MPEG-PLLA	10	80.7 ± 5.1	257	0.047

[a]The average size was measured with dynamic light scattering.
[b]Polydispersity factor for polymeric micelles.

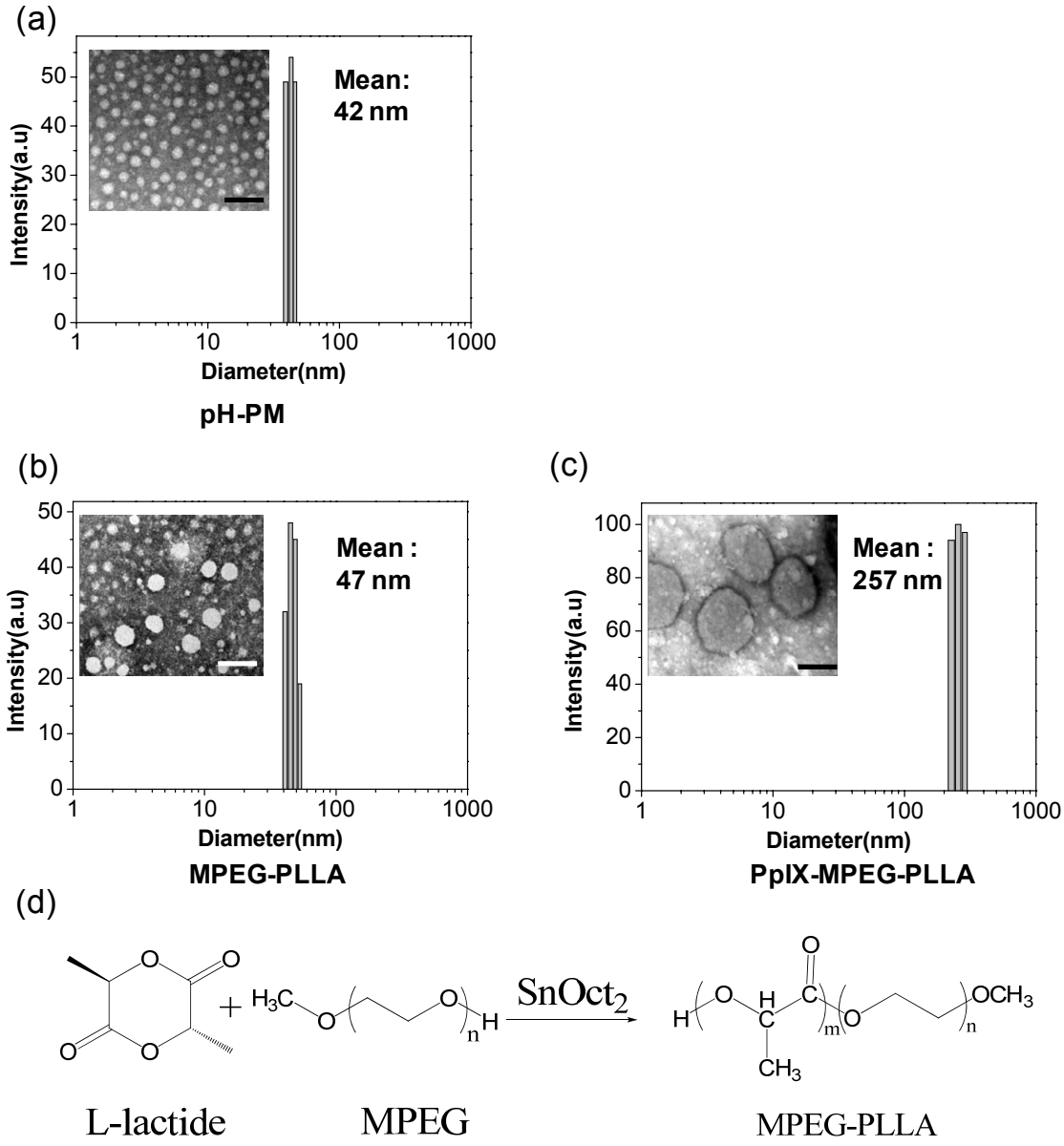

Fig.SI 1 Characteristics of polymeric micelles. The size distribution by dynamic light scattering (DLS) and transmission electron microscopy (TEM) images of (a) pH-PM (MPEG-poly (β-amino ester)) (b) MPEG-PLLA (c) PpIX-MPEG-PLLA. (d) Synthetic scheme of MPEG-PLLA. Scale bar is 200 nm (a, c) and 100 nm (b).

***In vitro* PpIX release and singlet oxygen generation profiles of PpIX-loaded polymeric micelles.** To determine the release profile of PpIX from micelles, lyophilized PpIX-loaded polymeric micelles (1 mg) were dispersed in 1 ml of PBS (pH 7.4) and the solution was sonicated at 90W for 3 min, with the pulse turned off for 1 s after 5 s sonication to prevent heat build-up. The dispersed PpIX-loaded polymeric micelles (1 mg/ml) was added to cellulose ester membrane tubes (molecular weight cutoff=8000, Spectrum®), and the dialysis tubes were incubated in 30 ml phosphate buffer saline (PBS, pH 6.4 or 7.4) at 37 °C in a water bath at 80 rpm. At given times, the solution was removed and the pH-dependant PpIX release profiles were determined by measuring the UV–vis absorbance.

The generation of singlet oxygen was observed chemically, using the disodium salt of p-nitroso-N,N′-dimethylaniline (RNO) as a singlet oxygen sensor. This sensor is bleached by singlet oxygen to the corresponding endoperoxide. The singlet oxygen generations of PpIX loaded and unloaded polymeric micelles were measured by the established method in aqueous solutions.[4] Briefly, RNO and histidine (250μM and 0.03M) was dissolved in distilled water. Free PpIX and PpIX loaded polymeric micelles were dissolved in pH 7.4 or pH 6.4 sodium phosphate buffer (20mM, 1 ml) containing 0.5 % of Tween 80. To measure the singlet oxygen generation, 1 ml of each solution was added into the RNO solution and it was irradiated with a 633 (He–Ne) laser. The optical density at 440 nm (λ_{max} of RNO) was monitored every 2 min in a spectrophotometer (ISS, Champaign, IL).

Cellular uptake and phototoxicity of PpIX from pH-responsive polymeric Micelles. Squamous cell carcinoma (SCC7) cells were originally obtained from the American Type Culture Collection (Rockville, MD) and cultured in RPMI 1640 (Gibco, Grand Island, NY) containing 10 % FBS (Gibco, Grand Island, NY) and 1% penicillin–streptomycin (Gibco) at

37 °C in a humidified 5 % CO_2–95 % air atmosphere, and 5×10^5 cells were grown on 60φ culture dishes. After 1 day, the medium was replaced by 1 ml of fresh medium, and free PpIX or PpIX-pH-PMs (5 μg/ml PpIX equiv.) incubated for 1hr at pH 6.4 or 7.4 were added. After 20 min, the culture dishes were washed with PBS (pH 7.4) 3 times, fixed with 4% (wt./vol.) formaldehyde in PBS (pH 7.4) for 30 min at 4 °C, and cellular uptake of free PpIX, and PpIX-pH-PMs at pH 6.4 or 7.4 was visualized with an Axioskope-2 imaging microscope (Carl Zeiss, Oberkochem, Germany) equipped with a fluorescence filter set for TRITC (Omega Optical) and an Axiocam black and white CCD camera (Carl Zeiss Vision, Hallbergmoos, Germany). Image acquisition and analysis were driven by KS400 software (Carl Zeiss Vision).

To determine the phototoxicity of PpIX-pH-PMs, SCC7 cells ($5x10^5$ cells) were seeded onto 60φ culture dishes and allowed to attach for 1 day. After cell attachment, the medium was replaced with 2 ml of serum-free culture medium containing free PpIX (5 μg/ml) or PpIX-pH-PMs (5 μg/ml of free PpIX) and then incubated for 20 min. The cells were then washed twice with PBS and then fresh culture medium was added. Free PpIX or PpIX-pH-PMs treated cells were illuminated with a He–Ne laser (633 nm, 3 mW/cm^2) for 12 min. Then, irradiated cells were incubated at 37 °C for 12 h. Apoptotic cells were identified by Annexin V-FITC fluorescence microscopy kit (BD pharmingen™).[5] We washed cells twice with 2 ml of 1 X PBS, and once with 2ml of Binding Buffer. Then, we stained cells with 1 ml of Annexin V-FITC diluted (1:10 w/v %) in Binding Buffer for 15 min at room temperature. After staining, we washed cells once with Binding Buffer, and added additional Binding Buffer and observed under Axioskope-2 imaging microscope (Carl Zeiss, Oberkochem, Germany) equipped with a fluorescence filter set for FITC (Omega Optical) and an Axiocam black and white CCD camera (Carl Zeiss Vision, Hallbergmoos, Germany). Image

acquisition and analysis were driven by KS400 software (Carl Zeiss Vision).

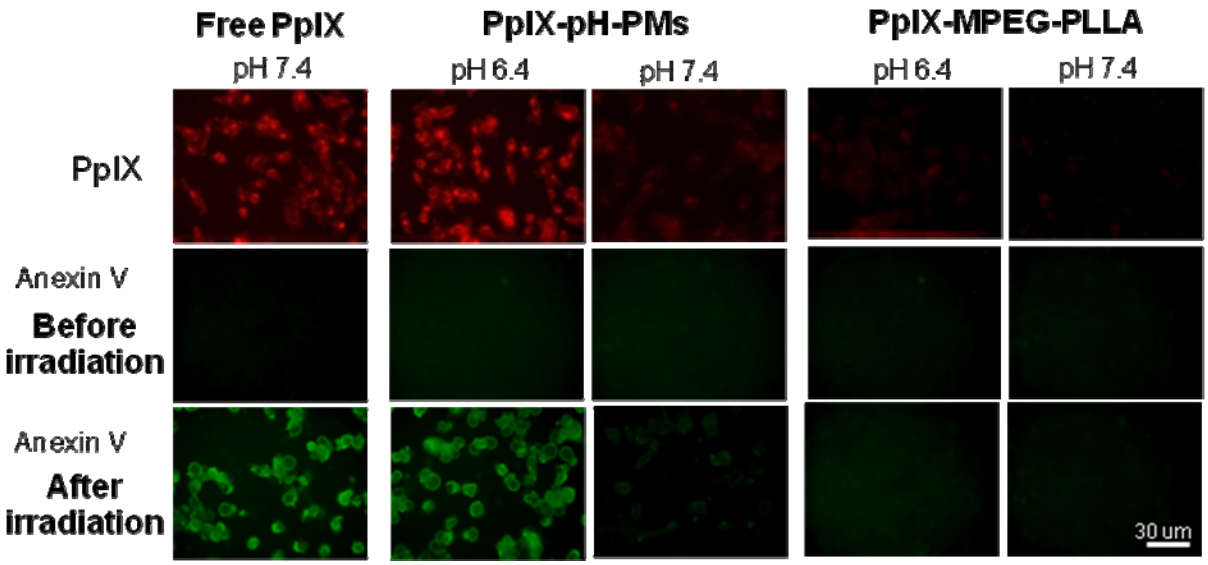

Fig. SI2 Cellular uptake and photocytotoxicity of free PpIX, PpIX- pH-PMs, PpIX- MPEG-PLLA micelles against SCC7 cells observed by confocal laser scanning microscope. Fluorescence images of PpIX were obtained with red color, Annexin V-FITC stain (green) depicts apoptotic cells, and differential interference contrast (DIC) depicts the cell morphology.

Tumor target specificity of PpIX-pH-PMs in SCC7 tumor-bearing mice. All experiments with live animals were performed in compliance with the relevant laws and institutional guidelines of Korea Institute of Science and Technology (KIST) and institutional committees have approved the experiments. Athymic nude mice (5-weeks old, Institute of Medical Science, Tokyo) were used for animal experiments. Subcutaneous tumors were established by inoculating SCC7 cells (1.0×10^6) into the backs of mice. When tumors grew to approximately 150–200 mm^3 in volume, PpIX-pH-PMs (5 mg/kg of free PpIX) in pH 7.4 PBS were intravenously injected via the tail vein into mice. Free PpIX (5 mg/kg) in DMSO was also intravenously injected into mice as control. After i.v. injection, the tumor specificities of free PpIX and PpIX-pH-PMs were imaged by positioning mice on an animal plate heated to

36.5 ℃ in the eXplore Optix System (Advanced Research Technologies Inc., Montreal, Canada).[6] During imaging, laser power and count time setting were optimized at 25 mW and 0.3 s per point. The excitation and emission spots were raster-scanned in 1 mm steps over the region of the interest to generate emission wavelength scans. A 670 nm pulsed laser diode was used to excite PpIX molecules. Long wave fluorescence emission (600–700 nm) was detected with a fast photomultiplier tube (Hamamatsu, Japan) and a time-correlated single photon counting system (Becker and Hickl GmbH, Berlin, Germany). The PpIX molecules in tumor tissues are quantified by measuring the region of interest (ROS) function of Analysis Workstation software (ART Advanced Research Technologies Inc., Canada). To compare tissue and tumor distributions of free PpIX and PpIX-pH-PMs, mice were sacrificed 2 days after i.v. injection. The excised tissues including livers, lungs, spleens, kidneys, and hearts, as well as the tumors, were determined using a 12-bit CCD camera (Image Station 4000 MM; Kodak, New Haven, CT) equipped with a special C-mount lens and a long wave emission filter (600–700 nm; Omega Optical). A quantification of in vivo tumor target specificity of free PpIX and PpIX-pH-PMs was recorded as total photons per centimeter squared per steradian (p/s/cm^2/sr) per each tumor.

Therapeutic efficacy of PpIX-pH-PMs in SCC7 tumor-bearing mice. To assay the therapeutic efficacy of PpIX-loaded polymeric micelles, a subcutaneous tumor was established by inoculating SCC-7 Squamous cell carcinoma (1.0×10^6 cells) into the back of athymic nude mice (5 weeks old). When approximate tumor volume became 150-200 mm^3, saline, free PpIX (5 mg/kg) and PpIX-loaded polymeric micelles (5 mg/kg of free PpIX) were filtered with syringe filter (0.45μm) and injected via a tail vein. Mice were divided into three groups treated with different injections, as follows; (1) saline (the control group, n=2) with irradiation, (2) PpIX with irradiation (n=2), (3) PpIX loaded polymeric micelles with

irradiation (n=2). After 1 day post-injection, solid tumors were irradiated with a 633 nm laser (He–Ne laser, 3mW/cm^2) twice for 30min. Then, we monitored the therapeutic efficacy of saline, free PpIX, and PpIX loaded polymeric micelles treated mice by monitoring the incidence of apoptosis and/or necrosis in tumor over time. Also, the tumor volumes were calculated as width x length x height x 1/2 with a caliper for 14 days.[7] Ten days after laser irradiation, tumor tissues were isolated from the mice, fixed with 4% paraformaldehyde, and embedded in paraffin. The sliced tumor tissues (6 μm) were stained with Hematoxylin and Eosin (H&E) to evaluate the induction of necrosis or apoptosis.

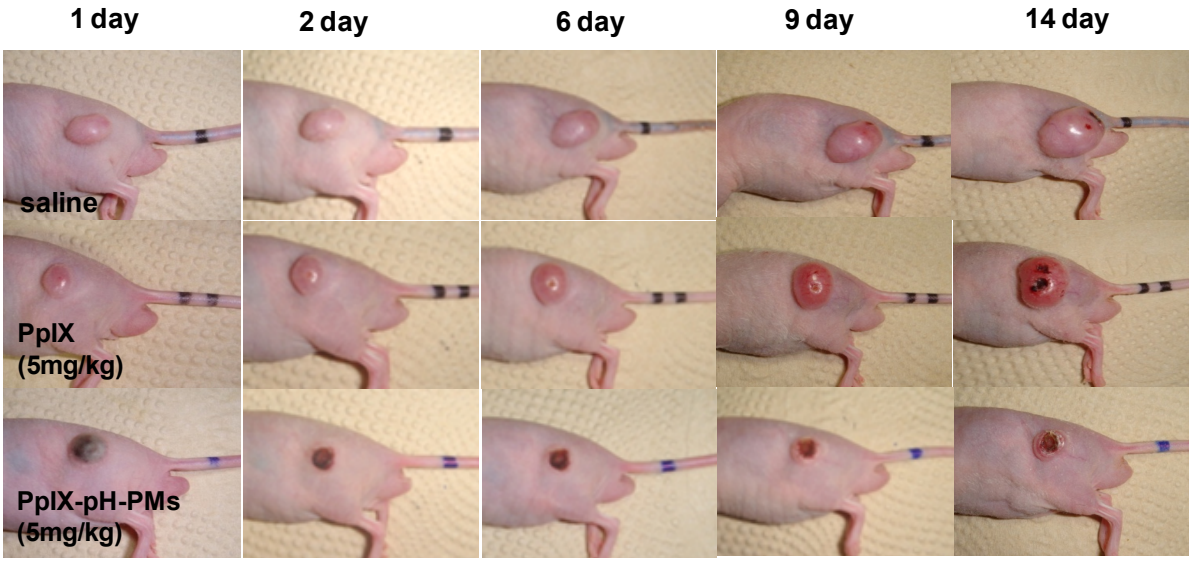

Fig. SI3 Photodynamic therapeutic efficacy of saline, free PpIX (5 mg/kg) and PpIX-pH-PMs (5 mg/kg of PpIX) in SCC7 tumor-bearing mice. One day after i.v. injection, we treated the animals with 633 nm light (3 mW/cm^2) twice for 30min. After the irradiation, therapeutic efficacy was monitored for 14 days.

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