Supporting Information

Enhanced Phototherapy by Nanoparticle-Enzyme *via* Generation and Photolysis of Hydrogen Peroxide

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EXPERIMENTAL SECTION

Materials. The conjugated polymers employed in this study are the polyfluorene derivative Poly[(9,9-dioctylfluorenyl-2,7-diyl)-alt-co-(1,4-benzo-{2,1′,3}-thiadiazole)] (PFBT, average MW 115,000, polydispersity 3.2), obtained from American Dye Source. The polymer poly (9,9-dioctylfluorene)-co-(4,7-di-2-thienyl-2,1,3-benzothiadiazole) (PF-5DTBT) was synthesized according to a previous report.[1] The functional copolymer poly(styrene-co-maleic anhydride) (PSMA, average MW ~1,700, styrene content 68%) and glucose oxidase (GOx, 160 kDa), Acridine orange (AO), Ethidium bromide (EB), Phosphate buffered saline (PBS) and Tetrahydrofuran (THF, anhydrous, 99.9%) were purchased from Sigma-Aldrich (Shanghai, China). 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC), Chlorin e6 (Ce6) and Rose Bengal sodium salt (RB) were purchased from J&K Scientific Ltd (Beijing, China). (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) (MTT) were purchased from BioSharp (Hefei, China). Terephthalic acid (TA) was purchased from Sinopharm Chemical Reagent Co., Ltd (Shanghai, China). Dimethyl sulfoxide (DMSO) and hydrogen peroxide (H_2O_2, 30%) were obtained from Beijing Chemical Works (Beijing, China). All chemicals were used as received without any further purification.

Preparation of Pdots. Pdots were prepared using a precipitation method described previously.[2] Briefly, PFBT, PF-5DTBT and PSMA (5:3:2 w/w) were dissolved in THF at a total polymer concentration of 50 μg/mL. The mixture solution of polymer was added quickly into water under sonication and the sonication last for 3 min. Then, the THF was removed by

nitrogen (N_2) stripping on a hot plate, followed by filtration through a 0.22 micrometer filter to remove larger aggregates, and concentrated by continuous heating.

Preparation of Pdot-GOx nanoparticles. We performed bionconjugation by utilizing the EDC catalyzed reaction between carboxyl groups on Pdots surface and amine groups on the GOx enzyme. In this bioconjugation reaction, 80 μL of concentrated PBS buffer (1 M, pH 7.4) were added to 4 mL of functionalized Pdots solution (50 μg/mL in MilliQ water), resulting in a Pdot solution in 20 mM PBS buffer with a pH of 7.4. Then, 125 μL of glucose oxidase (10 μM in 20 mM PBS) was added to the solution and mixed well on a vortex. 100 μL of freshly-prepared EDC solution (5 mg/mL in MilliQ water) was added to the solution, and the above mixture was left on a rotary shaker for 4 hours at room temperature. Finally, the resulting Pdot bioconjugates were separated from free biomolecules by gel filtration using sephacryl HR-300 gel media.

Characterizations. TEM characterizations of the samples were performed using a transmission electron microscope (TEM, Hitachi H-600). Dynamic light scattering (DLS) and surface Zeta potentials were recorded with Zeta-sizer NanoZS (Malvern, UK). Absorption spectra were recorded using an UV−Vis 2550 spectrophotometer with a 1.0 cm optical path length quartz cuvette. Emission spectra were measured using an F-4600 spectrophotometer.

Detection of hydroxyl radical (•OH) generated by Pdot-GOx nanoparticles. Terephthalic acid (TA, 6×10^{-2} M) was dissolved in NaOH (2×10^{-2} M) solution. In a typical process, Pdot-

GOx nanoparticles (0.6 mL, 100 μg/mL) was dispersed into a mixture solution (6 mL) containing glucose (5 mM) and TA (6 mM). A blue-LED array (460 nm with a bandwidth of 20 nm) with uniform illumination at a power density of 100 mW/cm^2 was used as the irradiation source. After the light irradiation for a designated time, the fluorescence spectra of TA were monitored for 60 minutes, and the emission intensity of TAOH at 426 nm was plotted as a function of the irradiation time. The oxidation of TA to TAOH by hydroxyl radical was confirmed by using dimethyl sulfoxide (DMSO) as a hydroxyl radical scavenger. With the addition of DMSO (0.6 M), the fluorescence spectra of TAOH induced by Pdot-GOx and glucose under 460 nm light irradiation were monitored for 60 minutes.

Detection of hydroxyl radical (•OH) generated by photolysis of H_2O_2. Terephthalic acid (TA, 6×10^{-2} M) was dissolved in NaOH (2×10^{-2} M) solution. In a typical process, H_2O_2 (0.4 mM) was mixed with a TA solution (6 mM, 6 mL). A blue-LED array (460 nm with a bandwidth of 20 nm) with a uniform illumination at a power density of 100 mW/cm^2 was used as the irradiation source. After the light irradiation for a designated time, the fluorescence spectra of TA were monitored at ten-minute intervals for 60 min, and the emission intensity of TAOH at 426 nm was plotted as a function of the irradiation time.

Cell culture and *in vitro* experiments. MCF-7 cancer cells were provided by American Type Culture Collection (ATCC) and cultured in Dulbecco modified eagle medium with 10% feat bovine serum FBS (Invitrogen) and 100 U mL-1 penicillin/streptomycin. The cell was

maintained at 37°C in humidified environment with 5% CO_2. For the cell culture, the medium was changed every 2 days.

The *in vitro* dark toxicity of Pdots, GOx and Pdot-GOx nanoparticles were assessed by a standard MTT assay. In a typical MTT assay, MCF-7 cells were seeded in a 96-well cell-culture plate at 1×10^4 $well^{-1}$ and cultured for 24 h. The cells were incubated with variable concentrations of Pdots, GOx and Pdot-GOx for 6 h, respectively. 20 μL of MTT (5 mg/mL) was introduced to the cell culture well and incubated for 4 h. Then, the culture medium was removed and 150 μL of DMSO was added to each plate well. After rocking for 10 min, the absorbance of each plate well was measured by a Bio-Tek Cytation 3 cell imaging multi-mode reader, and each data point represents a mean ± standard deviation (SD) from triplicate wells.

The enzyme-enhanced phototherapy (EEPT) effect was also evaluated using the MTT assay. MCF-7 cells were seeded in a 96-well plate and cultured in DMEM at 37°C for 24 h. Then, the Pdot-GOx nanoparticles at a series of concentrations (0, 0.0125, 0.025, 0.05, 0.1, 0.2, 0.4, 0.8 μg/mL) were added and incubated at 37°C for 6 h. Cell culture medium in each well was then replaced by 100 μL fresh medium. The cells were irradiated by a blue LED array (460 nm, 100 mW/cm^2) for 20 min, respectively. After 16 hours, MTT assay was performed according to the above-described procedure. The standard MTT assay was carried out to determine the cell viabilities relative to the control untreated cells. Meanwhile, the phototherapy effect of Pdots and GOx were measured by the standard MTT assay.

Intracellular H_2O_2 generation was detected by flow cytometry using a fluorescent probe 2,7-dichlorofluorescin diacetate (DCFH-DA). DCFH-DA was hydrolyzed enzymatically by intercellular esterases to non-fluorescence DCFH. DCFH could be oxidized by the intracellular

H_2O_2 to generate a fluorescent compound dichlorofluorescein (DCF). MCF-7 cells were incubated with different concentrations of Pdot-GOx, pure Pdots, and free GOx, respectively. The cells were then stained with DCFH-DA in DMEM for 30 min, and then washed three times, and finally analyzed using BD Accuri™ C6 Flow Cytometer.

Acridine orange/ethidium bromide (AO/EB) staining was used to detect apoptosis in MCF-7 cells. AO is a membrane-permeable dye that stains all the cells, whereas EB can only bind DNA in the dead or dying cells that have lost plasma membrane integrity. Green, yellow-orange and red fluorescence indicated live cells, early apoptotic cells and late apoptotic/necrotic cells respectively. MCF-7 cells were seeded in 35 mm plates at an initial cell density of $2x10^5$ cells per plate. After 24 h growth, the cells were incubated with Pdot-GOx nanoparticles (0.2 μg/mL) for 6 h. Then the samples were irradiated by the blue light (460 nm) at a power density of 100 mW/cm^2 for 20 min. Meanwhile, the cells without light irradiation were used as the control. Cells were stained with a mixture of AO/EB in PBS（100 μg/mL AO/100 μg/mL EB）for 10 min. Finally, the stained cells were washed with PBS buffer (pH = 7.4) for three times before imaging by confocal laser scanning microscope. The therapy effect of common photosensitizer such as Ce6 and RB were also evaluated by MTT and AO&EB cell staining.

Animal model. All animal experiments were conducted in agreement with all relevant guidelines and regulations set by Jilin University and with approved institutional protocols set by the China Association of Laboratory Animal Care. Male Balb/c mice were obtained from Beijing HFK Bioscience Co. Ltd. and used under protocols approved by Jilin University Laboratory Animal Center. For the MCF-7 xenograft model, MCF-7 cells (2×10^6) suspended in

PBS were subcutaneously injected into the right arm of each mouse. After one week, the tumors in the mice reached volumes of approximately 90-120 mm^3 for subsequent imaging and EMPT treatment.

***In vivo* imaging.** The tumor-bearing nude mice (~20 g) were intratumorally injected with Pdot-GOx nanoparticles (150 μL, 100 μg/mL). The mice without Pdot-GOx administration were used as controls. After 24 h, some mice were then anesthetized for whole-animal fluorescence imaging. Moreover, tumor masses and major organs (heart, liver, spleen, kidney, and lung) were collected for imaging and biodistribution analysis by a small animal imaging system. Meanwhile, after 14 and 28 days, the mice with Pdot-GOx injection were further imaged using a small animal imaging system to monitor the tumor retention effect of the Pdot-GOx nanoparticles in xenograft-bearing mice.

***In vivo* EEPT evaluation**. The tumor-bearing mice were randomly divided into six group (n=5, each group): i) Light, ii) Pdots + Light, iii) GOx, iv) GOx + Light, v) Pdot-GOx, vi) Pdot-GOx + Light. Each mouse was administered intratumorally with either Pdot-GOx (150 μL, 100 μg/mL) or PBS (150 μL). After the nanoparticle administration (24 h), the mice were anesthetized by intraperitoneal injection of 0.75 mg/kg chloral hydrate. For the experimental groups receiving light irradiation, the tumors were irradiated on the first and seventh day for 20 min at a power density of 100 mW/cm^2. Tumor volumes and body weight were measured weekly, and volume (mm^3) of tumors was calculated as (tumor length) × $(\text{tumor width}/2)^2$.

Relative tumor volume was calculated as V/V_0 (V_0 is the tumor volume when the treatment was initiated).

Tissue section staining. 30 days after intratumoral injection of Pdot-GOx nanoparticles, three mice from the treatment groups were sacrificed. Then tumor and major organs from these mice were collected, fixed in 10% neutral buffered formalin, embedded in paraffin, cut into 4 μm sections and stained using hematoxylin and eosin (H&E). Finally, the images of these histological tissue sections were obtained by optical microscope. Examined tissues included heart, liver, spleen, lung, kidney and tumor.

Statistical Analysis. Data were presented as mean ± standard deviation (SD). Statistical differences among experimental groups were analyzed using one-way analysis of variance (ANOVA) followed by two-tailed Student's t test. $P < 0.05$ was considered statistically significant.

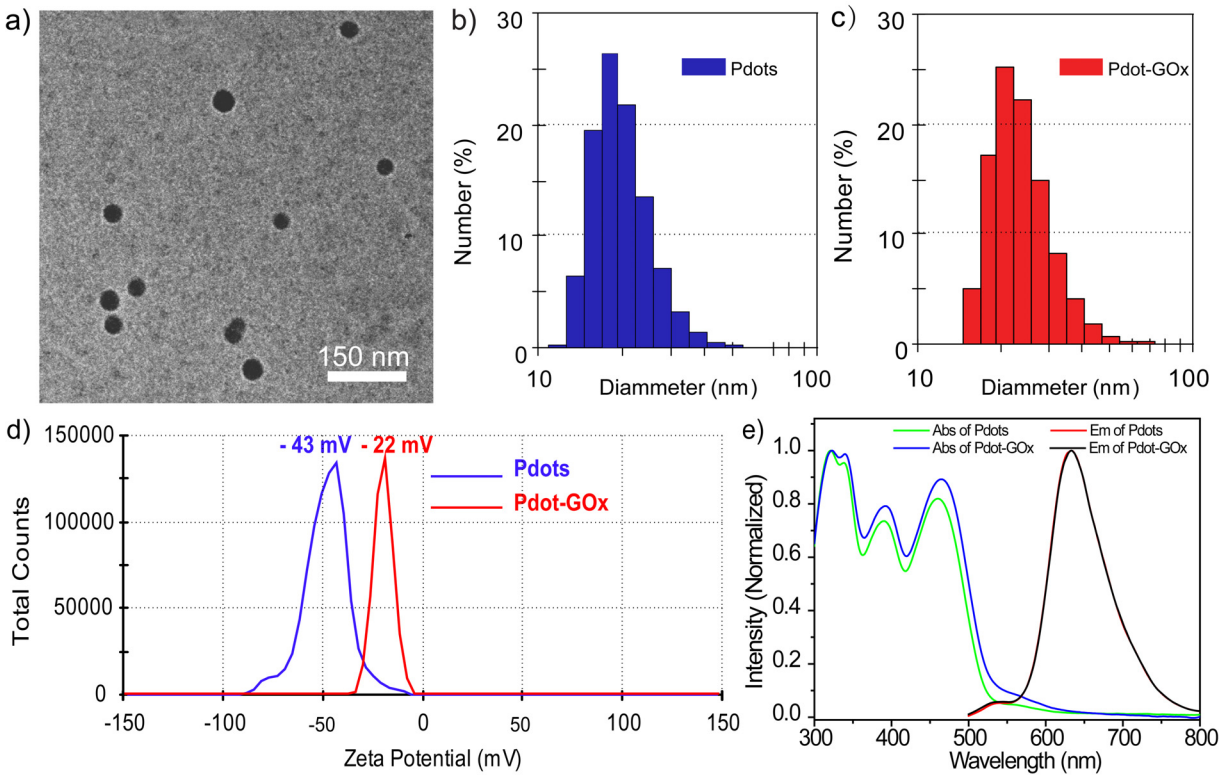

Figure S1. a) Typical TEM images of Pdot-GOx nanoparticles. b) and c) Size distributions of Pdots and Pdot-GOx nanoparticles in aqueous solution measured by dynamic light scattering (DLS). d) Zeta potential of Pdots and Pdot-GOx nanoparticles. e) Absorption and emission spectra of Pdots and Pdot-GOx nanoparticles.

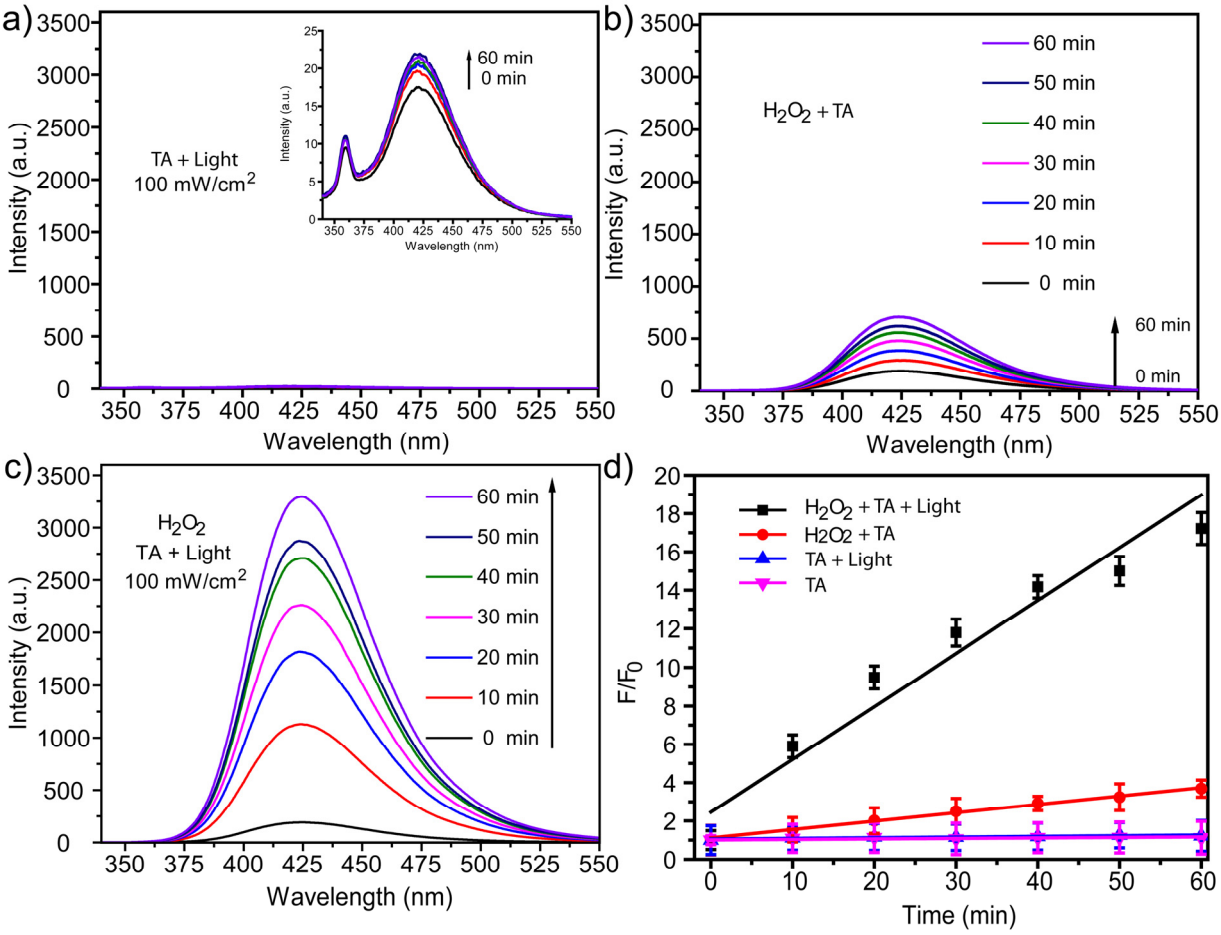

Figure S2. Detection of hydroxyl radical (•OH) generated by H_2O_2. Nonfluorescent compound Terephthalic acid (TA) reacts with hydroxyl radical (•OH) form stable fluorescent 2-hydroxy-TA (TAOH) so that TA was used to detect the •OH radicals generated by photolysis of H_2O_2. a) Fluorescence spectra of TAOH induced by TA and glucose under 460 nm light irradiation for different times (0-60 min). b) Fluorescence spectra of TAOH induced by H_2O_2 and glucose in absence of light irradiation (0-60 min). c) Fluorescence spectra of TAOH from the same solution in b) under 460 nm light irradiation for different times. d) Fluorescence intensity of TAOH at 426 nm as a function of irradiation time for the Pdot-GOx and different control treatments. F_0 and F were the fluorescence intensities of the system without or with treatment, respectively. The error bars represent the standard deviations of three separate measurements.

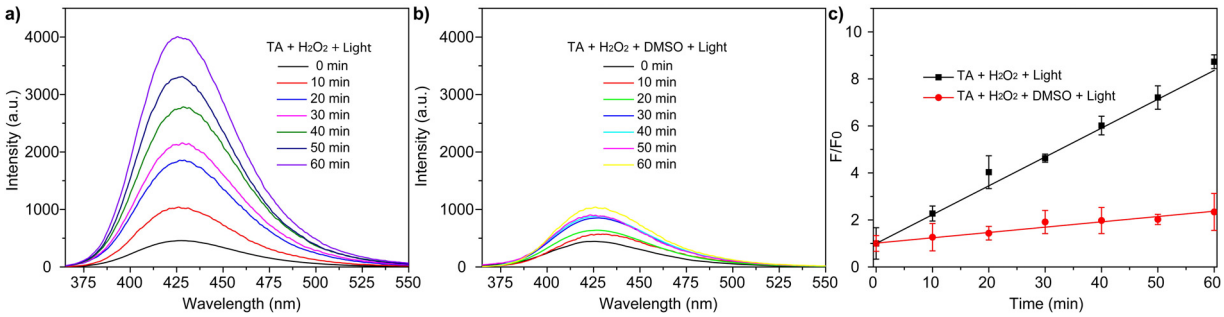

Figure S3. Effect of DMSO on the generation of hydroxyl radical (•OH) by photolysis of H_2O_2. a) Fluorescence spectra of TAOH oxidized by •OH via photolysis of H_2O_2 under 460 nm irradiation for different time (0-60 min); b) Fluorescence spectra of TAOH in the presence of DMSO. Other conditions are the same as those in (b); c) Fluorescence intensity of TAOH as a function of irradiation time. The two curves indicated the apparent inhibition of DMSO on the •OH generation. The error bars represent the standard deviations of three separate measurements.

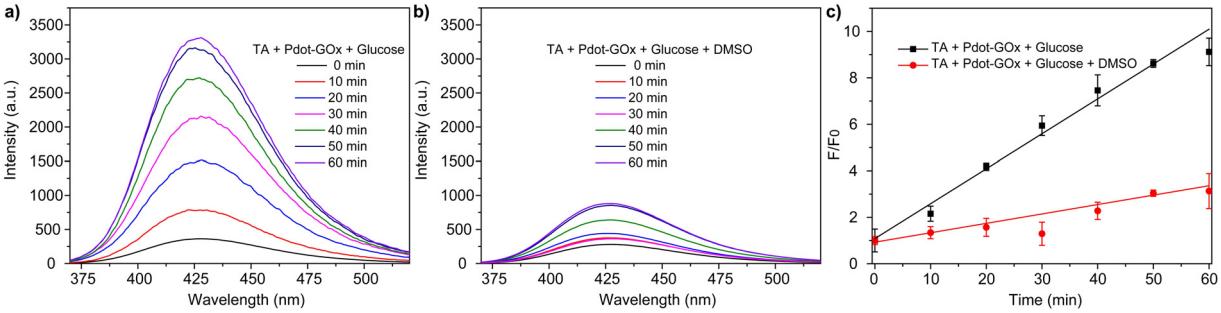

Figure S4. Effect of DMSO on the generation of hydroxyl radical (•OH) by using Pdot-GOx nanoparticles. a) Fluorescence spectra of TAOH by using Pdot-GOx nanoparticles under 460 nm irradiation for different time (0-60 min); b) Fluorescence spectra of TAOH by using Pdot-GOx nanoparticles in the presence of DMSO. Other conditions are the same as those in (b); c) Fluorescence intensity of TAOH as a function of irradiation time. The two curves indicated apparent inhibition of DMSO on the •OH generation. The error bars represent the standard deviations of three separate measurements.

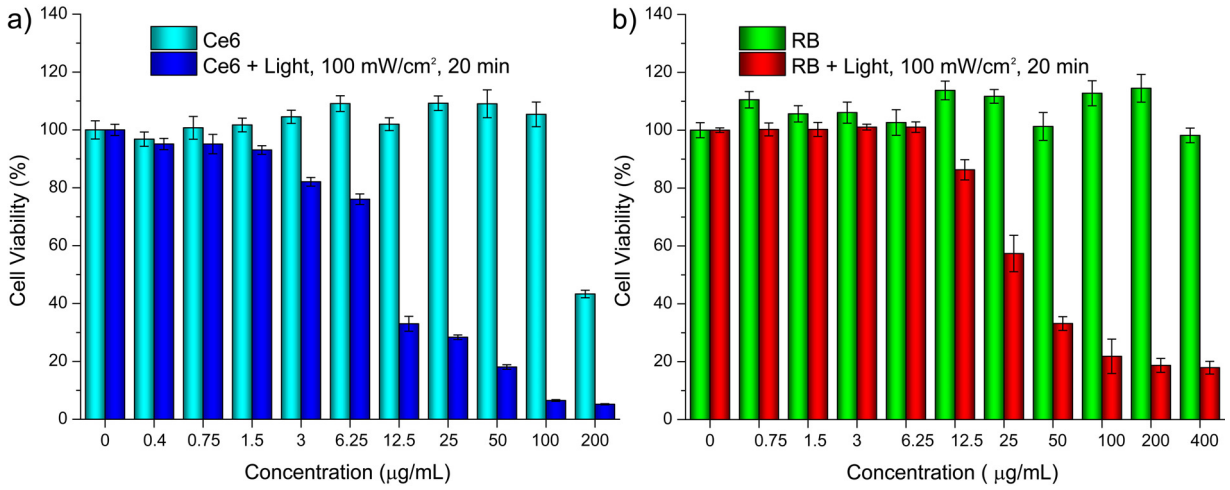

Figure S5. a) MTT cell viability values of MCF-7 cells incubated with different concentrations of Ce6 in presence and absence of light irradiation (650 nm, 120 J/cm^2). b) MTT cell viability values of MCF-7 cells incubated with different concentrations of RB in presence and absence of light irradiation (520 nm, 120 J/cm^2).

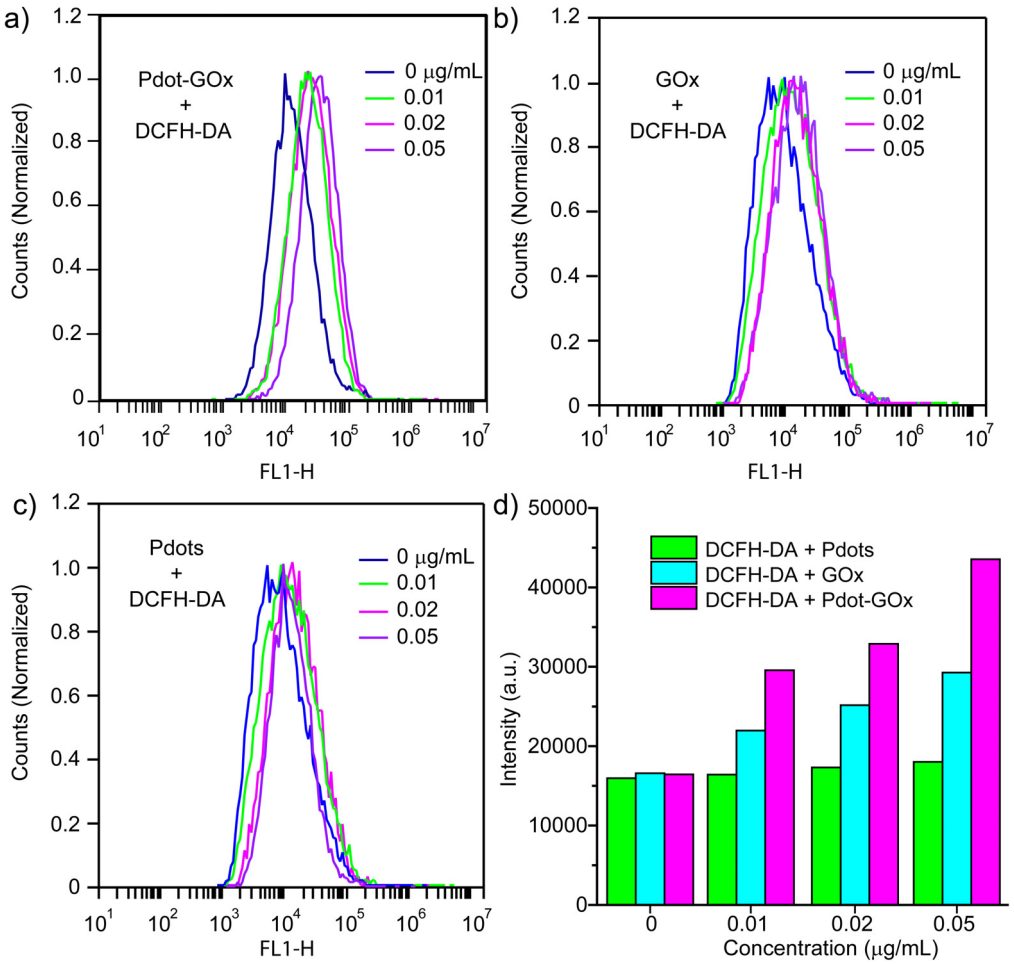

Figure S6. Measurement of intracellular H_2O_2 level in MCF-7 cells by using a fluorescent probe (dichlorofluorescein diacetate, DCFH-DA). MCF-7 cells were incubated with different concentrations of Pdot-GOx, bare Pdots, and free GOx, respectively. The cells were then stained with DCFH-DA for flow cytometry analysis. a) Flow cytometry of MCF-7 cells incubated with Pdot-GOx. b) Flow cytometry of MCF-7 cells incubated with bare Pdots. c) Flow cytometry of MCF-7 cells incubated with free GOx. d) Average fluorescence intensities of MCF-7 cells incubated with Pdot-GOx, bare Pdots, and free GOx, respectively.

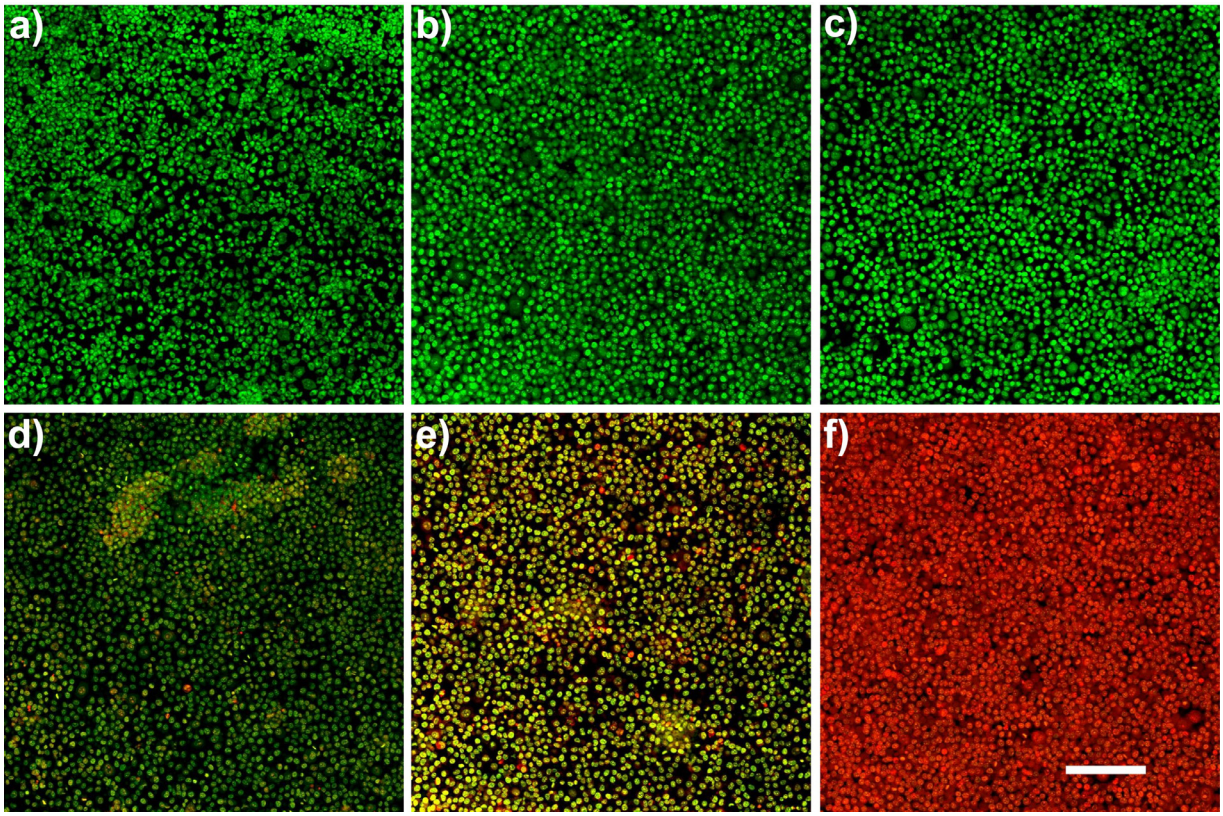

Figure S7. AO/EB staining of MCF-7 cells treated with different concentration of Ce6 and light irradiation (650 nm, 120 J/cm^2). a) 0 μg/mL; b) 0.75 μg/mL; c)1.5 μg/mL; d) 3 μg/mL; e) 6 μg/mL; f)12.5 μg/mL. Scale bar represents 250 μm.

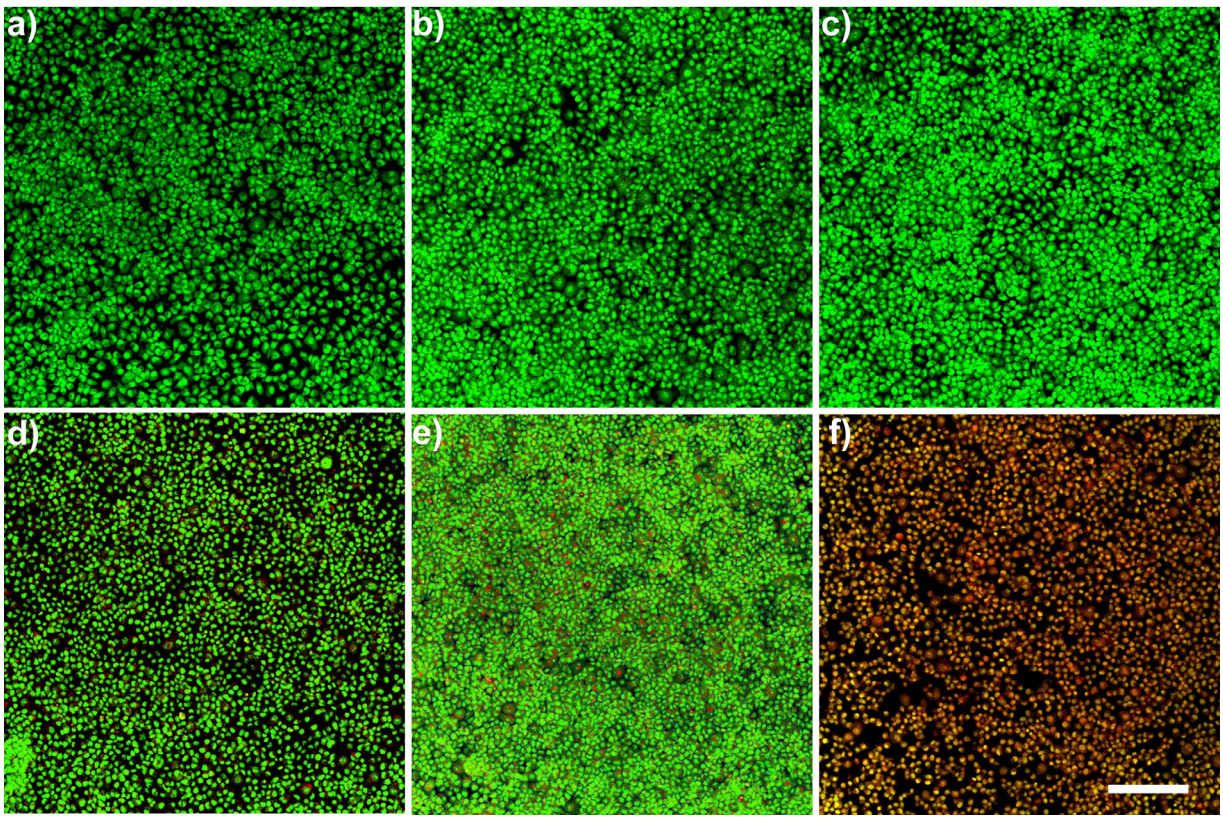

Figure S8. AO/EB staining of MCF-7 cells treated with different concentration of RB and light irradiation (520 nm, 120 J/cm^2). a) 0 μg/mL; b) 0.75 μg/mL; c)1.5 μg/mL; d) 3 μg/mL; e) 6 μg/mL; f)12.5 μg/mL. Scale bar represents 250 μm.

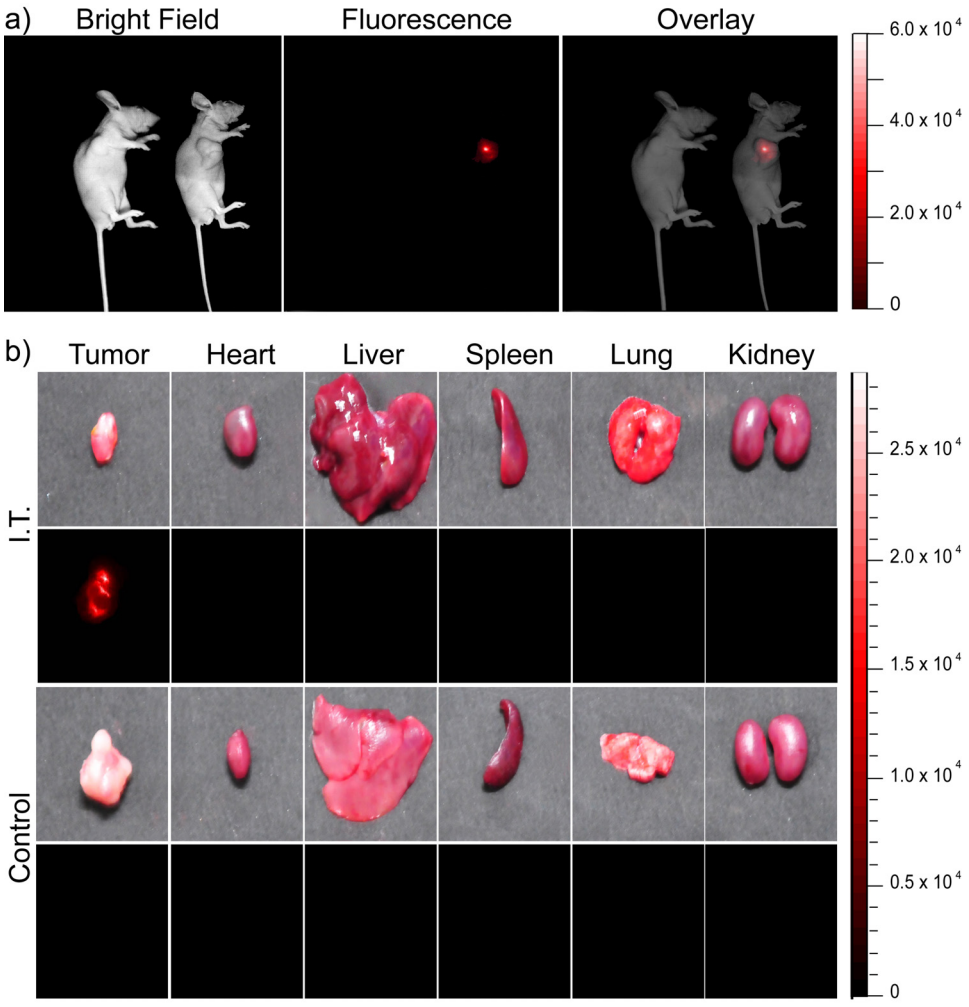

Figure S9. *In vivo* fluorescence imaging by Pdot-GOx nanoparticles. a) Bright-field and fluorescence images of whole-animal imaging for tumor-bearing mice with intratumoral injection of Pdot-GOx (24 h post injection). b) *Ex vivo* bright-field and fluorescence images of different mouse organs including tumor, heart, liver, spleen, lung, kidney. The top panel showed the organs collected from mice with intratumoral injection of Pdot-GOx, and the bottom panel showed the control group without Pdot-GOx administration. I. T. = Intratumoral Injection.

Table S1 A summary of recently published results on traditional photosensitizers for photodynamic therapy.

Photosensitizer	Photosensitizer Dose	Light Irradiation	Reference
Ce6	12 μg/mL	360 J/cm^2	ACS Nano, 2013, 7, 5320-5329.
Porphyrin	3 μg/mL	180 J/cm^2	Angew. Chem. Int. Ed. 2011, 123, 11622-11627.
Silicon naphthalocyanine (SiNc)	50 μg/mL	780 J/cm^2	Chem. Mater., 2015, 27, 6155-6165.
Ce6	12 μg/mL	600 J/cm^2	ACS Appl. Mater. Interfaces, 2015, 7, 17592−17597.
TPECM-TPP	4.5 μg/mL	120 J/cm^2	Chem. Sci., 2015, 6, 4580 –4586.
Ce6	12 μg/mL	120 J/cm^2	Biomaterials, 2013, 34, 4643-4645.

(1) Q. Hou, Q. Zhou, Y. Zhang, W. Yang, R. Yang, Y. Cao, *Macromolecules* **2004**, *37*, 6299-6305.

(2) C. Wu, C. Szymanski, J. McNeill, *Langmuir* **2006**, *22*, 2956-2960.