ISCI, Volume *9*

Supplemental Information

Photo-Enhanced Singlet Oxygen Generation of Prussian Blue-Based Nanocatalyst for Augmented Photodynamic Therapy

Dongdong Wang, Ruohong Shi, Jiajia Zhou, Sixiang Shi, Huihui Wu, Pengping Xu, Hui Wang, Guoliang Xia, Todd E. Barnhart, Weibo Cai, Zhen Guo, and Qianwang Chen

Supplemental Data Items

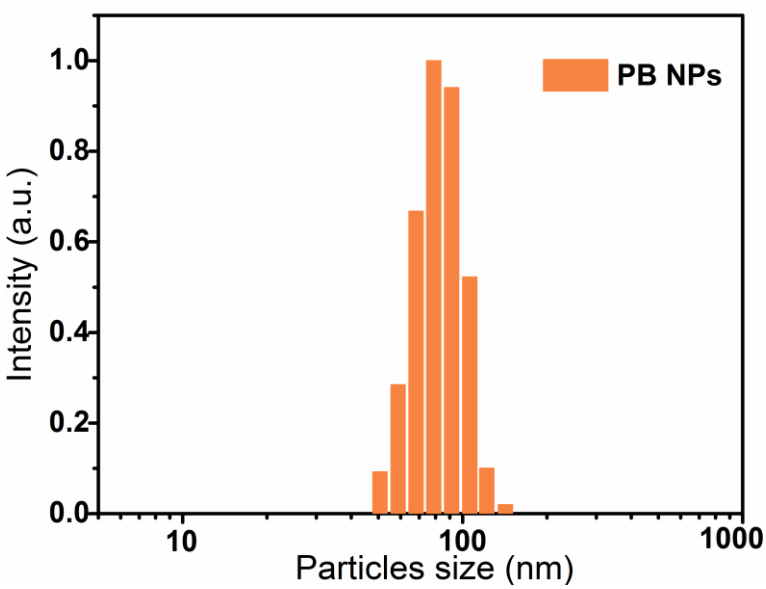

Figure S1. Dynamic light scattering (DLS) size distribution of Prussian Blue (PB) NPs, related to Figure 1.

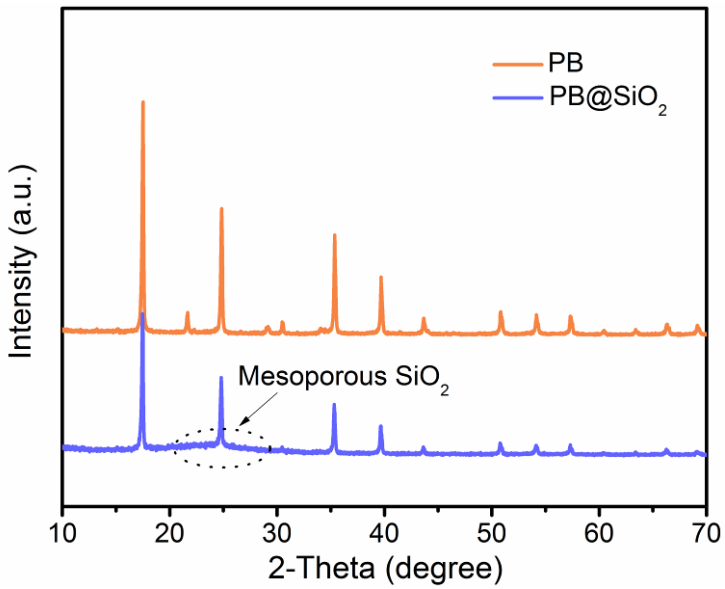

Figure S2. Powder X-ray diffraction (PXRD) pattern of PB and PB@SiO_2 NPs, related to Figure 1.

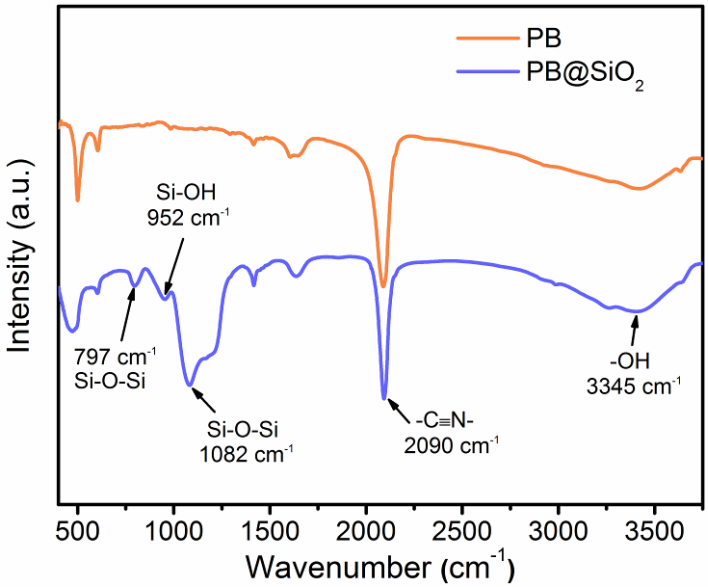

Figure S3. FT-IR spectra of PB and PB@SiO_2 NPs, related to Figure 1.

In the FT-IR spectra (Figure S3), a broad band around 3345 cm^{-1} is due to the -OH stretching and bending vibrations of water. After further mesoporous SiO_2 coating, several broad absorptions assigned to asymmetric stretching (1082 cm^{-1}) and symmetric vibration (797 cm^{-1}) of Si-O-Si and Si-OH (952 cm^{-1}) show the generation of $mSiO_2$ due to the hydrolysis of TEOS. A sharp peak centered at 2090 cm^{-1} should be assign to -C≡N- of PB NPs.

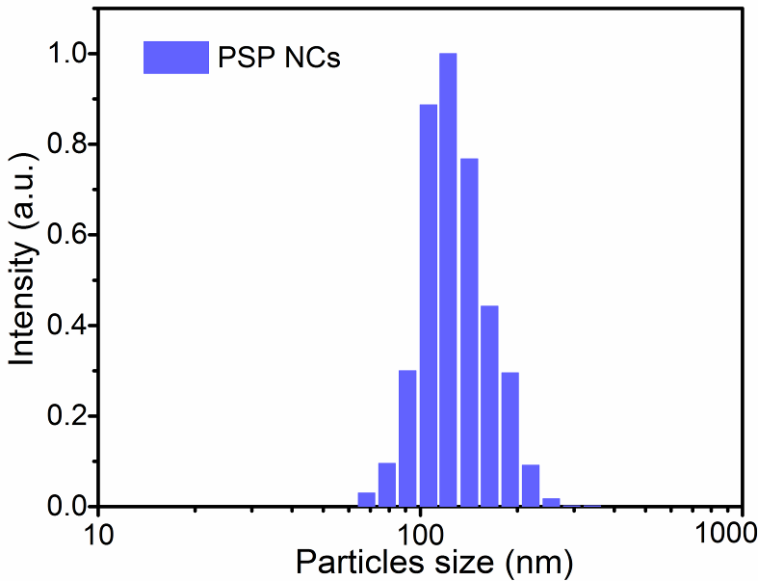

Figure S4. Dynamic light scattering (DLS) size distribution of final PSP NCs, related to Figure 1.

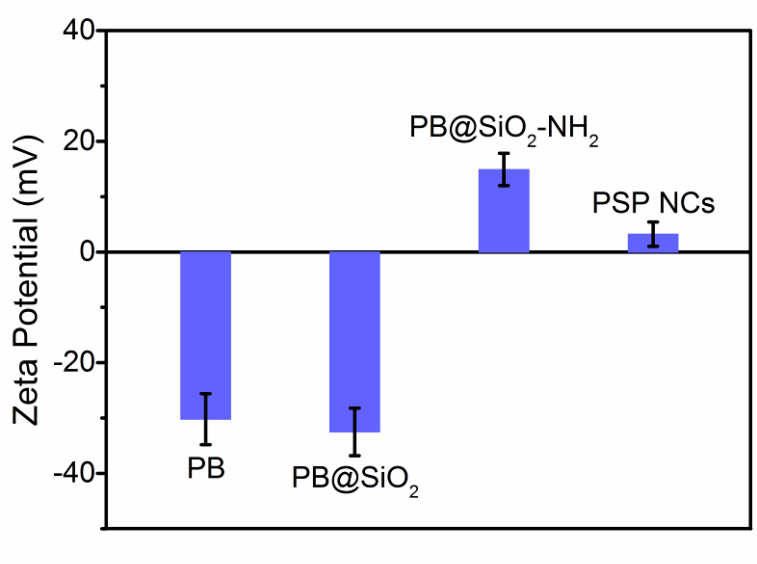

Figure S5. Zeta potential of PB, PB@SiO_2, PB@SiO_2-NH_2 NPs and final PSP NCs, related to Figure 1.

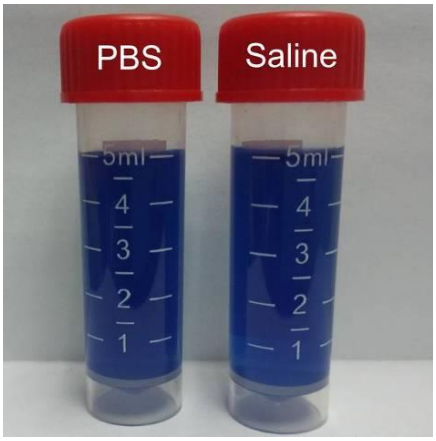

Figure S6. Photos of PSP NCs twenty months after dispersing in PBS and Saline solutions, related to Figure 1.

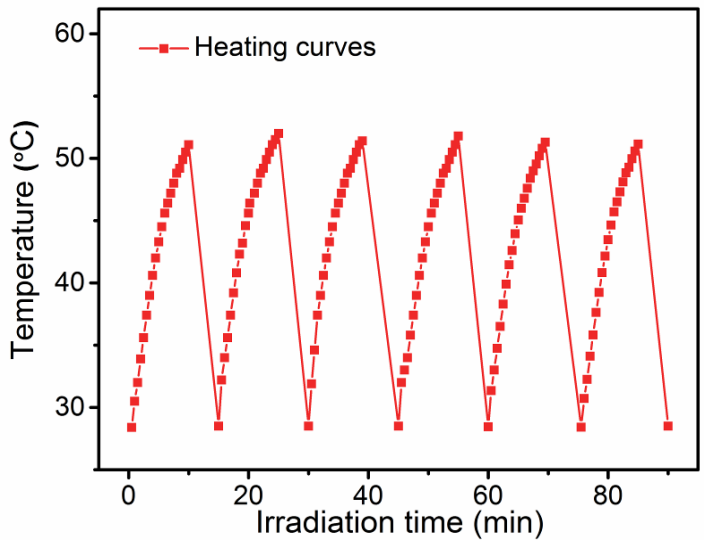

Figure S7. Temperature variations of PSP NCs solution for six heating-cooling cycles, related to Figure 2.

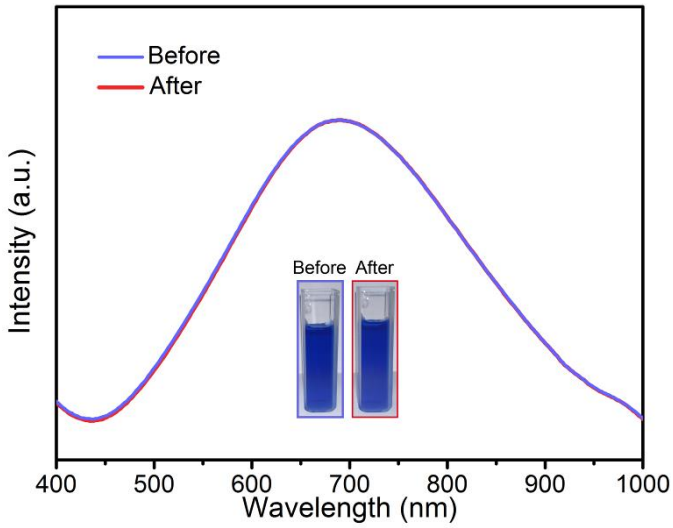

Figure S8. UV-vis-NIR spectroscopy of PSP NCs under the continuous irradiations of 671 nm laser. Inset: the representative photos of PSP NCs solution before and after irradiation for six heating-cooling cycles (the total irradiation time is 60 min), related to Figure 2.

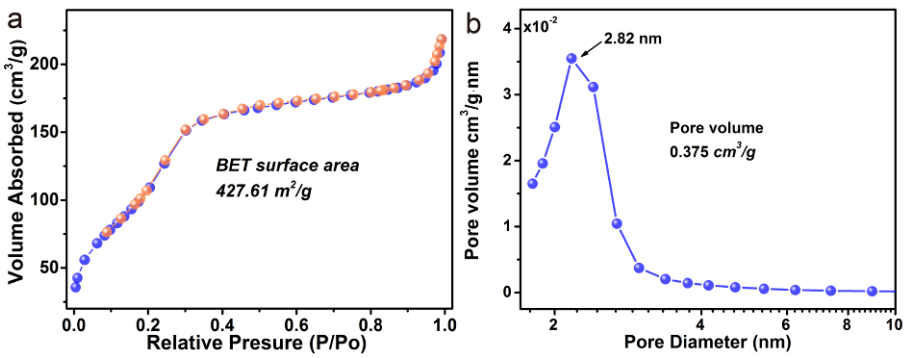

Figure S9. Nitrogen adsorption-desorption isotherms and pore size distributions of PSP NCs, related to Figure 2.

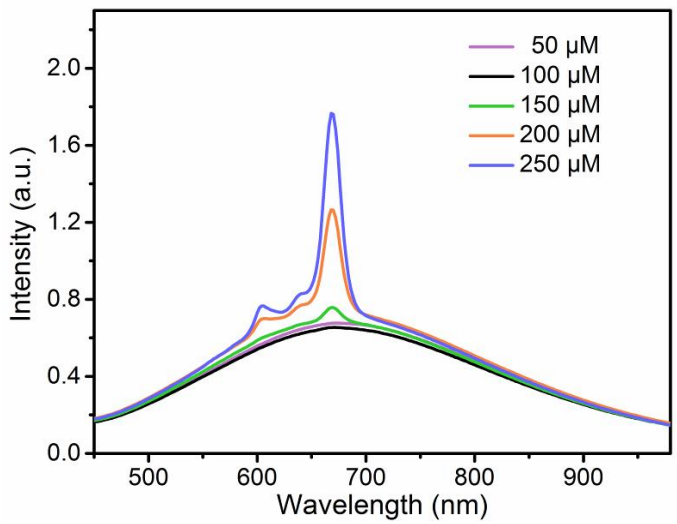

Figure S10. UV-vis spectra of PSPZP after loading ZnPc at different concentrations (5 mg of PSP NCs mixed with 1 mL of photosensitizer in DMSO solutions), related to Figure 2.

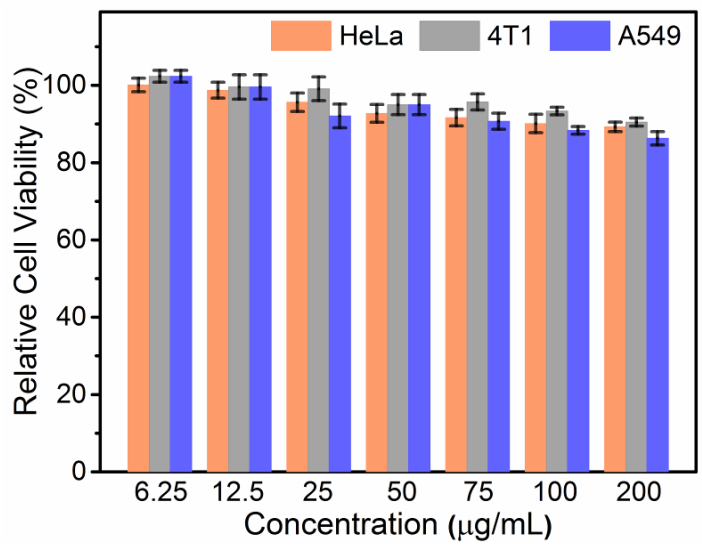

Figure S11. Viability of HeLa, 4T1 and A549 cells treated with PSP NCs at different concentrations, related to Figure 4.

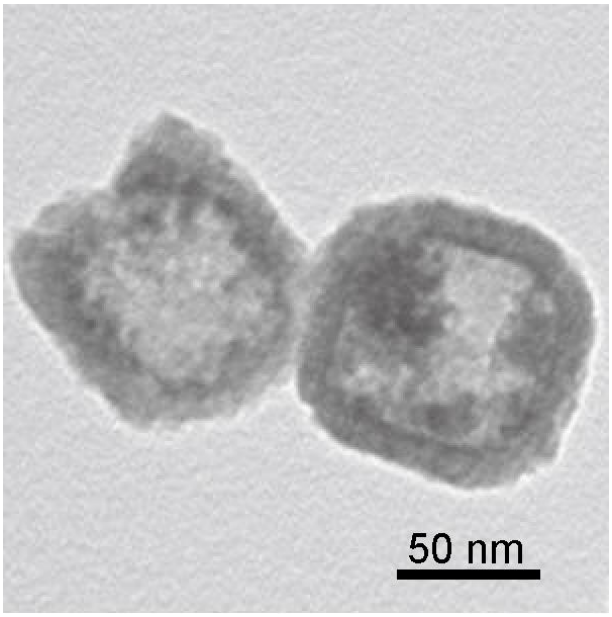

Figure S12. TEM images of mesoporous silica after the removal of inner Prussian blue, related to Figure 4.

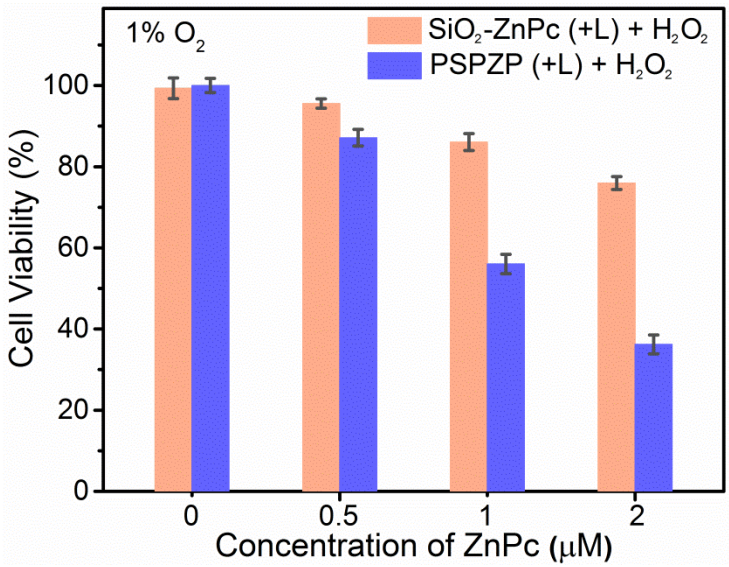

Figure S13. Viabilities of 4T1 cells treated with SiO_2-ZnPc or PSPZP NCs under NIR irradiation (671 nm, 0.4 W cm^{-2}, 5 min) in hypoxic condition, related to Figure 4.

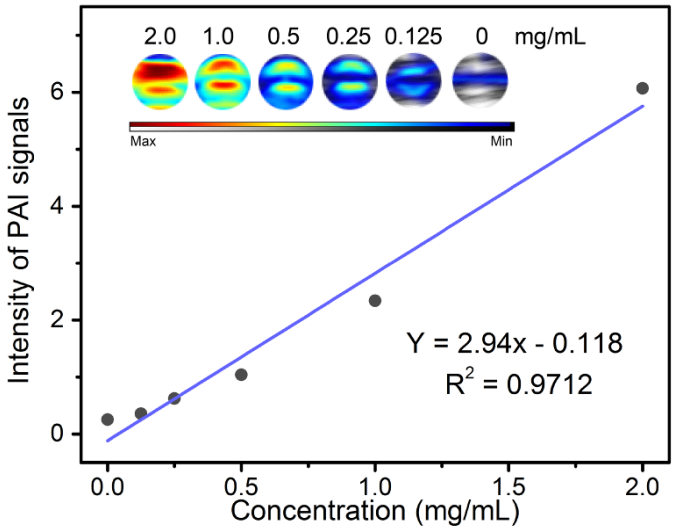

Figure S14. PA images of a series of PSP NCs solutions with different concentrations, related to Figure 5.

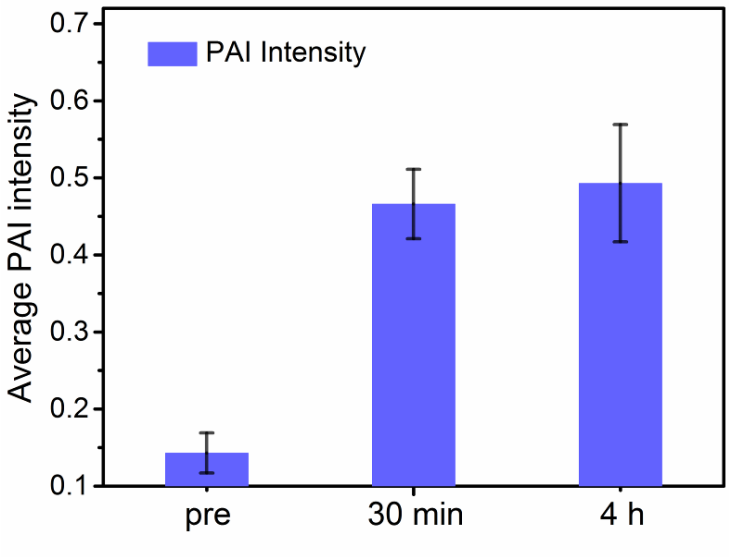

Figure S15. Temporal evolution of PA imaging signal of the tumor site, related to Figure 5.

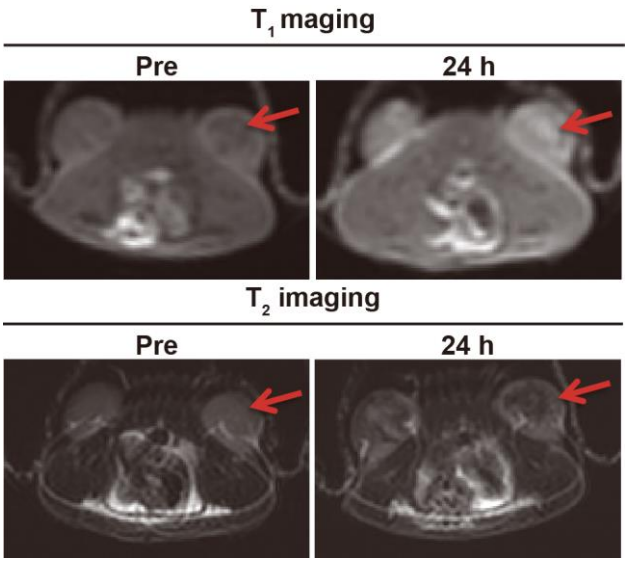

Figure S16. T_1- and T_2-weighted MR images of 4T1 tumor-bearing mice before and 24 hr after intravenous injection of PSP NCs, tumor marked by red arrows, related to Figure 7.

Transparent Methods

Materials Synthesis

All starting materials were obtained from commercial supplies and used as received. Potassium hexacyanoferrate ($K_3[Fe(CN)_6]$) and Zinc phthalocyanine (ZnPc) were purchased from Sigma-Aldrich (St. Louis, MO). 3-Aminopropyltriethoxysilane (APTES) was purchased from Alfa Aesar Reagent Company. Poly (vinylpyrrolidone (PVP, K-30), ammonia water ($NH_3 \cdot H_2O$), tetraethylorthosilicate (TEOS) and concentrated hydrochloric acid were purchased from Sinopharm Co. (Shanghai, China). Methoxy polyethylene glycol-maleimide (MAL-PEG-SCM, MW = 5000) was obtained from ToYongBio, Inc (China). 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were purchased from Keygen Biotech. Co. (China).

Preparation of PSP NCs

First, PB nanoparticles were obtained according to our previous reports (Wang et al., 2016). ***Attention!!!:*** $C{\equiv}N^-$ containing chemicals may be produced and released during the acidic hydrothermal process. Typically, 50 mg Prussian blue nanoparticles (PB NPs) and 2 mL water were added in 20 mL of ethanol. Subsequently, 1 mL of ammonia (25%) were added. Then, 25 μL of TEOS were added quickly for preparing PB@sSiO_2. Thereafter, mesoporous silica was coated on the PB@sSiO_2 with the assistance of CTAB as the template. Typically, CTAB (0.12 g) and water (15 mL) were added in the above PB@sSiO_2 solution and stirred for 12 hr. Subsequently, 200 μL of TEOS and 280 μL of ammonia (25%) were added. After stirring at room temperature for 2 hr, the products were centrifuged and washed with ethanol three times. Finally, the PB@SiO_2 was refluxed and extracted in NH_4NO_3 ethanol solution (6 g/L) for 2 hr to remove the CTAB for three times. To further functionalize the mesoporous SiO_2 surface with -NH_2 groups, 10 mg of PB@SiO_2 NPs were first dispersed in 20 mL of absolute ethanol, followed by adding 1 mL of APTES. The system was sealed and kept at 80~90℃ in an oil bath for 48 hr. Afterward, the mixture was centrifuged and washed with ethanol several times to remove the residual APTES. Afterward, the 20 mg of PB@SiO_2-NH_2 was dispersed in a mixture containing MAL-PEG-SCM (50 mg) and ethanol (10 mL) and shook for 12 hr at room temperature. Finally, PB@SiO_2-PEG nanocatalyst (named as PSP NC) were obtained after washing with ethanol twice and dispersed in phosphate buffer solution (PBS) before subsequent studies. Also, the as-prepared PB@SiO_2 NPs were used to construct mesoporous silica through an etching process under alkaline solution. Then, the PEGylation modification process was the same as the PSP NCs.

Decomposition of H_2O_2 catalyzed by PSP NCs

The catalytic effect of PSP NCs was performed by mixing 1 mM of H_2O_2 and 2 mM of PSP NCs in PBS at 25, 37, 40 and 43℃. Then, 50 μL of the solution was added to 100 μL of $Ti(SO_4)_2$ solution (1.33 mL of 24% $Ti(SO_4)_2$+ 8.33 mL of H_2SO_4 in 50 mL of distilled water) every 30 min. The concentration of H_2O_2 was acquired by measuring the absorbance at 405 nm. The continuous catalytic effect was verified by repetitive addition of 1 mM of H_2O_2 to the solution, followed by measuring the concentration of H_2O_2 after incubation with PSP NCs under different temperature. To investigate the capability of evolving oxygen with the intracellular concentration of H_2O_2, 150 μM of H_2O_2 was incubated with 250 μM of PSP NCs in PBS, followed by measuring the O_2 concentration with an oxygen probe (JPBJ-608 portable Dissolved Oxygen Meters, Shanghai REX Instrument Factory).

Photosensitizer ZnPc loading

Zinc phthalocyanine (ZnPc) was chosen and dissolved in DMSO for further loading treatment. Loading

of ZnPc into PSP NCs was carried out by dispersing 5 mg of PSP in 1 mL of photosensitizer solutions (50~250 μM) and stirred for 12 hr without light interference at room temperature. The formed complexes PSP@ZnPc (named as PSPZP) were collected by centrifugation, washed with PBS 3 times. The contents of ZnPc were determined by the characteristic absorption at 671 nm. The weight amount of ZnPc loaded into PSP NCs was calculated by subtracting the ZnPc content in the supernatant from the total.

Singlet oxygen ($^1O_2$) detection

A chemical probe DPBF was used to confirm singlet oxygen by detecting its absorption intensity at 421 nm via UV-Vis spectroscopy (Lu et al., 2015). In a typical DPBF experiment, 30 μL DPBF in DMSO (10 mmol/L) was added to PSPZP solution (3 mL, 0.5 mmol/L). The solution was saturated with Ar atmosphere and kept in the dark and irradiated with a 671 nm laser (0.4 W cm^{-2}) for 30 min, and the absorption intensity of DPBF at 421 nm was recorded every 2 min. In the control experiments, DPBF absorption was recorded in water, and PSPZP plus H_2O_2 counterparts with or without 671 nm irradiation under different temperatures. Herein, hypoxic condition was achieved by Ar blowing into the solution for 30 min before test.

In vitro cellular experiments

HeLa human cervical cancer cells (HeLa), Human lung cancer cells (A549) and 4T1 murine breast cancer cells were originally obtained from American Type Culture Collection (ATCC) and cultured in standard cell media into 96-well plates and incubated in 5% CO_2 at 37℃ for 24 hr. Then different concentrations of PSP NCs were added into the 96-well plates and continued to be cultured for 24 hr. Cell viability was determined by the standard MTT assay. To study the cell killing efficacy of PTT, 4T1 cells seeded in 96-well plates were incubated with various concentrations of free PSP NCs for 4 hr with 671 nm laser at a power density of 0.4 W cm^{-2} for 5 min. For PDT, 4T1 cells seeded in 96-well plates were mixed with various concentrations of PSPZP and SiO_2-ZnPc with or without 100 μM H_2O_2 under normal (21% O_2, 5% CO_2 and 74% N_2) or hypoxic (1% O_2, 5% CO_2 and 94% N_2) environments. After 4 h, the 96-well plates were exposed to the 671 nm laser for 5 min. Then cells were transferred into fresh medium and further incubated for 24 hr. The standard MTT tests were then conducted to measure the relative cell viabilities. To further confirm the heat-assisted PDT efficacy, 4T1 cells were incubated with various concentrations of PSPZP with 100 μM H_2O_2 under different incubation temperatures. After 4 hr of incubation, the 96-well plates were exposed to the 671 nm light at a power density of 0.4 W cm^{-2} for 5 min. Then, cells were transferred into fresh medium and further incubated for 24 hr before the MTT assay.

Intracellular catalysis ability towards H_2O_2

Intracellular H_2O_2 assay (MAK164, Sigma Aldrich) was used to evaluate the intracellular H_2O_2 concentration after PSP NCs treatment. 100 μM of H_2O_2 was treated to 4T1 pre-incubated with PSP NCs for 24 hr, followed by incubation for 1 hr. Then, cell media was replaced by assay buffer and incubated for another 1 hr. After washing with PBS, intracellular H_2O_2 concentration was measured by fluorescent images (ex/em = 490/520).

Detection of intracellular O_2 generation

4T1 cells were seeded on Chambered cover glass (Lab-Tek Chambered 1.0 Borosilicate Cover Glass system, Nunc) (Xia et al., 2014). After 24 hr, the cells were cultured to 50-60% confluency, and incubated with PBS or PSP NCs at 37℃ under normal or hypoxic condition for 24 hr. Afterward, the cells in each group experienced their corresponding treatments. After 12 hr, these cells were then incubated with $[Ru(dpp)_3]Cl_2$ (Sigma-Aldrich, Co. Ltd.) at a concentration of 10 μg/mL for another 12

hr in a hypoxic incubator so as to evaluate the outcomes of continuous hypoxia modulation within 24 hr, followed by rinsing with PBS three times to remove the free $[Ru(dpp)_3]Cl_2$ and residual particles. The level of intracellular O_2 was evaluated by detecting the fluorescence of $[Ru(dpp)_3]^{2+}$ (λex = 450 nm, λem = 610 nm) by confocal laser scanning microscope (CLSM).

In vivo PA imaging, PET imaging and magnetic resonance imaging

For PA imaging, the 4T1 tumor-bearing mice were anesthetized and then injected with PSP NCs at a dose of 20 mg kg^{-1}. Then, at different time intervals post-injection (p.i.), the tumor region was imaged using the Endra Nexus 128 LAZER system with an excitation wavelength at 730 nm. For PET Imaging, ^{89}Zr-oxalate was produced according to previously reported procedures by the University of Wisconsin–Madison cyclotron group (Zhang et al., 2012). For chelator-free ^{89}Zr labeling, 1 mL of as-prepared PSP NCs in HEPES buffer (pH 7.0; 0.1 M) were mixed with 111 MBq ^{89}Zr oxalate and the pH was adjusted to 8-9 using Na_2CO_3 solution (2 M). The mixture was incubated at 70°C for 4 hr. After labeling, the labeling yield was determined by TLC at different time points. Final labeled products were collected by centrifugation. For *in vivo* PET imaging, 4T1 murine breast cancer cells were used for 4T1 tumor implantation when they reached ~80% confluence. All animal studies for PET imaging were conducted under a protocol approved by the University of Wisconsin Institutional Animal Care and Use Committee. The 4T1 tumor-bearing mice were used for *in vivo* experiments when the tumor diameter reached 6–8 mm. PET scanning at various time points (0.5, 3, 6, and 24 hr) after intravenous injection of 2~5 MBq of ^{89}Zr-PSP NCs were performed using a microPET/microCT Inveon rodent model scanner (Siemens Medical Solutions USA, Inc.). After the last scan at 24 hr p.i., biodistribution studies were carried out to confirm that the %ID/g values based on PET imaging truly represented the radioactivity distribution in mice. For *in vivo* MRI, 4T1-bearing mice were injected with PSP NCs (20 mg kg^{-1}) through the tail vein. Then, T_1 and T_2* MR imaging were performed before and 24 h after the intravenous injection with clinical magnetic resonance scanner (GE Signa HDxt 3.0 Tesla MRI system) equipped with a special animal imaging coil.

In vivo PTT assisted PDT therapy

The 4T1 tumor-bearing mice were randomly allocated into six group (n = 6): 1 is saline, 2 is PSPZP, 3 is NIR (671 nm, 0.4 W cm^{-2}, 5 min), 4 is ZnPc + NIR, 5 is PSP + NIR, 6 is PSPZP + NIR. The mice in the treatment groups were injected with saline, free ZnPc, PSP NCs, PSPZP solutions (total dose = 100 µL, $C_{[PSPZP]}$ = 2 mg mL^{-1}, the concentration of ZnPc and PSP NCs were based on the loading capacity) via the tail vein respectively. After 24 hr, the tumors from group 2, 3, 4 and 5 were irradiated with 671 nm laser (0.4 W cm^{-2}) for 5 min. The tumor dimensions were measured with a caliper, and the tumor volume was calculated according to the equation: Volume of tumor was calculated as $V = a \times b^2/2$, where “a” and “b” were the longest and shortest diameters of the tumor, respectively. The body weight of mice was recorded at 2 day intervals. Relative tumor volume was normalized to its initial size before administration and laser irradiation. All mice were sacrificed after experiment and tumors of each group were collected.

Histology examination

After *in vivo* therapy experiments, the tumor tissues harvested from the administrated mice were fixed in 10% formalin, dehydrated with gradient ethanol, embedded in paraffin and sectioned. Then the tumor sections were stained with hematoxylin and eosin (H&E) for the detection of changes in the cellular integrity and tissue morphology and anti-Ki-67 rabbit polyclonal antibody (Abcam, ab15580, America) for the detection of proliferative cells. Finally, the images of the stained tumor slices were characterized using Olympus IX-70 microscope.

Immunofluorescence staining

To study the change of hypoxia within tumor microenvironment after intravenous injection with PSP NCs (10 mg kg^{-1}). 1.5 hr before tumors surgically excised from the mice (24 hr post injection of PSP solution), the mice were intraperitoneally injected with pimonidazole hydrochloride (Hypoxyprobe, USA) at a dose of 30 mg kg^{-1} according to the procedure provided by the manufacturer. The collected tumor slices were firstly stained with mouse anti-pimonidazole monoclonal antibody and rat anti-mouse CD31 antibody as primary antibodies to label tumor hypoxia regions and blood vessels, respectively. Then the slices were stained with Alex 488-conjugated goat anti-mouse secondary antibody and rhodamine-conjugated donkey anti-rat secondary antibody, respectively. Finally, the images of stained slices were characterized using CLSM (Zeiss LSM 710). The hypoxia region and the density of blood vessels of each slice were statistically analyzed using the Imaging-J software.

Hematological analysis and in vivo biocompatibility test

Healthy female Bale/c mice were divided into two groups and injected with saline and PSPZP (100 μL, 2 mg mL^{-1}), respectively. At 7 and 15 days after intravenous injection, blood samples were collected from the tail artery for blood chemistry tests (ALT, ALP, AST and BUN) and routine blood analysis (RBC, WBC, HGB, MCHC, PLT, MCH, MPV, MCV, HCT). For testing *in vivo* biocompatibility of PSPZP. 4T1 tumor bearing mice were treated with saline, PB, mesoporous SiO_2, PSP or PSPZP for 120 hr, respectively. Then the sections of main organs (heart, kidney, liver, lung, spleen) were stained with hematoxylin and eosin (H&E) for the detection of changes in the cellular integrity and tissue morphology.

Characterization

The interaction and microstructure of particles were analyzed by an FT-IR spectrometer (Nicolet 8700, Nicolet Co., USA) and X-ray diffraction (XRD) (TTR-III, Rigaku Co., Japan). The morphology was analyzed with an H-7650 TEM (Hitachi Co., Japan) and a JSM-6700 M SEM (JEOL Co., Japan). The elements distribution was characterized using a STEM with EDX spectroscopy (JEM-2100F, JEOL Co., Japan). ICP measurements were carried out on an ICP-AES (Optima 7300DV, PerkinElmer Co., USA). The BET specific surface areas and pore size distribution were measured using a porosimetry analyzer (ASAP 2020, Micromeritics, USA). The UV-Vis absorption spectra were measured on an ultraviolet-visible absorption spectrometer (SOLID 3700, Shimadzu, Japan). The temperature was measured using a digital non-contact Infrared Thermometer with a resolution of 0.1℃. Particle size distribution measurements were conducted on a DLS detector (Zetasizer uV, Malvern, UK).

Supplemental References:

Wang, D., Zhou, J., Chen, R., Shi, R., Zhao, G., Xia, G., Li, R., Liu, Z., Tian, J., Wang, H., Guo Z., Wang, H. and Chen, Q. (2016). Biomaterials *100*, 27-40.

Lu, S., Tu, D., Hu, P., Xu, J., Li, R., Wang, M., Chen, Z., Huang, M., and Chen, X. (2015). Angew. Chem., Int. Ed. *54*, 7915-7919.

Xia, P., Zhou, J., Song, X., Wu, B., Liu, X., Li, D., Zhang, S., Wang, Z., Yu, H., Ward, T., Zhang, J., Li, Y., Wang, X., Chen, Y., Guo, Z., Yao, X. (2014). Journal of molecular cell biology *6*, 240-254.

Zhang, Y., Hong, H., Severin, G.W., Engle, J.W., Yang, Y., Goel, S., Nathanson, A.J., Liu, G., Nickles, R.J., and Leigh, B.R. (2012). Am J Transl Res *4*, 333-346.