Supporting Information

Polymer Nanoparticles Regulate Macrophage Repolarization for Antitumor Treatment

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Experimental

Materials: The poly(phenylene vinylene) derivative poly[2-methoxy-5-(2-ethylhexyloxy)-1,4-phenylenevinylene] (PPV; MW: 150,000-250,000 Da), poly(styreneco-maleic anhydride) (PSMA), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2',7'-dichlorofluorescein diacetate (DCFH-DA), lipopolysaccharide (LPS), and the solvent tetrahydrofuran (THF, anhydrous,99.9%) were all purchased from Sigma-Aldrich (Shanghai, China). All the above reagents and solvents were used without further purification. Raw 264.7, 4T1, and NIH/3T3 were purchased from Procell Life Science&Technology Company (Wuhan, China). Fetal bovine serum (FBS), Dulbecco's modified Eagle medium (DMEM), and Roswell Park Memorial Institute 1640 (RPMI 1640) were purchased from Sijiqing Biological Engineering Materials (Hangzhou, China). Lyso tracker, Hoechst 33342, and DAPI were purchased from Invitrogen (Beijing, China). Recombinant murine IL-4, cell

staining buffer and Ki-67 antibody, active caspase-3 antibody, Alexa Fluor 488 rabbit anti-mouse IgG, anti-CD86 APC, and anti-CD80 APC were purchased from Thermo Fisher Scientific. 0.4 μm-sized transwell system was purchased from Corning.

Measurements: The morphology of PPV-PSMA-NPs was determined by a Hitachi HT7700 transmission electron microscopy (TEM). UV-Vis absorption spectra were collected by JASCO V-550 Spectrophotometer. Fluorescence emission spectra were recorded on a Hitachi F-4500 spectrofluorometer equipped with a Xenon lamp excitation source. The dynamic light scattering (DLS) and zeta potential measurements were conducted with Malvern Zetasizer Nano ZS90. MTT analysis was performed on a microplate reader (BIO-TEK Synergy HT). Confocal laser scanning microscopy (CLSM) characterizations were captured by the confocal laser scanning biological microscope (FV1000-IX81, Olympus, Japan). The flow cytometric analysis was performed by flow cytometer (BD Accuri C6).

Preparation of PSMA-NPs: As the previous reports, PSMA-NPs were prepared through the nano-precipitation method.[1] The PSMA was totally dissolved in THF with a concentration of 2 mg/mL. 5 mL of THF containing PSMA (1 mg/mL) was dispersed into 15 mL of Milli-Q water under vigorous sonication. THF and water (5 mL) were removed by Rotary Evaporator. The THF-free dispersion nanoparticles were filtered through a 0.22 μm cellulose membrane filter to obtain PSMA-NPs at the final concentrations of 500 μg/mL.

Preparation of PPV-PSMA-NPs: PPV-PSMA-NPs were prepared following the above method. The PPV was totally dissolved in THF with a concentration of 0.25 mg/mL. 5 mL of THF containing PPV (100 μg/mL) and PSMA (1 mg/mL) was dispersed into 15 ml of Milli-Q water under vigorous sonication. THF and water (5 mL) were removed by Rotary Evaporator. The THF-free dispersion nanoparticles were filtered through a 0.22 μm cellulose membrane filter to obtain PPV-PSMA-NPs at final concentrations of 500 μg/mL. The concentration of PPV-PSMA-NPs was defined as

the concentration of PSMA.

Cell culture and passage: Raw264.7 and NIH/3T3 cells were both cultured in DMEM medium with 10% FBS at 37 ℃ in a humified atmosphere containing 5% CO_2. 4T1 cells were cultured in RPMI 1640 medium with 10% FBS at 37 ℃ in a humified atmosphere containing 5% CO_2.

Preparation of TAMs (M2-like macrophages): M2-like phenotype TAMs were prepared by treating primary macrophages with cytokines IL-4. Raw 264.7 were seeded in a 100 mm dish with a density of 2×10^6. After 12h later, the supernatant was removed, and the medium was replaced with DMEM containing 10 ng/mL IL-4 and co-cultured for an additional 24 h to obtain TAMs (M2-like macrophages).

Cell viability assay of TAMs: TAMs were seeded in 96-well plates at a density of 5000 cells/well until adherent. Cells were co-incubated with various concentrations of different groups in fresh culture medium for 24 h. After removing the supernatant in the plates, MTT (0.5 mg·mL-1 in medium, 100 μL/well) was subsequently added to the plates followed by incubation at 37℃ for 4h. Then, the supernatant was removed and 100 μL DMSO was added to each well to dissolve the produced formazan. After shaking the plates for 60s, absorbance values at 570 nm of each well were recorded by microplate reader.

***In vitro* optical imaging and co-localization of TAMs:** TAMs were seeded in a 30 mm confocal dish at a density of 3×10^4 cells/dish, until adherent. The cells were cultured with a medium containing PPV-PSMA-NPs (50 μg/mL) for 4 h, 72 h. Then, the supernatant was removed, and dishes were washed with PBS three times. Lyso Tracker was added at a final concentration of 200 nM. After culturing for 30 mins, the medium was removed, and cell nuclei were stained by Hoechst 33342. The CLSM

images were taken. The wavelength of the stimulation laser of Hoechst 33342 was 405 nm. Lyso Tracker and PPV-PSMA-NPs was 488 nm.

Flow cytometry analysis of the re-polarization of TAMs *in vitro*: TAMs were seeded into 6-well plates (10^6 cells/well). The LPS (1 μg/mL), PSMA-NPs (50 μg/mL), PPV-PSMA-NPs (50 μg/mL), PSMA (50 μg/mL) and PPV (50 μg/mL) were added to plates, respectively. After 24 h later, the cells were harvested and washed with PBS buffer for three times. TAMs were counterstained with anti-APC CD86 and anti-APC CD80. The cells were washed again and detected by flow cytometry analysis.

Immunofluorescence analysis of 4T1 cells: 4T1 cancer cells were seeded in the lower chamber with a density of 10^5 cells/well until adherent. After 24 h, 0.4 μm-sized transwell plate was added, and 3×10^5 cells/mL of TAMs, TAMs (PPV-PSMA-NPs), and medium (blank) were seeded in the upperranswell chamber for 4 h, respectively. Then, the upper chamber was removed and 4T1 cells were washed with PBS three times. 4% paraformaldehyde in PBS buffer was added to fix 4T1 cells for 15 min at room temperature. Then cells were prepared for immunofluorescence staining of Ki-67 and active caspase 3 followed by instructions. The images were observed by CLSM, and the wavelength of the stimulating laser of Alexa Fluor was 488 nm. Cell nuclei were stained by DAPI.

***In vivo* anti-tumor:** BALB/c mice were obtained from GemPharmatch Co. Ltd. All animal procedures were approved by Animal Ethics Committee of Jiangsu University. The female BALB/c mice of 6 weeks were used to establish a breast tumor model. 4T1 cells (10^6 cells per mouse) were subcutaneously injected into mice for 7 days. Then, the mice were divided into 3 groups (n =5) and intratumoral injected with PBS, PPV-PSMA-NPs (50 μg/mL), PSMA (50 μg/mL) at a volume of 100 μL per mouse, respectively, repeated every other day. The volumes of tumor and body weigh were measured every two days for 14 days since the first treatment. The tumor volume was determined as follows: $V = W^2 \times L/2$, where W and L were the lengths in minor and

major of the tumor, respectively. After 14 days post-injection, the mice were mercifully sacrificed and representative images of tumor tissues from each group were collected.

Evaluation of ROS generation ability of PPV-PSMA-NPs: 200 μL of 40 mM activated DCFH, 4 μL of PPV-PSMA-NPs (500 μg/mL) were added into a black 96-well plate, respectively. Under white light irradiation, fluorescent intensity was immediately recorded by a microplate reader with emission at 528 nm and excitation at 488 nm every minute.

PDT cytotoxicity experiments towards cancer cells: 4T1 were seeded in 96-well plates at a density of 4000 cells/well until adherent. Cells were co-incubated with various concentrations of different groups in fresh culture medium for 24 h. Then the cells were exposed to white light (30 min, 25 mM/cm^2). After another 24 h incubation, MTT (0.5 mg·mL^{-1} in medium, 100 μL/well) was subsequently added to the plates followed by incubation at 37 ℃ for 4 h. Then, the supernatant was removed and 100 μL DMSO was added to each well to dissolve the produced formazan. After shaking the plates for 60 s, absorbance values at 570 nm of each well were recorded by microplate reader.

Reference

[1] Y. Wang, S. Li, L. Liu, F. Lv, S. Wang, *Angew. Chem., Int. Ed.* 2017, **56**, 5308–5311.

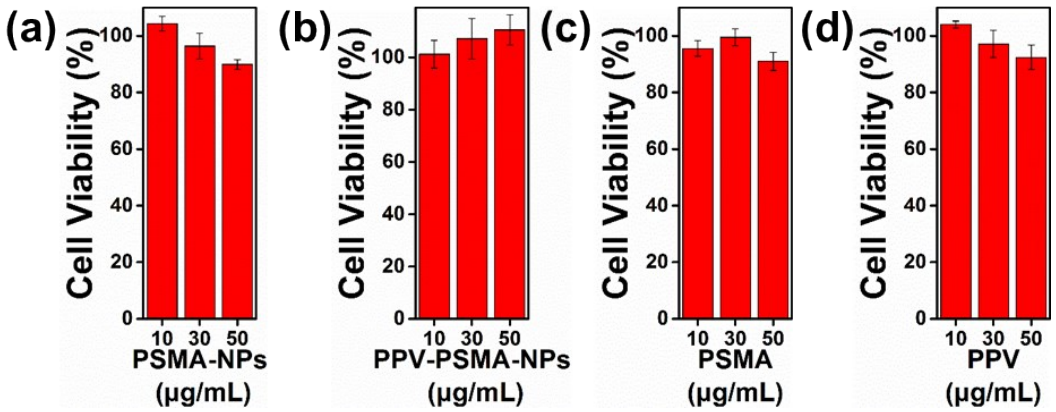

Figure S1. Cell viabilities of TAMs treated with different concentrations of PSMA or PPV.

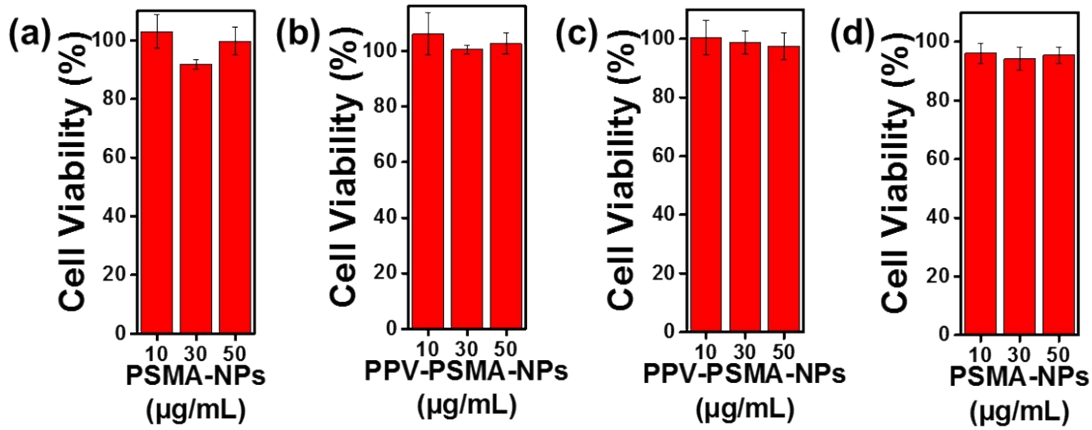

Figure S2. Cell viabilities of 4T1 cells treated with different concentrations of PPV-PSMA-NPs (a) and PPV-PSMA-NPs (b). Cell viabilities of NIH/3T3 cells treated with different concentrations of PPV-PSMA-NPs (c) and PPV-PSMA-NPs (d).

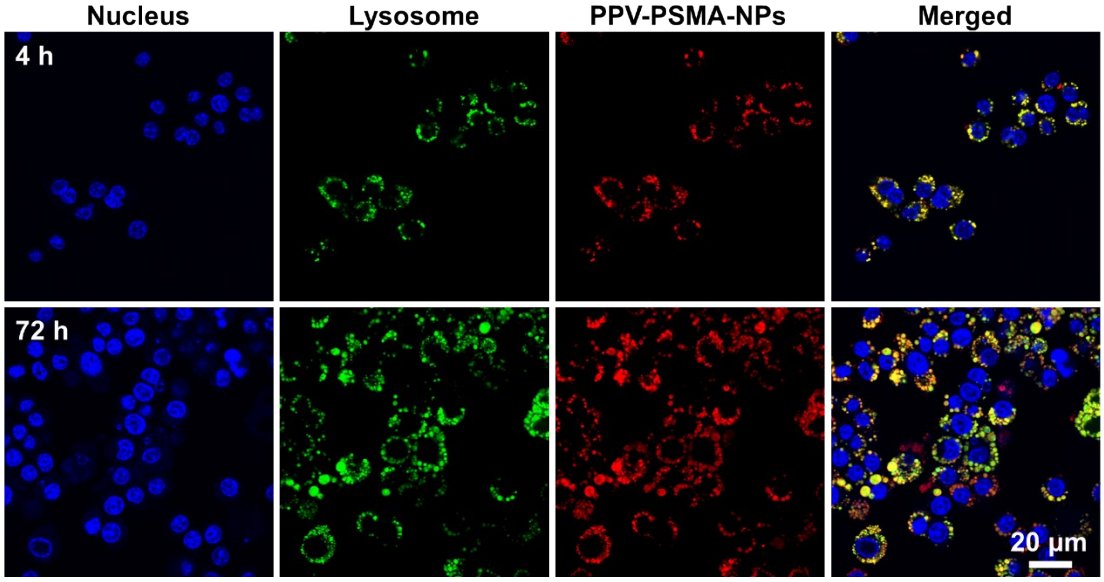

Figure S3. Co-localization analysis of PPV-PSMA-NPs (red) with Lysosome Tracker (green) in tumor-associated macrophages (TAMs) at 4 h and 72 h.

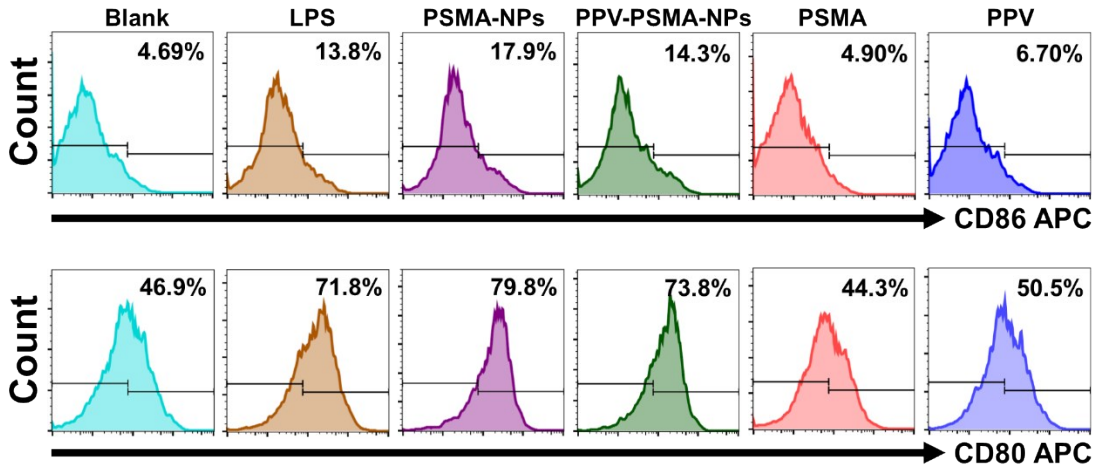

Figure S4. *In vitro* re-polarization of M2-subtype tumor-associated macrophages (TAMs) by P-NPs. Flow cytometric analysis showed the expression level of M1-related markers CD86 and CD80.

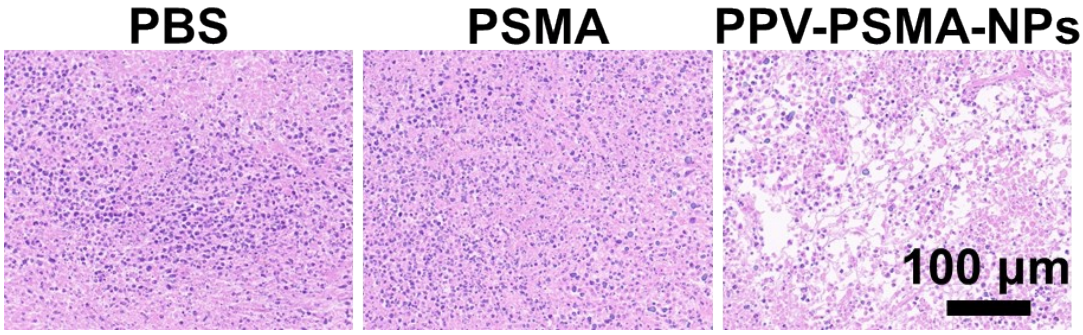

Figure S5. H&E staining analysis of tumor slices in different groups at 14 d post-treatments.

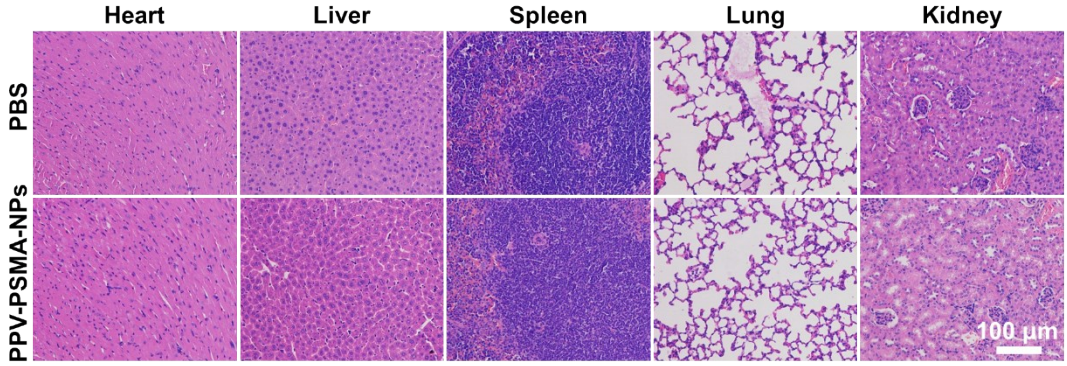

Figure S6. H&E staining images of heart, liver, spleen, lung and kidney at 14d post-treatments.

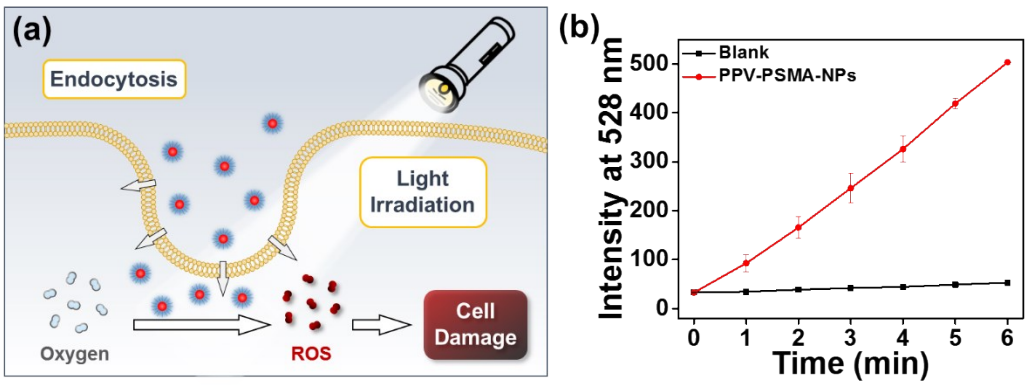

Figure S7. (a) Schematic illustration of the PDT-induced cell death of PPV-PSMA-NPs inside cancer cells. (b) Evaluation of ROS generation under white light irradiation.

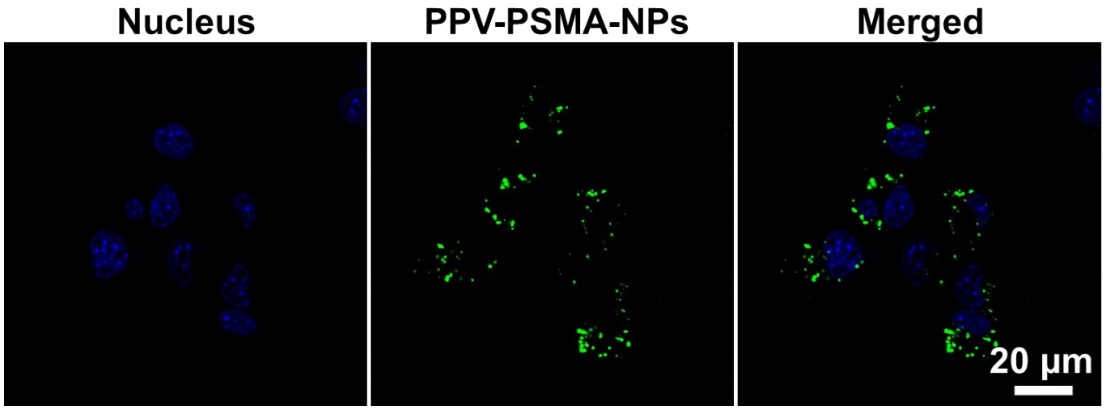

Figure S8. CLSM images of 4T1 cells incubated with PPV-PSMA-NPs for 24 h.

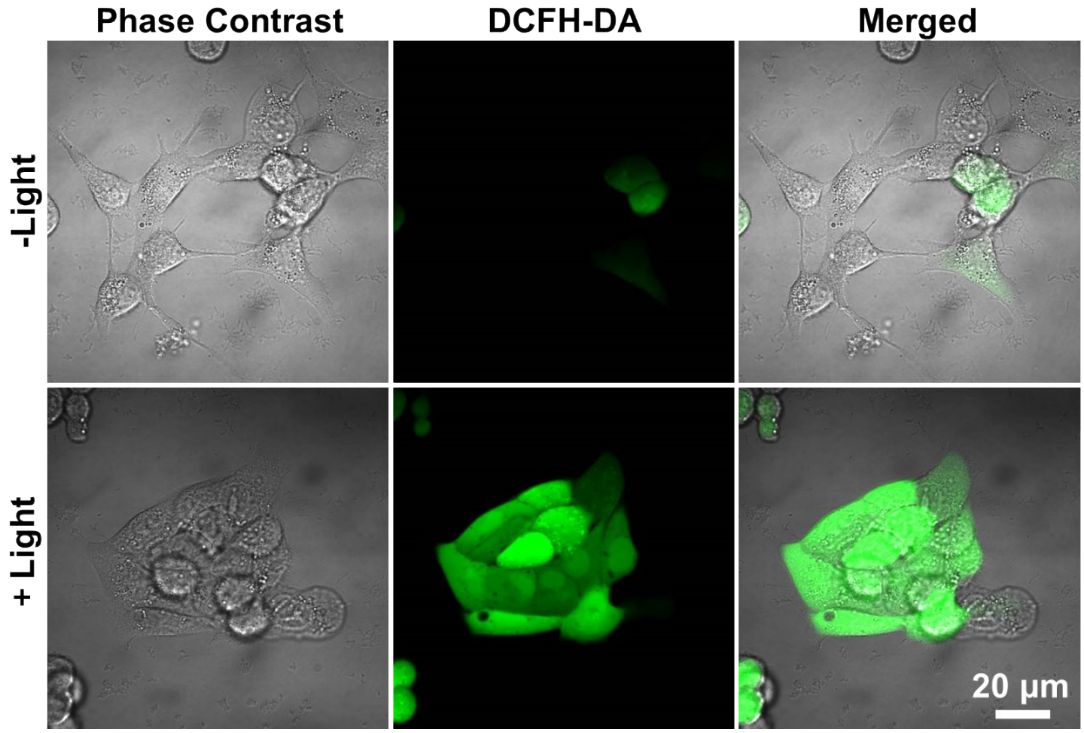

Figure S9. Intracellular ROS level before and after PDT therapy detected by DCFH-DA probe.