Supporting Information

Near-Infrared Laser-Driven in Situ Self-Assembly as a General Strategy for Deep Tumor Therapy

Fu-Hua Liu,[§] Yong Cong,[§] Guo-Bin Qi, Lei Ji, Zeng-Ying Qiao, Hao Wang**

CAS Center for Excellence in Nanoscience, CAS Key Laboratory for Biomedical Effects of Nanomaterials and Nanosafety, National Center for Nanoscience and Technology (NCNST), Beijing, 100190, China

E-mail: qiaozy@nanoctr.cn or wanghao@nanoctr.cn; Tel: +86-10-82545759

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Materials.

Poly(ethylene glycol) diacrylate (PEGDA, average Mw 700, 575, 302), dithiothreitol (DTT), acryloyl chloride, indocyanine green (ICG)-NHS and 4-(2-aminoethylamino)-7-(N, N-dimethylsulfamoyl) benzofurazan (DBD-ED) were purchased from Sigma-Aldrich Chemical Corporation. Fmoc-Cys(Trt)-Wang resin and amino acids such as Fmoc-Arg-OH, Fmoc-Gln(Trt)-OH, Fmoc-Gly-OH, Fmoc-Tyr(But)-OH and HBTU for peptide synthesis were obtained from GL Biochem (Shanghai) Ltd. Cell culture medium included of Fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI-1640) and Benzylpenicillin/streptomycin were obtained from HyClone/Thermo fisher (Beijing, China). Female BALB/c mice (6-8 weeks, about 16 g) were purchased from Vital River laboratory animal technology Co, Ltd. (Beijing, China). All the other reagents and solvents for peptide-polymer conjugates synthesis in the research were purchased from Sinopharm Chemical Reagent Beijing Co. Ltd or Beijing Chemical Company (China) and used without further purification unless otherwise stated.

Preparation of main chain polymer MP1.

PEGDA (M_r= 700, 0.35 g, 0.5 mmol), PEGDA (M_r = 575, 0.2875 g, 0.5 mmol) and DTT (0.154 g, 1 mmol) were added in single-necked round-bottomed flask, and then 3 mL of DMSO solvent (with impurities removed through a filter: 220 nm) containing 30μL of triethylamine was added. After 15 minutes stirring under the protection of N_2, the solution was stirred at 50 ^{o}C in the oil bath and 800 rpm for 3 days. After the reaction, the sample was dialyzed (MWCO: 2000 Da) for 48 h in deionized water and the dialysate was changed every 2-8 h. After the dialysis, and solution was frozen in a refrigerator at -20 °C and lyophilized for 2 days to obtain a white gelatinous MP1.

Preparation of acrylated MP1

MP1 (0.100 g) in DMF solvent (10 mL) was added into a 10 mL single-necked round-bottomed flask, the then triethylamine (633.9 μL, 4.55 mmol) was added. Then acryloyl

chloride (122.5 μL, 1.52 mmol) was slowly added drop-wisely at 0 ^{o}C and stirred overnight at room temperature. After the reaction, the sample was dialyzed (MWCO: 2000 Da) for 48 h in deionized water and the dialysate was changed every 2-8 h. After the dialysis, and solution was frozen in a refrigerator at -20 °C and lyophilized for 2 days to obtain yellow-brown gelatinous acrylated MP1.

Preparation of polymer-peptide conjugates P1

ICG-SH was first synthesized via the stirring of ICG-NHS and cysteamine in pH 8.0 buffer solution. Acrylated MP1 (0.01 g), ICG-SH (0.0015 g, 0.0017 mmol) (KLAK (0.0217 g, 0.0101 mmol) and TAT (0.0134 g, 0.0067 mmol) in DMSO solvent (5 mL) were added into a single-necked round-bottomed flask and then were stirred under the protection of N_2 for 15 minutes. The solution was stirred at 50 ^{o}C in the oil bath and 800 rpm for 2 days. After the reaction, the sample was dialyzed (MWCO: 2000 Da) for 48 h in deionized water and the dialysate (PBS, pH 7.4) was changed every 2-8 h. After the dialysis, and solution was frozen in a refrigerator at -20 °C and lyophilized for 2 days to obtain yellow solid P1. P2 and P3 were synthesized with similar protocols.

Characterization of PPCs.

The chemical structures of PPCs in d_6-DMSO were proved by ^{1}H NMR measurements on a Bruker ARX 400 MHz spectrometer. The molecular weights and dispersity index (PDI) of copolymers were determined by gel permeation chromatography (GPC) equipment (Shimadzu LC-20A). The measurements were performed at 40 ^{o}C in DMF at a flow rate of 1.0 mL min^{-1}. A family of narrow dispersed polystyrenes was used as the standards for calibration.

Turbidimetric measurements of main chain polymers.

The main chain polymers MP1, MP2 and MP3 were dissolved in a phosphate buffer saline (PBS, pH 7.4) and were gradually diluted to different concentrations (2.0 mg/mL, 1.0

mg/mL, 0.5 mg/mL, 0.2 mg/mL and 0.1 mg/mL). The resulting solutions were characterized by UV-vis spectrophotometer equipped with a thermoelectric temperature controller.

Particle size and morphology of PPCs.

The sizes of P1, P2 and P3 were measured on dynamic light scattering (DLS) analyzer (Zetasizer Nano ZS). 1.0 mL of P1 solutions was added to a quartz cell with different concentrations (0.4 mg/mL, 0.3 mg/mL, 0.2 mg/mL, and 0.1 mg/mL). The DLS were used to test the particle size at a reflection angle of 173^{o} and the change trends of particle sizes at 37-43 ^{o}C. Particle sizes of P2 and P3 with different concentrations were tested with the same method at different temperatures.

The morphology of PPCs were examined using a transmission electron microscopy (TEM, Tecnai G2 20 S-TWIN) operating on an accelerating voltage of 200 KeV. The TEM samples were prepared by contacting the droplets with copper grids for 5 min at 37 ^{o}C and 41 ^{o}C, and then the excess droplets were removed, followed by staining with uranyl acetate for 5 min.

For atomic force microscopy (AFM) characterization, P1 (0.2mg/mL) was deposited onto freshly cleaved mica and then incubated at 37 ^{o}C and 41 ^{o}C for 5 min. After carefully rinsed with water, the samples were air dried before measurements. AFM measurements were performed with Dimension 3100 AFM (Bruker, MA, US) in tapping mode in air.

Fluorescence Spectroscopy

Polar properties of temperature-responsive polymers were measured using DBD-ED as a fluorescence probe. 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) (20 mg, 0.1043 mmol) and 4-dimethylaminopyridine (DMAP) (5 mg, 0. 0409 mmol) were added to the P1 solution in DMF solvent. The mixed solution was stirred at 0 ^{o}C for 10 min, and 0.5 mL DCM containing 1 mg DBD was slowly added. The mixture was kept in dark overnight at room temperature, and then it was dialyzed against deionized water (MWCO: 2000 Da) for 24 h to obtain a bright-yellow solution. The fluorescence characteristic of P1-DBD in 0.2 mg/mL at

different temperatures was characterized by F-280 fluorescence spectrophotometer. The excitation wavelength was set to 406 nm and the emission wavelength range of the sample was set to 450-650 nm.

Multicellular Spheroid (MCS) Model.

The three-dimensional multicellular culture system has been extensively studied to imitate the growth pattern of tumor cells in complex microenvironments in vivo and is often used to study the permeability of tissues. Destroying the adherent conditions of SKBR-3 tumor cells in vitro can achieve three-dimensional growth of SKBR-3 cells. In this paper, MCS were constructed using agar-liquid overlay method. The 2% agarose gel solution was placed to coat 96-well plate to prevented the cells adhesion, and SKBR-3 cells (600 cells per well) suspended in RPMI-1640 media were seeded in the 96-well plate. The cells were cultured for one week to form SKBR-3 MCS model.

Penetration of PPCs into SKBR-3 MCS

The SKBR-3 MCS were incubated with P1-Cy5 or P2-Cy5 (KLAK concentration: 10 μM) in the 96-well plate. After 6 h, the MCS was shifted to a confocal microscope dish for confocal laser scanning microscopy (CLSM) observation using a Zeiss LSM710 CLSM with a 10× objective lens. Cy5 fluorescence signals were obtained in the Z-stack mode with a 10 μm interval between each slice from the top of the MCS to the center.

In vivo photoacoustic (PA) Imaging.

All animal experiments were performed complying with the NIH guidelines for the care and use of laboratory animals of National Center for Nanoscience and Technology. To built-up the SKBR-3 tumor model, female BALB/c mice (6-8 weeks, about 16 g) were subcutaneously inoculated in the right leg with 5×10^6 SKBR-3 cells. After tumor SKBR-3 cells were developed for 7 days, the tumor volume of the mice was approximately 200 mm^3. For in vivo PA imaging, SKBR-3 tumor-bearing mice were injected intravenously with 200 μL PPCs (2 mg/mL, pH 7.4, PBS) and detected by PA imaging (MOST 128, 800 nm) at 4 h.

Considering that ICG molecules are easy to be quenched after exposed to NIR light, the right hind leg bearing the tumor of P1/41 ^{o}C group is pre-heated to 41 ^{o}C for 30 min by soaking it into water bath instead of NIR irritation. P2 and PBS are also injected into mice as control groups.

In vivo penetration ability in tumor tissue.

To evaluate the penetration of PPCs in tumor tissue, PPCs (2 mg/mL, 200 μL) were injected through a tail vein of mice bearing SKBR-3 tumor. Tumors were first excised after 24 h injection and solidified in 4% paraformaldehyde for 4 h at 4 ℃. The vessels were stained with FITC-CD31 and the nuclei were labeled with DAPI. The stained tissues were ultrathin sectioned and scanned to observe the fluorescence distribution in tumor tissue. To trace the location of PPCs, the measurement of the distance between PPCs and tumor vessels thourgh scatter diagrams were charted based on the green (tumor vessels) and red (biomaterials) fluorescence images.

In vivo photothermal imaging.

P1 (2 mg/mL, 200 μL) was injected intravenously into the tail of mice bearing SKBR-3 tumor. The temperature of tumor was recorded by infrared thermograph Thermal Imager of FLIR E64501. After irradiation with 808 nm lasers for 5 s, the temperature of tumor was observed at 4 h, 8 h, 12 h, and 24 h after administration.

Fluorescence Imaging of PPCs in vivo

When tumors reached 200 mm^3, the peptide-polymers PPCs (2 mg/mL, 200 μL) were injected intravenously into the mice. Near-infrared imaging of mice was carried out after 4 h, 8 h, 12 h and 24 h injection using a CRI Maestro II *in vivo* spectrum imaging system (795-845 nm) to observe the distribution of PPCs. To further understand the accumulation of PPCs in each vital organ, we randomly selected one mouse from each group to dissect for 24 h after injection. The animals were sacrificed and tumor, heart, liver, spleen, lung and kidney were excised for *ex vivo* fluorescence imaging.

Cell culture

SKBR-3 cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% antibiotic solution (penicillin and streptomycin) at 37 °C in an atmosphere containing 5% CO_2.

Colocalization analysis in Cells

1 mL of SKBR-3 suspension with a cell density of 2×10^4 cells/mL was added in a confocal dish for incubation of 24 h. The P1-Cy5, P2-Cy5, and P3-Cy5 (KLAK 10 μM) were added to 1 mL of RPMI-1640 medium cultured SKBR-3 cells at 37 ^{o}C and 41 ^{o}C for 2 h, and then Lyso-Tracker Green DND-26 (500 nM) was added to incubate with cells for 30 minutes. Finally, the samples were observed under a 63× oil microscope in a confocal microscope (Zeiss LSM710).

Biological TEM observation

PPCs treated cells as above mentioned were harvested and fixed with 2.5% glutaraldehyde in PBS buffer for at least 6 h. After washing with PBS three times, the samples were fixed with 1% osmium containing PBS. Subsequently, samples were dehydrated with a graded series of ethanol solutions. Finally, the cells were sectioned, stained, and observed by HITACHI HT7700 TEM at an acceleration voltage of 80 kV.

Mitochondria-regulated apoptosis by JC-1 assay.

SKBR-3 cells were cultured as above mentioned. For the JC-1 assay, P1 at 37 °C and 41 °C were dispersed in RPMI-1640 media and added into cells grown in a confocal microscope dish. The cells were further incubated at 37 °C in a humidified atmosphere containing 5% CO_2 for 24 h. The mitochondria were stained with JC-1 in RPMI-1640 media for another 15 min. Images of the cells were observed under a 63×objective lens in a confocal microscope (Zeiss LSM710).

Cytotoxicity Assay

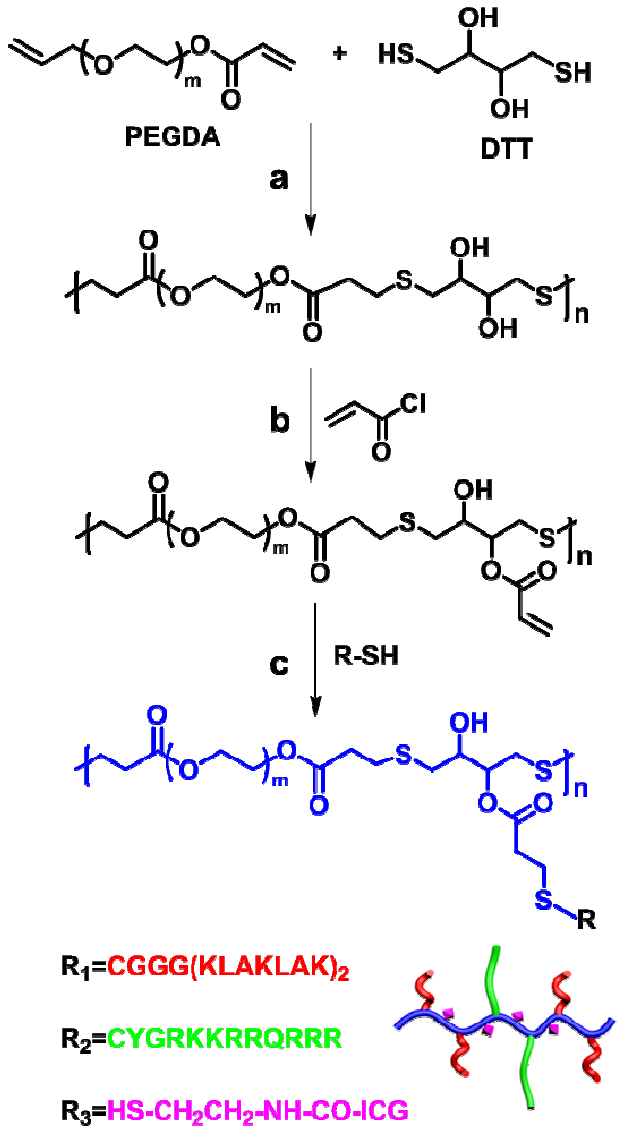
P1, P2, P3, P4 and KLAK with difference peptide concentrations at 37 °C and 41 °C were added to each well, and the SKBR-3 cells were incubated for an additional 24 h. Then 10 μL of CCK-8 solution was added to each well and the cells were cultured for another 2-4 h. The UV−vis absorptions were tested on a Tecan infinite M200 continuous spectrophotometer. All the experiments were performed in triplicate.

Antitumor ability in SKBR-3 MCS

The SKBR-3 MCS were treated with P1 (KLAK concentration: 60 μM) at 37 ^{o}C and 41 ^{o}C. At day1, day 5 and day 9, the MCS was shifted to a confocal microscope dish using a Zeiss LSM710 CLSM with a 10× objective lens to trace the MCS morphology change.

In vivo evaluation of PPCs for tumor therapy

After the SKBR-3 tumor sizes reached about 50 mm^3, the nude mice were randomly divided into six groups. The mice bearing SKBR-3 tumor were administered with P1+laser, P1, P1+laser, P2, P3+laser, P4+laser (2 mg/mL, 200 μL) and PBS by intravenous injection, and tumor volume changes and body weights were measured every other day. For groups with laser irritation, the mice were exposed to NIR 808 nm laser (2.0 W/cm^2) for 20 min per day. The temperature changes of tumor were recorded by Thermal Imager of FLIR E64501, which is controlled between 40-42 ^{o}C. The tumor volumes were measured by the caliper and calculated by the equation: $1/2\times(\text{length of tumor})\times(\text{width of tumor})^2$.



Scheme S1. Synthesis routes of PPCs. (a) Michael-type addition, Et_3N, DMSO, 50°C, 3 d. (b) Acryloyl chloride, DMF, 25°C, overnight. (c) $CGGG(KLAKLAK)_2$, CYGRKKRRQRRR and ICG-SH, Michael-type addition, Et_3N, DMSO, 50 °C, 2 d.

Table S1. Synthesis and Characterization of PPCs.

	PEGDA1	PEGDA2	Molar ratio	KLAK:TAT	M_n	PDI (M_w/M_n)
P1	M_n=700	M_n=575	0.5:0.5	3:2	-	-
P2	M_n=700	M_n=300	0.5:0.5	3:2	-	-
P3	M_n=700	M_n=575	0.75:0.25	3:2	-	-
P4	M_n=700	M_n=575	0.5:0.5	0:2	-	-
MP1	M_n=700	M_n=575	0.5:0.5	0	142200	1.65
MP2	M_n=700	M_n=300	0.5:0.5	0	131300	1.39
MP3	M_n=700	M_n=575	0.75:0.25	0	135900	1.37

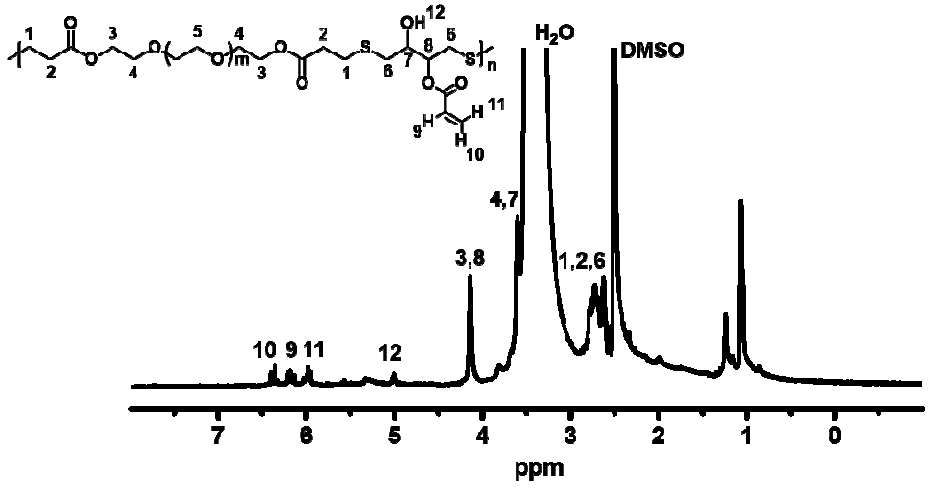


Figure S1. ^{1}H NMR spectrum of MP1 in d_6-DMSO. Polymer concentration: 10 mg/mL.

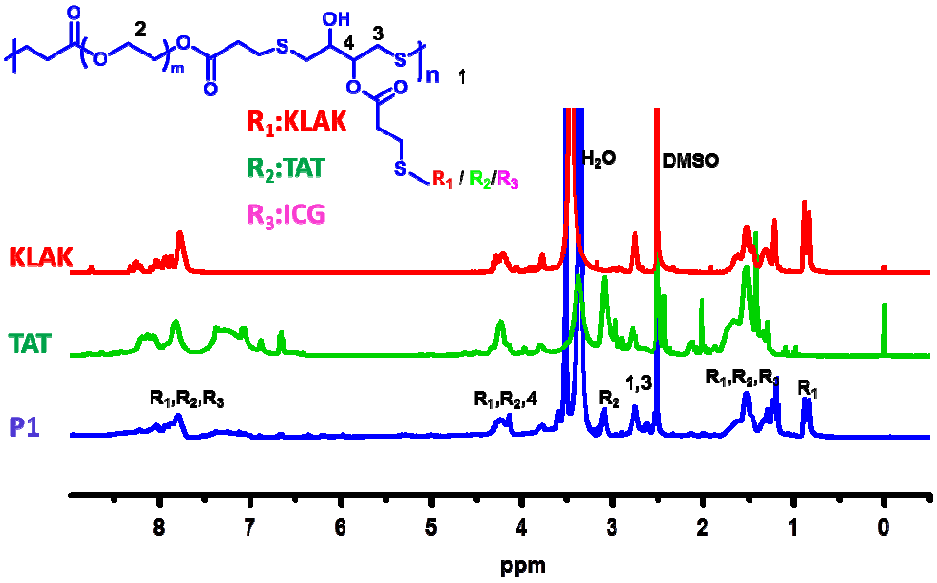


Figure S2. ^{1}H NMR spectrum of PPCs in d_6-DMSO. Polymer concentration: 10 mg/mL.

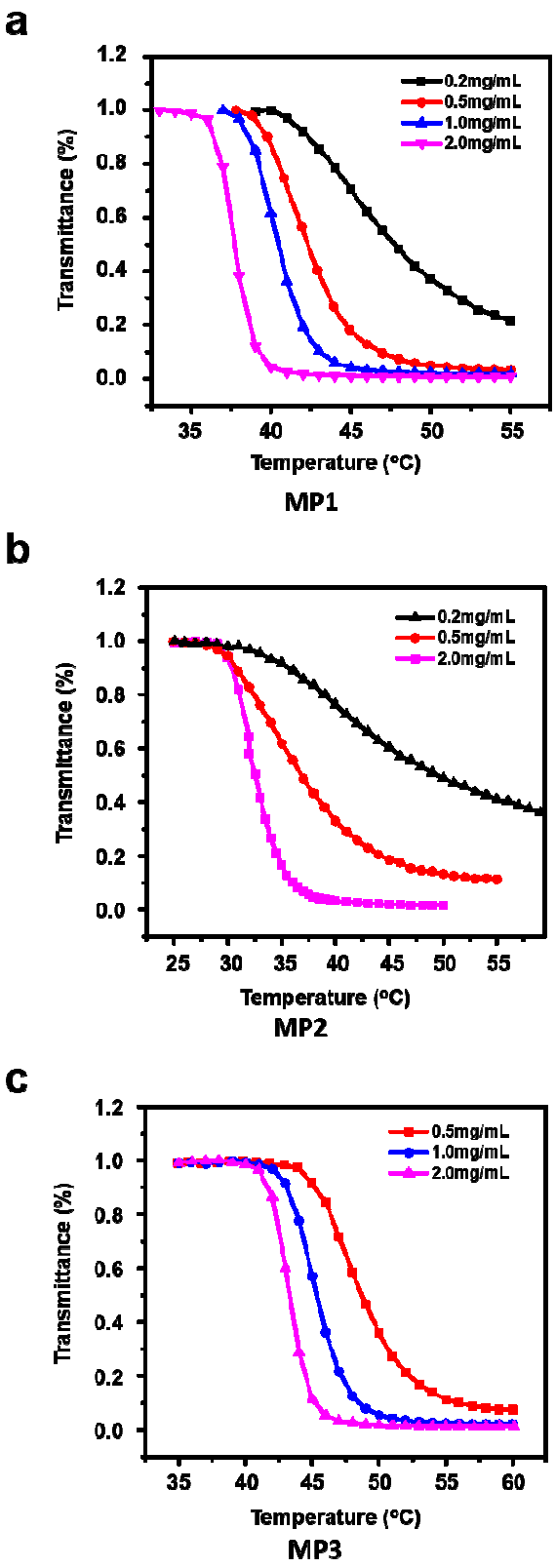


Figure S3. Turbidimetric measurements of main chain polymers a) MP1, b) MP2, and c) MP3 in PBS.

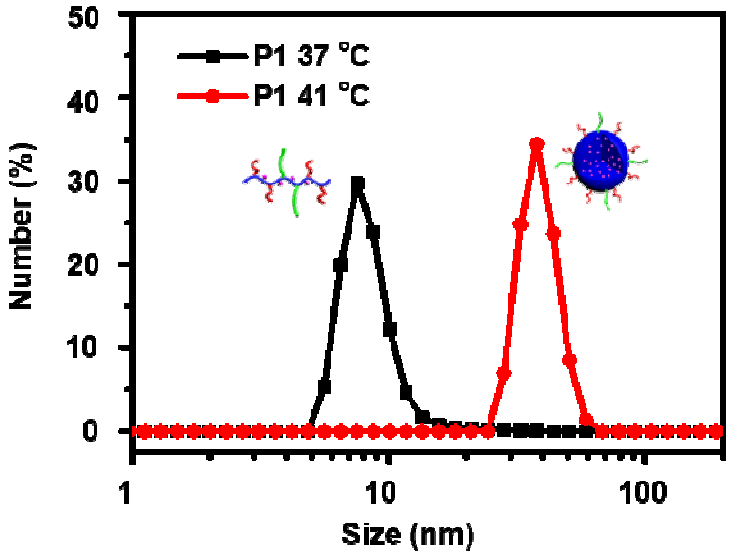


Figure S4. Number size distribution of P1 in PBS solutions (pH 7.4) at 37 °C and 41 °C measured by DLS. Copolymer concentration: 0.2 mg/mL.

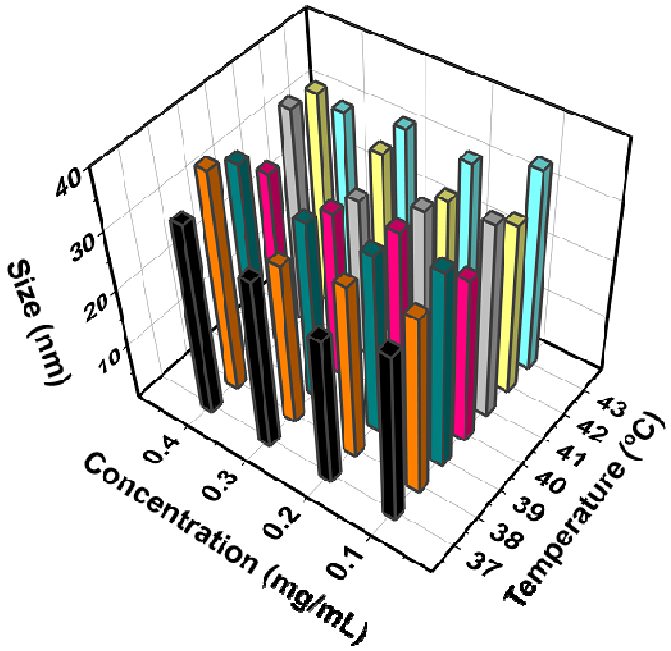


Figure S5. The particle size of P2 at different concentrations in PBS at 37 °C-43 °C measured by DLS.

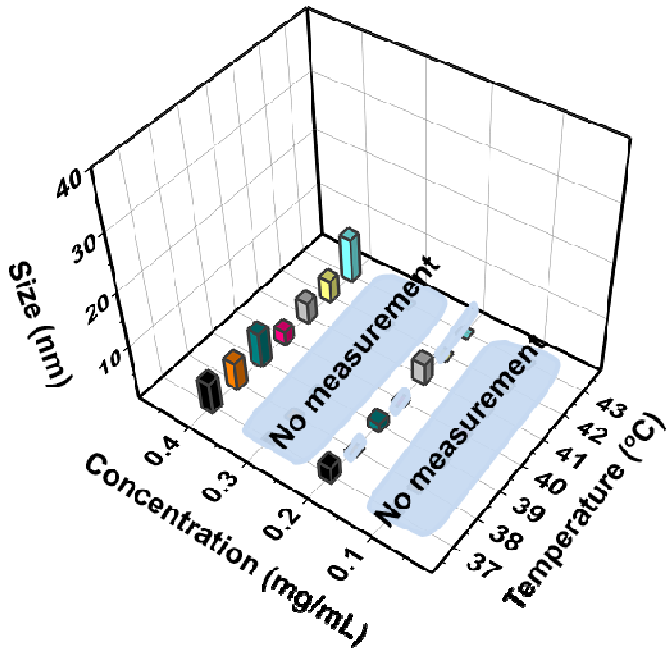


Figure S6. The particle size of P3 at different concentrations in PBS at 37 °C-43 °C measured by DLS.

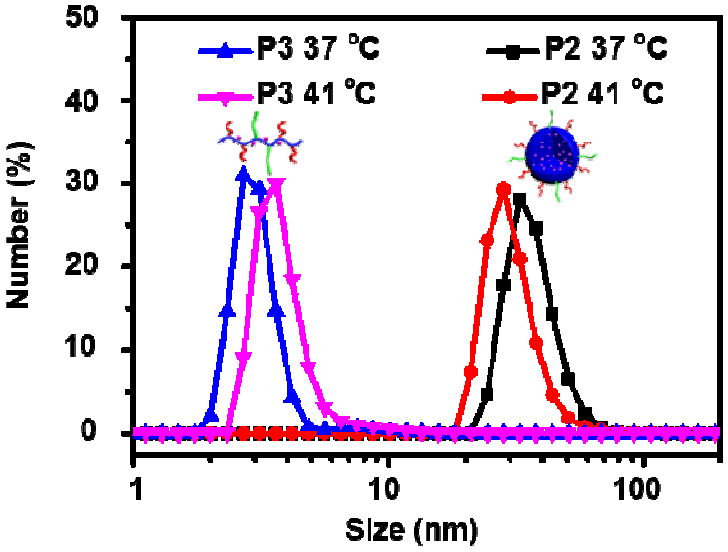


Figure S7. Number size distribution of P2 and P3 in PBS solutions (pH 7.4) at 37 °C and 41 °C measured by DLS. Copolymer concentration: 0.2 mg/mL.

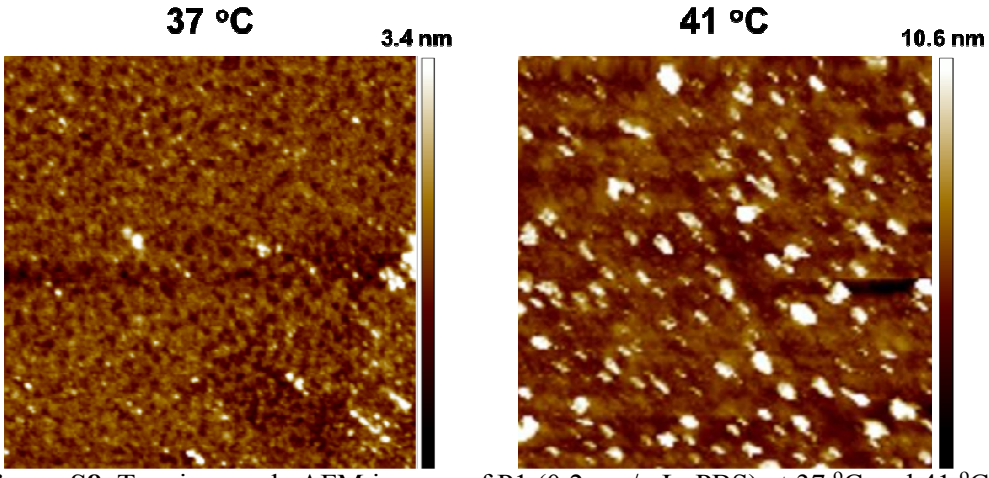


Figure S8. Tapping mode AFM images of P1 (0.2 mg/mL, PBS) at 37 °C and 41 °C.

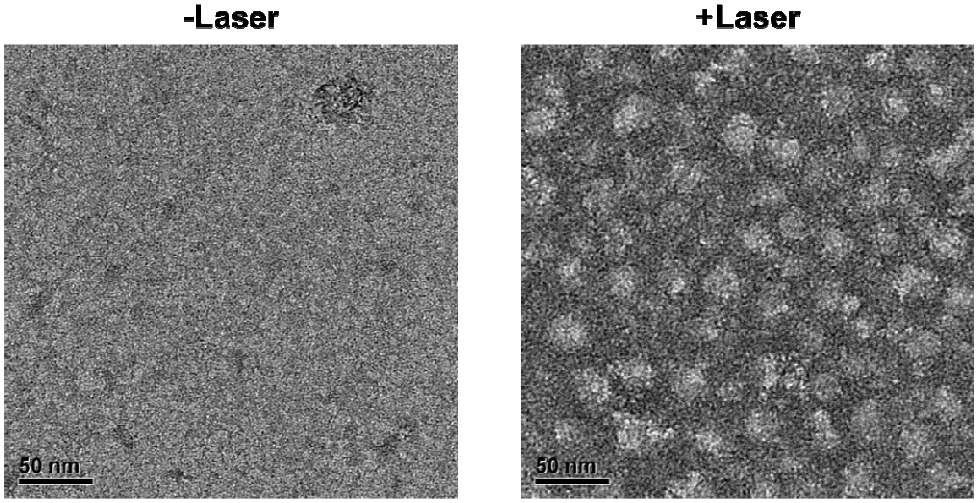


Figure S9. TEM images of the P1 (0.2 mg/mL, PBS) before and after the NIR laser irritation (808 nm, 2.0 W/cm^2, 5 min).

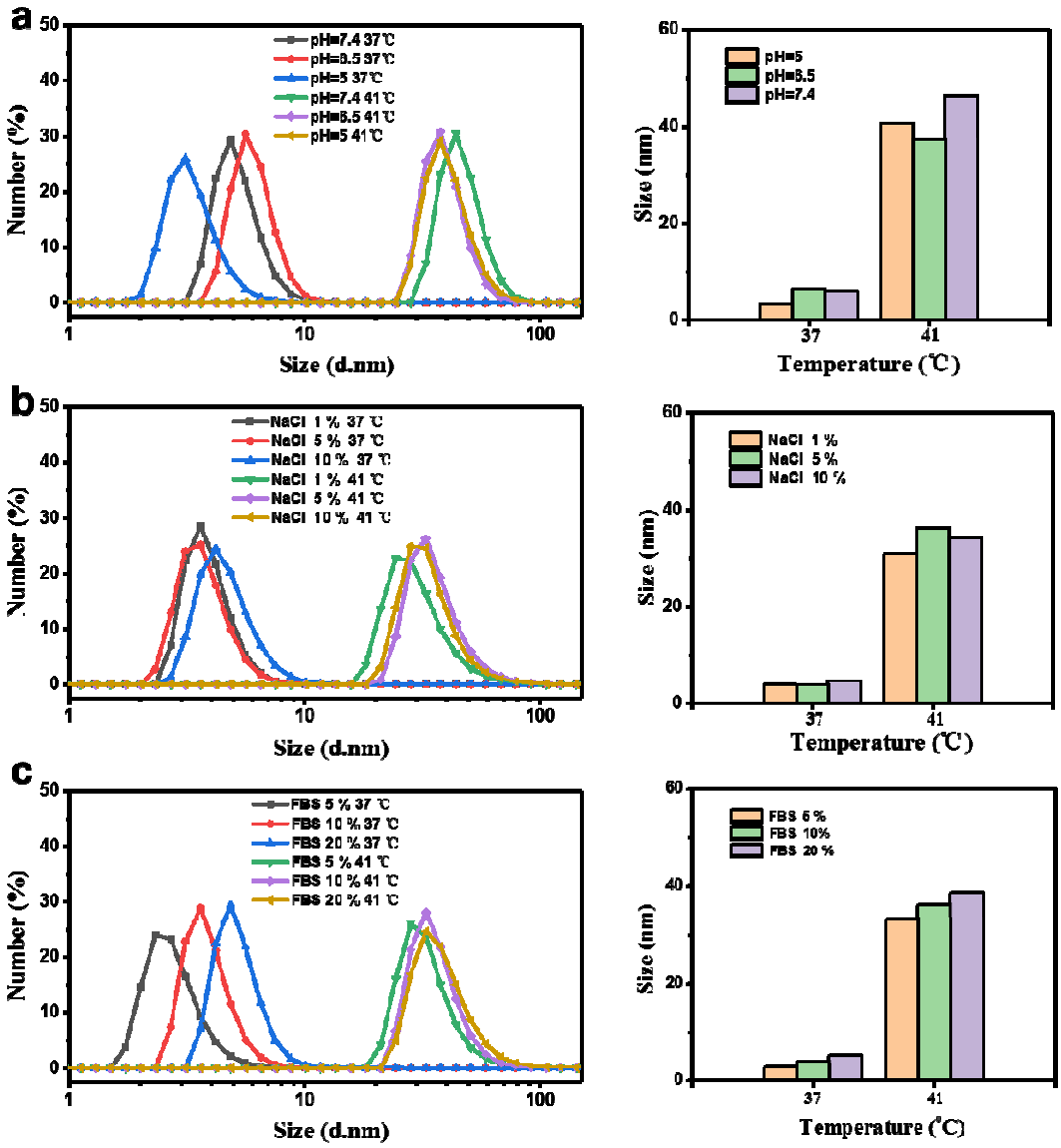


Figure S10. The particle size of P1 (0.2 mg/mL, PBS) in different physicochemical conditions at 37 °C and 41 °C measured by DLS. a) pH 5, pH 6.5 and pH 7.4; b) NaCl 1%, 5% and 10%; c) fetal bovine serum (FBS, 5%, 10% and 20%).

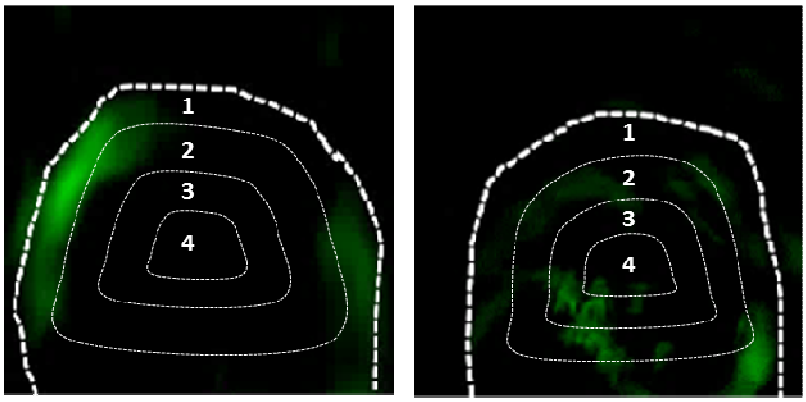


Figure S11. Method of calculation for penetration distance of P1 through average PA signal intensity from tumor edge to center. 1: 25%; 2: 50%; 3: 75%; 4: 100%.

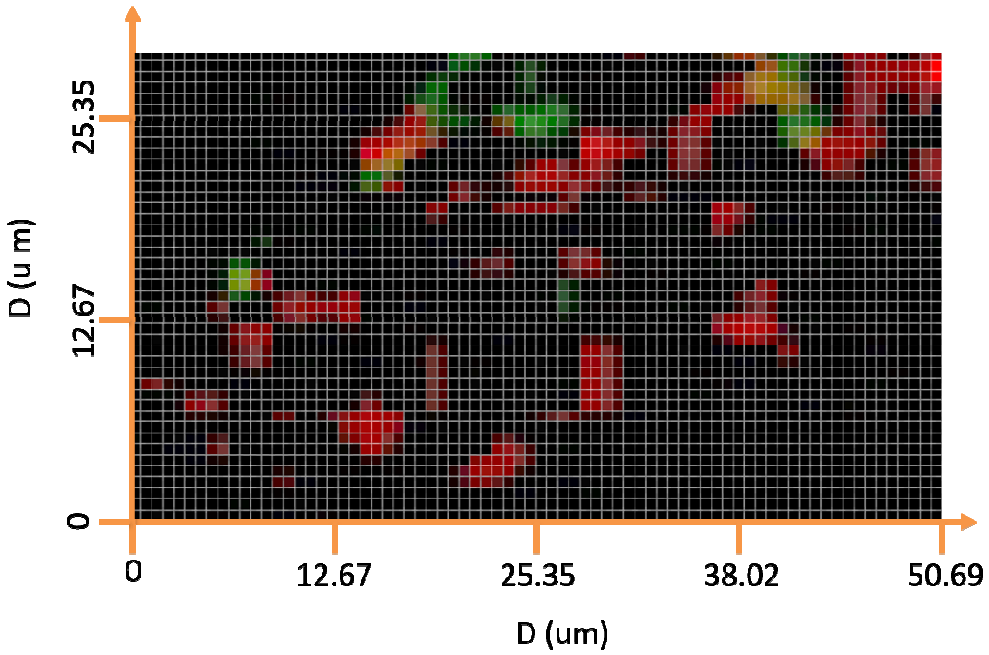


Figure S12. Method of pixel calculation for penetration distance of PPCs.

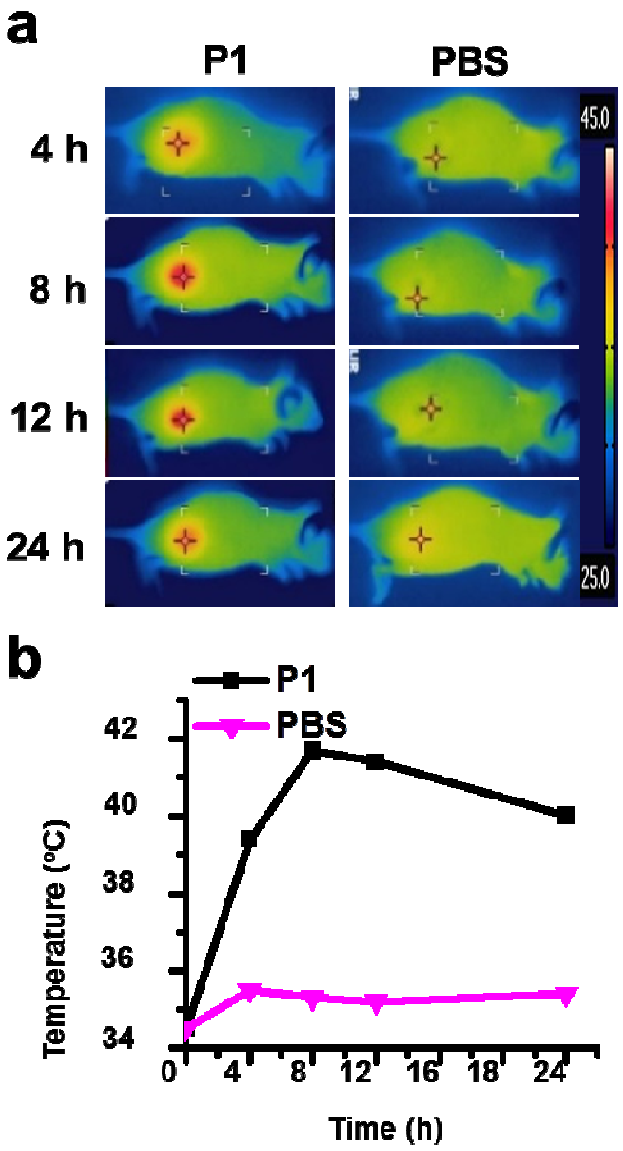


Figure S13. a) Thermal imaging and b) quantitative data of SKBR-3 tumor-bearing mice after intravenously injected by P1 and PBS for 4 h, 8 h, 12 h and 24 h.

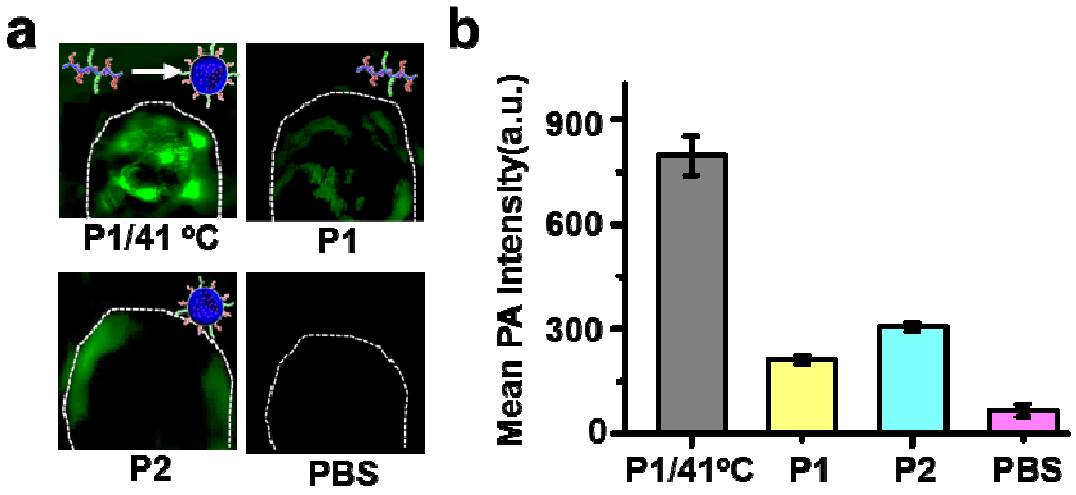


Figure S14. a) In vivo PA imaging of tumors after injection of PPCs via tail vein. b) Quantification of PA intensity in tumor. Results are presented as the mean ±SD in triplicate.

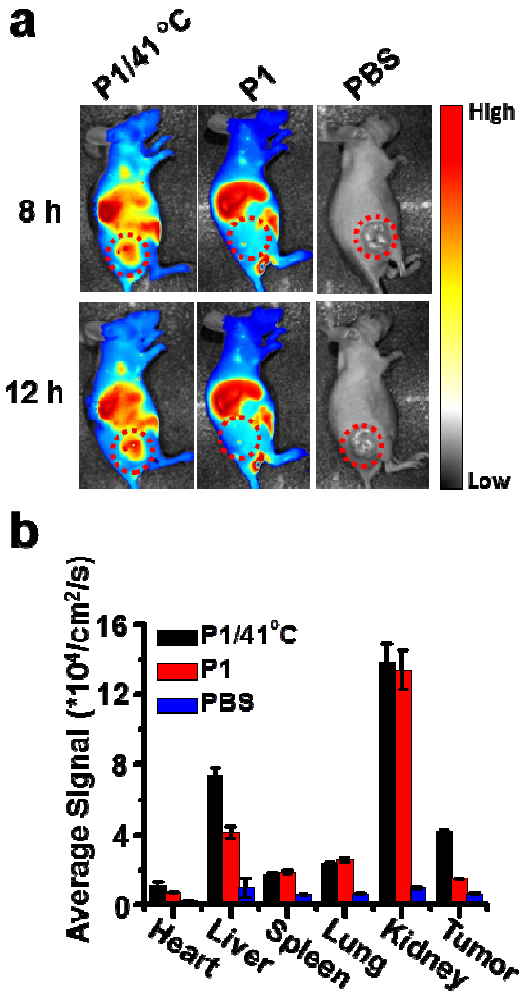


Figure S15. a) Fluorescence imaging of SKBR-3 tumor-bearing mice after intravenous injection with P1 at different local tumor temperatures. b) Quantitative results of fluorescence imaging of the ex vivo biodistribution of P1, P1/41 °C, P2 and PBS in major organs (heart, liver, spleen, lung, kidney and tumor). Results are presented as the mean ±SD in triplicate.

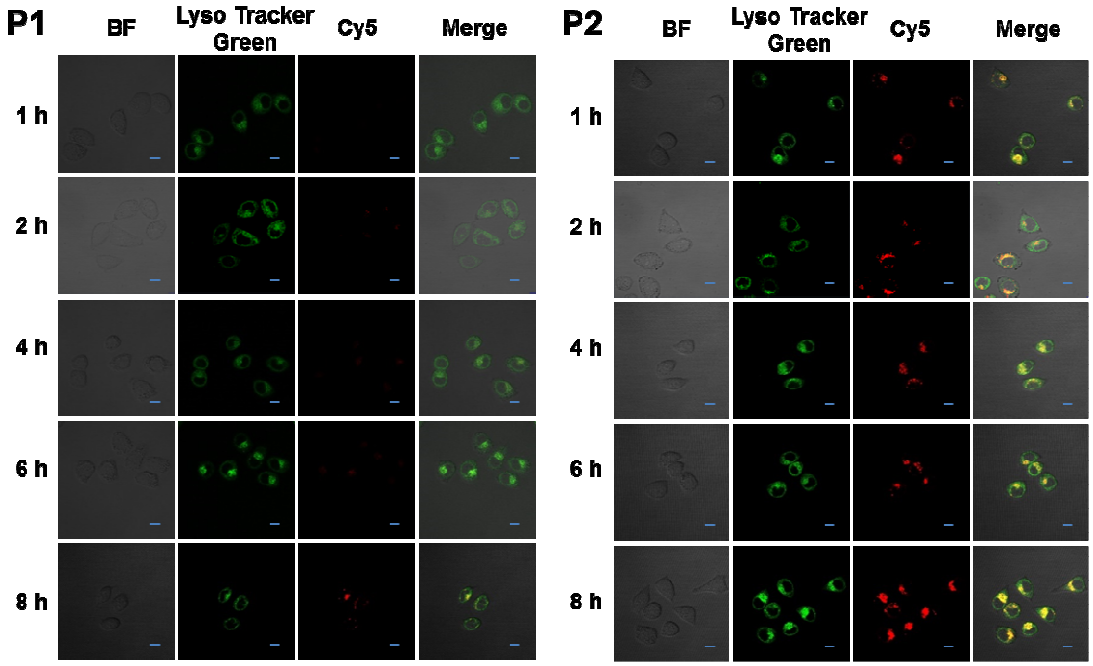


Figure S16. CLSM microscopy of living SKBR-3 cells that were incubated with Cy5 labeled P1 and P2 for different times (KLAK concentration: 10 μM). Lysosomes were labeled with Lyso Tracker Green DND-26 for 30 min before imaging. Scale bar: 10 μm.

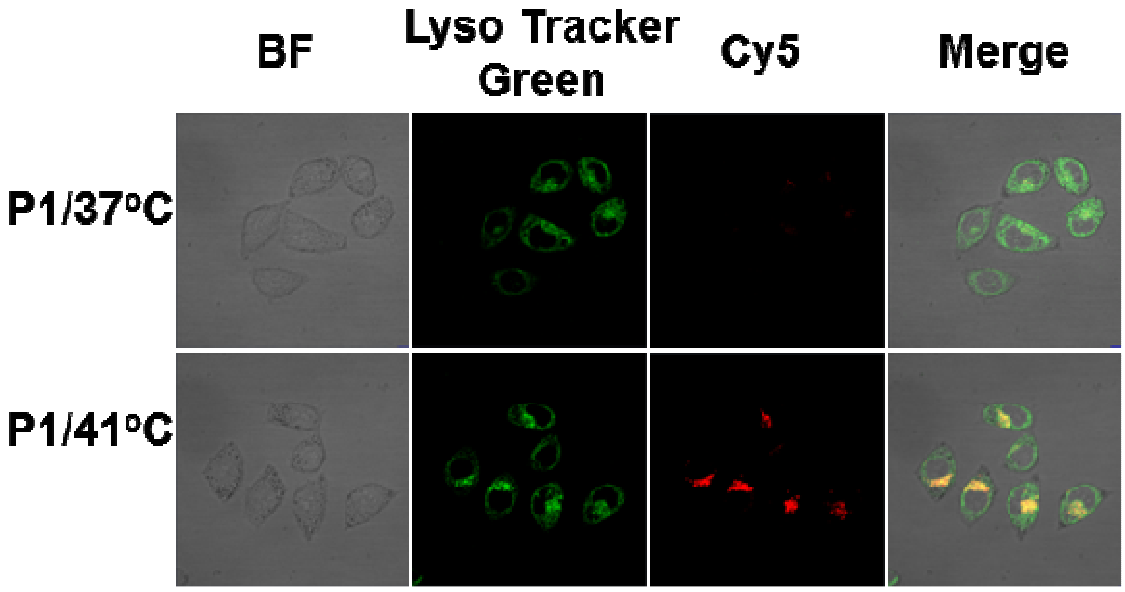


Figure S17. The enlarged images of Figure 3e.

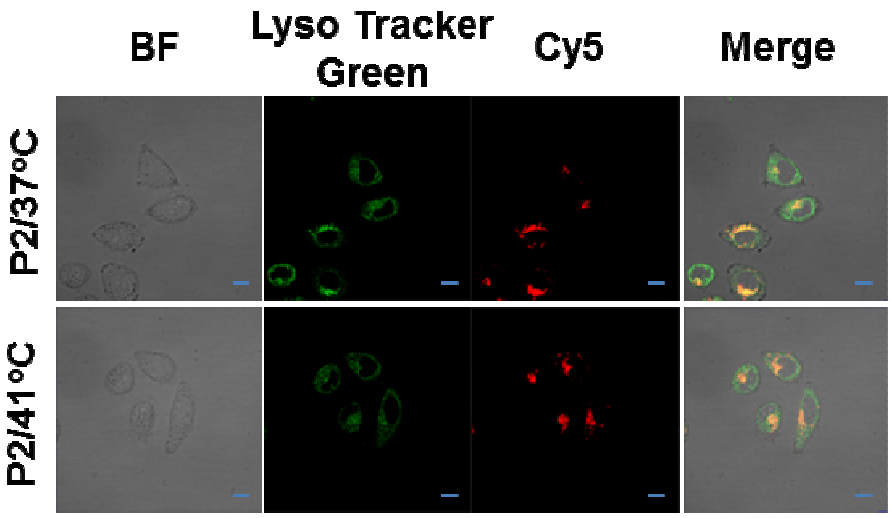


Figure S18. CLSM microscopy of living SKBR-3 cells that were incubated with P2-Cy5 for 2 h (KLAK concentration: 10 μM). Lysosomes were labeled with Lyso Tracker Green DND-26 for 30 min before imaging. Scale bar: 10 μm.

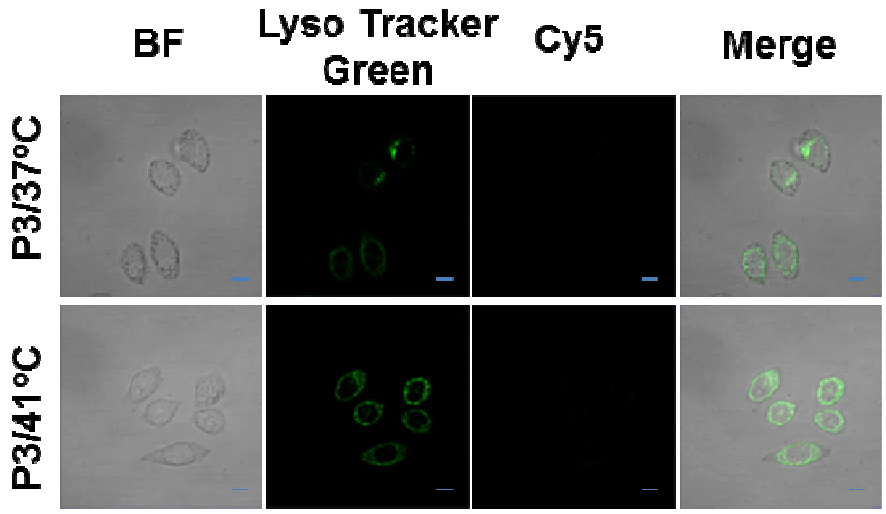


Figure S19. CLSM microscopy of living SKBR-3 cells that were incubated with P3-Cy5 for 2 h (KLAK concentration: 10 μM). Lysosomes were labeled with Lyso Tracker Green DND-26 for 30 min before imaging. Scale bar: 10 μm.

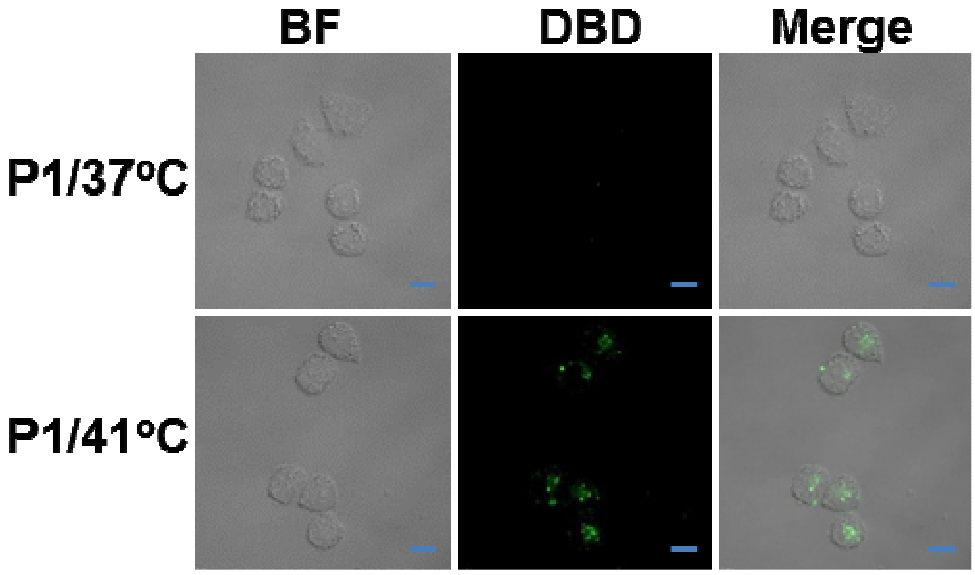


Figure S20. The enlarged images of Figure 3e.

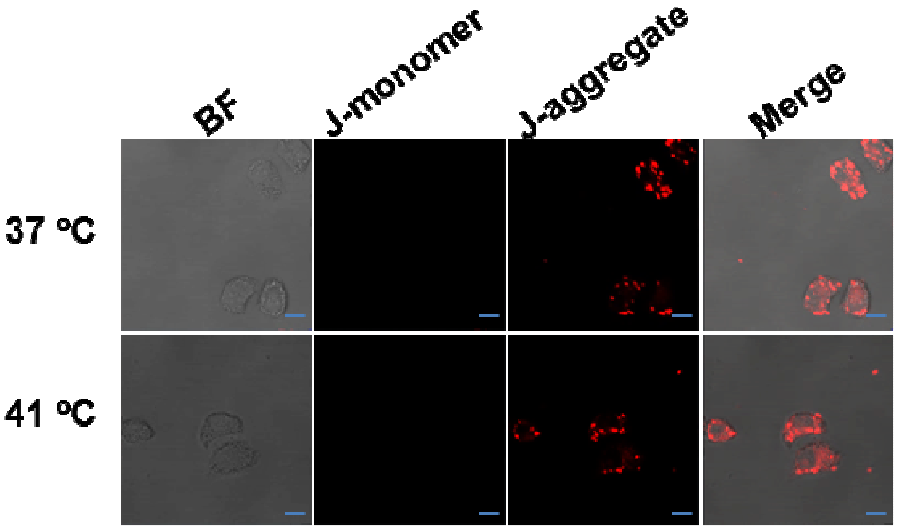


Figure S21. Mitochondrial membrane potentials (JC-1 assay) of SKBR-3 cells incubated with PBS at 37 ^{o}C and 41 ^{o}C. Scale bar: 10 μm.

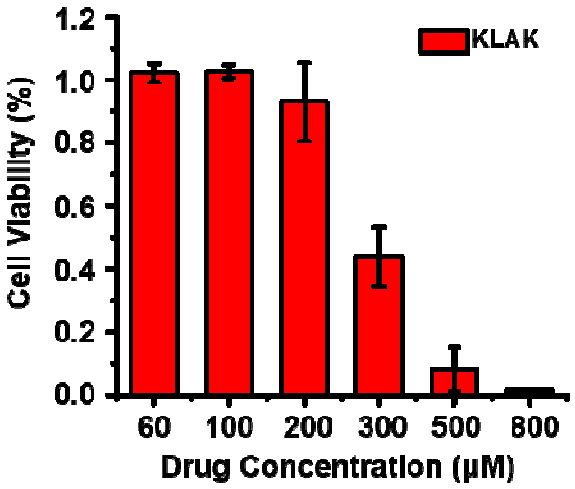


Figure S22. Viability of SKBR-3 cells incubated with KLAK at 37 °C measured by the CCK-8 assay. Results are presented as the mean ±SD in triplicate.

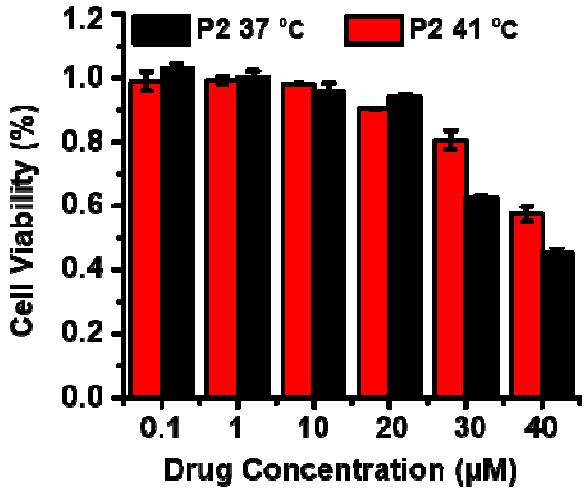


Figure S23. Viability of SKBR-3 cells incubated with P2 at 37 °C and 41 °C measured by the CCK-8 assay. Results are presented as the mean ±SD in triplicate.

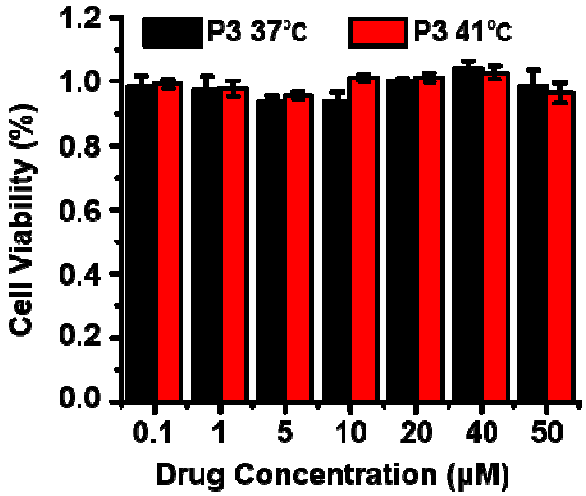


Figure S24. Viability of SKBR-3 cells incubated with P3 at 37 ^{o}C and 41 ^{o}C measured by the CCK-8 assay. Results are presented as the mean ±SD in triplicate.

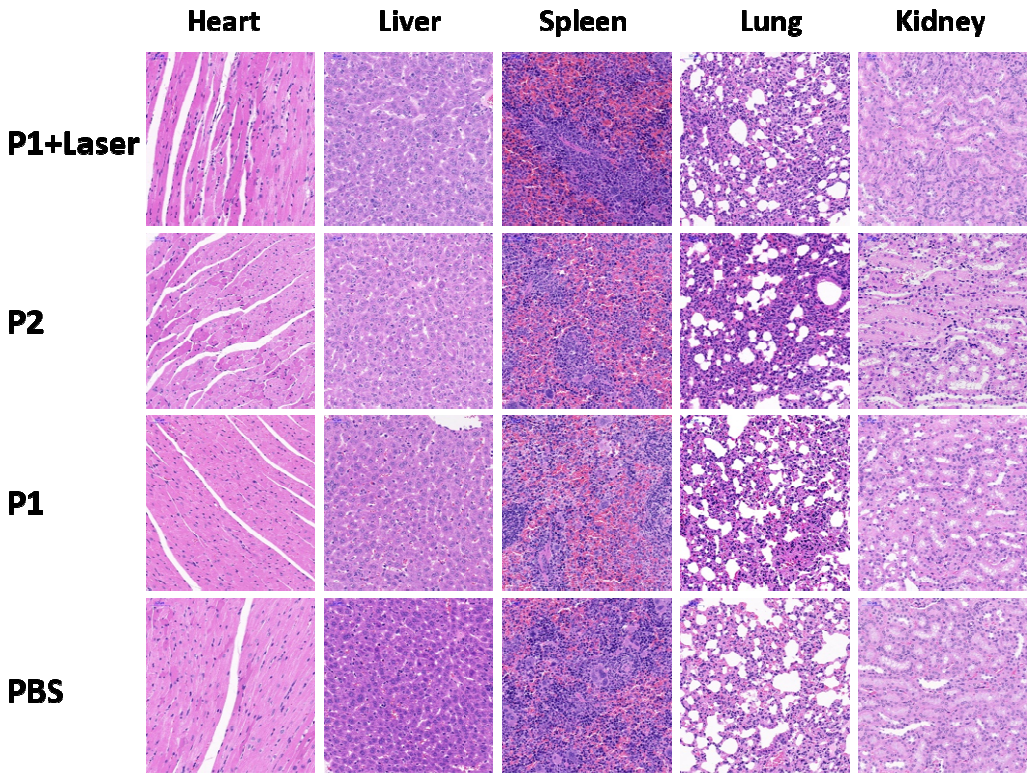


Figure S25. Representative photomicrographs of the heart, liver, spleen, lung and kidney sections (H&E staining) of tumor-bearing mice.