Electronic Supplementary Material (ESI) for Chemical Science.
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Functional Black Phosphorus Nanosheets for Mitochondria-Targeting Photothermal/Photodynamic Synergistic Cancer Therapy

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Experimental Section

1. Materials

Black phosphorus (BP) was prepared according to literature.[1] N-methyl-2-pyrrolidone (NMP), dopamine hydrochloride, (5-carboxypentyl)triphenylphosphonium bromide (TPP), and 4-Morpholineethanesulfonic acid (MES) were purchased from Adamas. Chlorin e6 (Ce6) was purchased from Frontier Scientific. Tris(hydroxymethyl)aminomethane (Tris) was purchased from Sinopharm. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (EDC•HCl), 9,10-Anthracenediyl-bis(methylene)dimalonic acid (ABDA), and N-hydroxysuccinimide (NHS) were purchased from Sigma-Aldrich. SOSG was purchased from Thermo Fisher Scientific.

2. Instruments

XRD measurements were measured on a SmartLab 3kW diffractometer with Cu Kα radiation. Raman measurements were performed on a WITEC Alpha300M+ Raman microspectrometer with 633 nm excitation. The absorption spectra were recorded on a Shimadzu UV-3600 UV-Vis-NIR spectrophotometer. The fluorescence spectra were acquired on a HITACHI F-4600 fluorescent spectrophotometer. The size of nanosheets was measured by a NanoPlus-3 DLS instrument. The morphology characterizations of BP nanosheets were carried out on a transmission electron microscope (JEM-1400PLUS) and an atomic force microscope (Dimension ICON). The photothermal temperature was recorded with an E50 infrared camera (FLIR, Arlington, VA).

3. Preparation of black phosphorus

A mixture of red phosphorus (300 mg), Sn (10 mg) and SnI_4 (5 mg) was sealed into an ampoule tube. The temperature of the ampoule was firstly raised to 873 K within one hour and kept for one hour, then cooled down to 823 K with a rate of 1 K/min. After that, the ampoule tube was cooled down to 773 K with a slower rate of 0.1 K/min, followed by naturally cooling to room temperature. Finally, the black phosphorus crystal was washed by hot toluene and alcohol thoroughly, then dried and conserved in N_2 gas for further use.

4. Preparation of BP nanosheets

Bulk BP dispersed in NMP was tip sonicated in ice bath for 10 h (period of 1 s, 1 s interval, 250 W), and then was sonicated in 0 ˚C for 10 h. The resulted brown suspension was firstly centrifuged at 7000 rpm for 20 min, then 12000 rpm for 20 min. The precipitate was rinsed with water and dried to obtain the BP NSs.

5. Synthesis of polydopamine-modified BP NSs (BP@PDA NSs)

For the synthesis of polydopamine-modified BP NSs, 6 mg of dopamine hydrochloride in Tris solution (2 mg/mL, 7.2 mL, pH = 8.5) was added dropwise to a BP NSs solution (1 mg, 0.5 mL) under vigorous stirring for 1.5 h. Then the resulting BP@PDA NSs were collected by centrifugation (12,000 rpm, 20 min), and rinsed with water thrice. The final BP@PDA NSs were resuspended in water.

6. Preparation of BP@PDA-Ce6&TPP NSs

Ce6 (4 mg) was dissolved in a mixture of THF and DMSO, and activated by EDC/NHS for 3 h.[2] On the other hand, TPP (3 mg) was dissolved in MES[3] and activated by EDC/NHS for 3 h. Then the above solutions were mixed together and then added by BP@PDA NSs solution (1.5 mL, 600 μg/mL) under stirring for 24 h. The crude products were collected by

centrifugation (12000 rpm, 10 min), and washed thrice with PBS and water to obtain BP@PDA-Ce6&TPP NSs.

BP@PDA-Ce6 NSs were obtained via a similar method in the absence of TPP.

7. Photothermal properties of BP based nanomaterials

BP@PDA NSs or BP NSs (1.0 mL) with the same absorption at 660 nm were added into a 1.5 mL centrifugation tube fixed on a retort stand. The temperature was monitored by thermal infrared imaging camera under the laser irradiation (660 nm, 1 W/cm^2). The photothermal conversion efficiency (η) was measured through the following formula:[4]

$$\eta = \frac{hS\Delta T_{max} - Q_S}{I(1 - 10^{-A_{660}})}$$

where h refers to the heat transfer coefficient, S means the container surface area, ΔT_{max} is the difference between the equilibrium and the ambient temperature, Q_S is the heat dissipated from light absorbed by the centrifuge tube containing PBS (calculated to be 6.3 mW), I means the laser power density, and A_{660} refers to the absorbance at 660 nm. $hS = mc/\tau$, where m refers to the solution mass, c means the specific heat capacity of the solution and τ is the ratio of time to -lnθ in the cooling process.

BP@PDA NSs, BP@PDA-Ce6 NSs, BP@PDA-Ce6&TPP NSs or Ce6, with the same BP@PDA or Ce6 concentration, was added into 1.5 mL centrifugation tubes, respectively. The temperature was monitored by thermal infrared imaging camera under Laser irradiation (660 nm, 1 W/cm^2).

8. Photodynamic properties of BP@PDA based nanoplatform

ABDA (DMF, 50 μL, 10^{-4} mol/L) was added into BP@PDA NSs, BP@PDA-Ce6 NSs, BP@PDA-Ce6&TPP NSs, or Ce6 with the same BP@PDA or Ce6 concentration (PBS) in

dark, respectively. The samples were irradiated by laser (660 nm, 0.10 W/cm^2), then measured by UV-vis at different time points.

SOSG (CH_3OH, 50 μL, 60 μg/mL) was added into BP@PDA NSs, BP@PDA-Ce6&TPP NSs, or Ce6 with same concentration of BP@PDA or Ce6, respectively. The samples were irradiated by laser (660 nm, 0.01 W/cm^2), measured by fluorescent spectrophotometer at different time.

9. Cell culture

Hela cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) media with 1% double-antibody and 10% fetal bovine serum (Gibco) at 37 °C in 5% CO_2.

10. Confocal Fluorescence Imaging

HeLa cells were cultured in a confocal dish (1 × 10^6 cells/well), containing 2 mL culture medium with BP@PDA NSs, BP@PDA-Ce6 NSs and BP@PDA-Ce6&TPP NSs (BP@PDA concentration of 10 μg/mL), at 37 °C for 12 h. Then, the cells were rinsed with PBS, fixed by 4% paraformaldehyde for 25 min and stained with DAPI for 3 min. The cellular uptake was examined by two-photon laser confocal microscope (LSM880NLO).

In Co-localization experiments, HeLa cells cultured with BP@PDA-Ce6 NSs or BP@PDA-Ce6&TPP NSs were rinsed with PBS, then incubated with Mito-Tracker Green (5.0 μM) for 30 min. The cells were imaged by two-photon laser confocal microscope (LSM880NLO).

In cellular ROS study, HeLa cells were cultured in a confocal dish (1 × 10^6 cells/well), containing 2 mL culture medium with BP@PDA NSs, BP@PDA-Ce6 NSs and BP@PDA-Ce6&TPP NSs (BP@PDA concentration of 10 μg/mL), at 37 °C for 12 h. Then, the cells

were rinsed with PBS, cultured with DCFH-DA[5] (1 mL, V/V = 1:1000) for 20 min. Subsequently, the cells were fixed by 4% paraformaldehyde for 25min and stained with DAPI for 3 min, followed by PBS washing for three times. Finally, after irradiated by laser (660 nm, 0.5 W/cm^2, 5 min), the cells were imaged under two-photon laser confocal microscope (LSM880NLO).

11. Cytotoxicity assay

The cytotoxicity was determined by MTT assay. Cells seeded into the 96-well plates (1 × 10^4 cells/well) were cultured for 24 h. Next, the medium was replaced with medium (200 μL) containing BP@PDA NSs, BP@PDA-Ce6 NSs and BP@PDA-Ce6&TPP NSs (BP@PDA concentration of 1, 2.5, 5, 10, 15 and 25 μg/mL). After 24 h incubation, the cells of phototherapy group were irradiated by laser (660 nm, 0.5 W/cm^2, 5 min) and cultured at 37 °C for another 10 h. For MTT assay, the cells were added by MTT solution (20 μL) and cultured for another 4 h. Then the supernatants were carefully sucked up, and DMSO (150 μL) was added. The absorbance at 492 nm (OD_{492nm}) was measured on microplate reader (Bio-Tek, USA). For the live/dead assay, the cells were cultured with calcein AM and PI for 30 min, next, captured by inverted fluorescence microscope (Nikon ECLIPSE Ts2R).

12. Flow Cytometry Study

Cells seeded into the 6-well plates were cultured for 24 h. Next, the medium was replaced with medium (3 mL) containing BP@PDA NSs, BP@PDA-Ce6 NSs and BP@PDA-Ce6&TPP NSs (BP@PDA concentration of 10 μg/mL), at 37 °C for 24 h. After irradiated by laser (660 nm, 0.5 W/cm^2, 5 min), the cells were incubated for another 8 h, then

collected by centrifugation and resuspended in binding buffer containing propidium iodide (PI, 5 μL) and annexin-V FITC (5 μL) for 15 min in darkness.[6] The signal was collected by a BD FACSCalibur flow cytometer (PerkinElmer).

13. *In vivo* fluorescence imaging

The nude mice bearing Hela tumor was intravenously injected with BP@PDA-Ce6&TPP NSs (200 μL, 50 μg/mL) and examined by a fluorescence imaging system (FluorVivo 200, INDEC BioSystems) for 24 h. Then the tumor, as well as main organs were collected and imaged immediately.

14. *In vivo* antitumor efficiency and biosafety of BP@PDA-Ce6&TPP NSs

Female nude mice (5 weeks old) were purchased from Comparative medicine center of Yangzhou University and used under regulation approved by the School of Pharmaceutical Sciences, Nanjing Tech University (Nanjing, China).

The tumor model was generated via subcutaneous injection of saline containing 1×10^6 Hela cells (100 μL) on the side of each mouse. The tumor volume was measured by the equation:

$$\text{Tumor volume } (mm^3) = 1/2 \times \text{length} \times \text{width}^2.$$

After 10 days, the tumors grew to about 150 mm^3, the mice were divided for different treatments (receiving injection and irradiation) every other day (n = 4): (1) saline, (2) BP@PDA NSs, (3) BP@PDA-Ce6&TPP NSs, (4) BP@PDA NSs with 660 nm irradiation, (5) BP@PDA-Ce6&TPP NSs with 660 nm irradiation. For group 1, the mice were obtained an intravenous injection of saline (200 μL). For group 2 and 4, the mice were given an intravenous injection of BP@PDA NSs (200 μL, 0.56 mg/kg). For group 3 and 5, the mice were intravenously injected by BP@PDA-Ce6&TPP NSs (200 μL, at an equivalent

BP@PDA dose of 0.56 mg/kg). For group 4 and 5, after 12 h injection, the mice were irradiated by laser (660 nm, 0.5 W/cm^2, 10 min). The tumor temperature was obtained *via* infrared camera during the irradiation. The main tissues were excised for histological section.

References

1. M. Köpf, N. Eckstein, D. Pfister, C. Grotz, I. Krüger, M. Greiwe, T. Hansen, H. Kohlmann and T. Nilges, *J. Cryst. Growth*, 2014, **405**, 6-10.

2. D. Zhang, M. Wu, Y. Zeng, L. Wu, Q. Wang, X. Han, X. Liu and J. Liu, *ACS Appl. Mater. Interfaces*, 2015, **7**, 8176-8187.

3. D. Chen, J. Zhang, Y. Tang, X. Huang, J. Shao, W. Si, J. Ji, Q. Zhang, W. Huang and X. Dong, *J. Mater. Chem. B*, 2018, **6**, 4522-4530.

4. Q. Tian, F. Jiang, R. Zou, Q. Liu, Z. Chen, M. Zhu, S. Yang, J. L. Wang, J. H. Wang and J. Hu, *ACS nano*, 2011, **5**, 9761-9771.

5. Y. Cai, P. Liang, Q. Tang, X. Yang, W. Si, W. Huang, Q. Zhang and X. Dong, *ACS nano*, 2017, **11**, 1054-1063.

6. G. Liu, J. Zou, Q. Tang, X. Yang, Y. Zhang, Q. Zhang, W. Huang, P. Chen, J. Shao and X. Dong, *ACS Appl. Mater. Interfaces*, 2017, **9**, 40077-40086.

Supplementary Figures

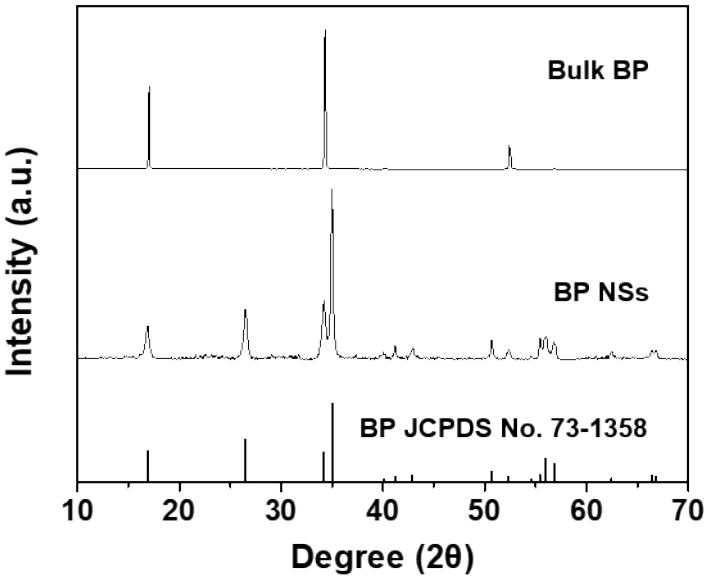

Figure S1. XRD patterns of bulk BP and BP NSs.

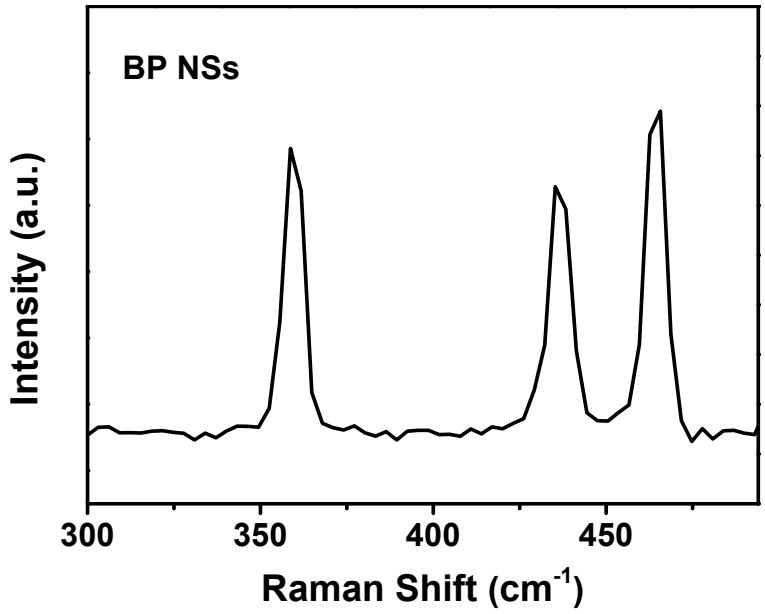

Figure S2. Raman spectrum of BP NSs.

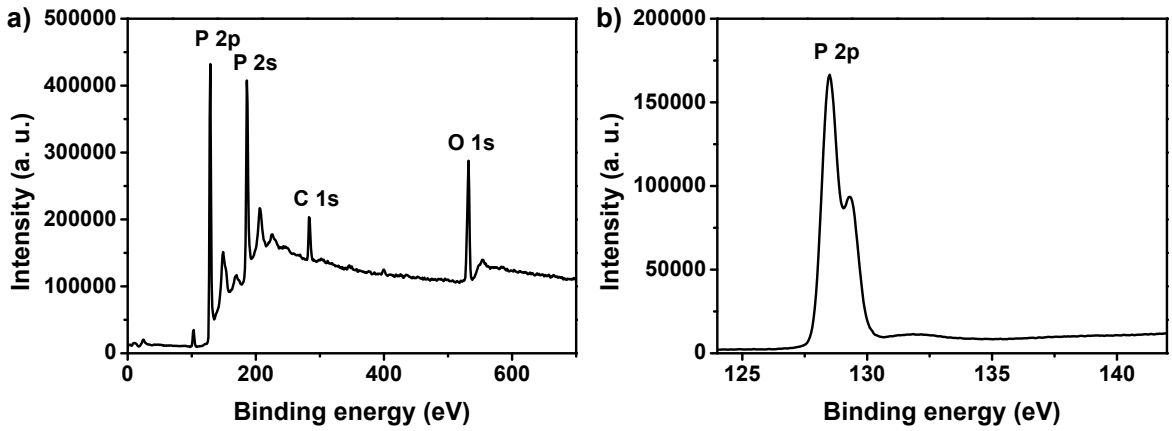

Figure S3. XPS spectra of BP NSs. (a) Survey spectrum. (b) P2p spectrum.

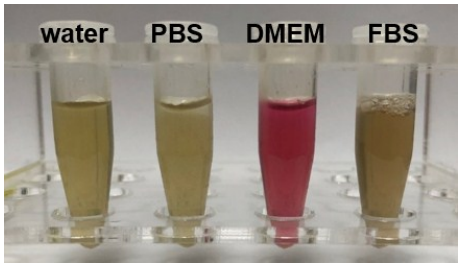

Figure S4. Photograph of BP@PDA-Ce6&TPP in H_2O, PBS, DMEM, and FBS, respectively, after 4 h.

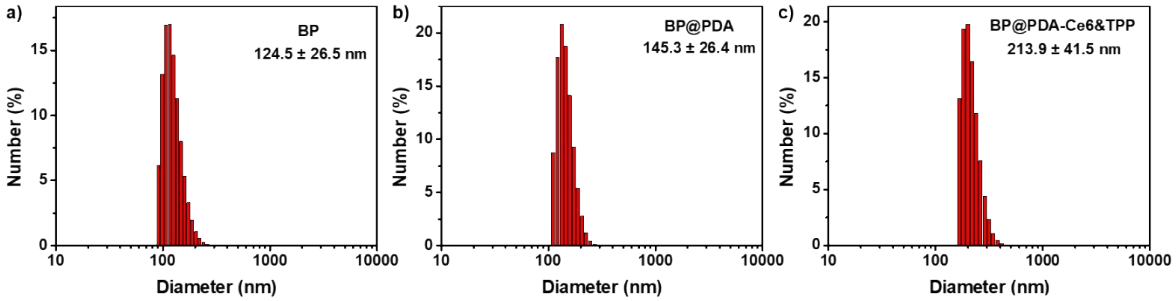

Figure S5. DLS size distributions of (a) BP NSs, (b) BP@PDA NSs, and (c) BP@PDA-Ce6&TPP NSs.

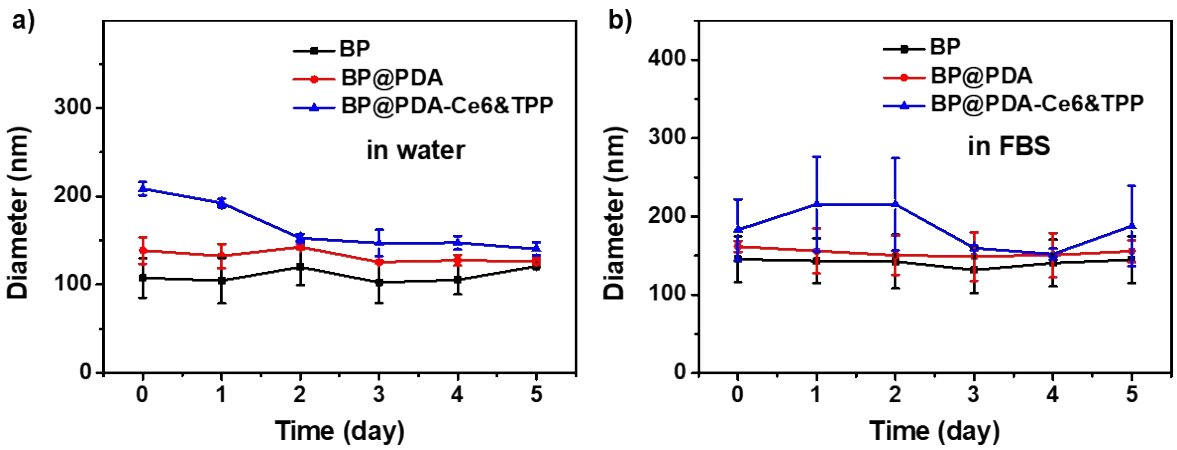

Figure S6. The DLS sizes of BP-based nanosystems in water and FBS, respectively, for 5 days.

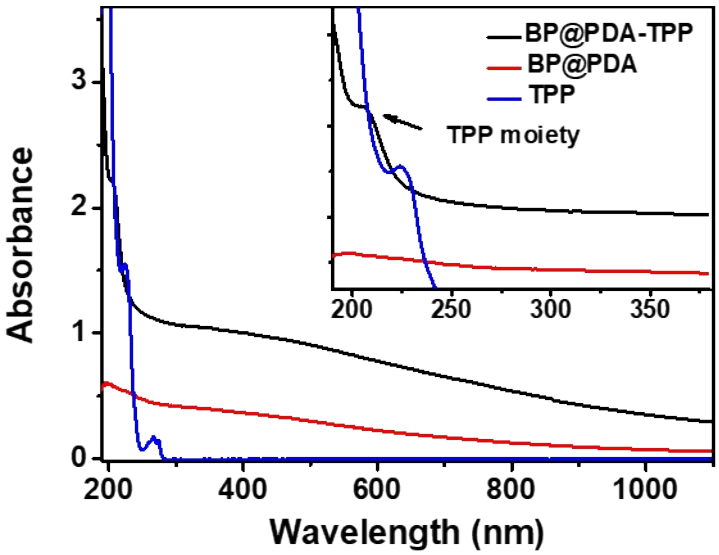

Figure S7. UV-vis-NIR spectra of BP@PDA-based nanomaterials without Ce6.

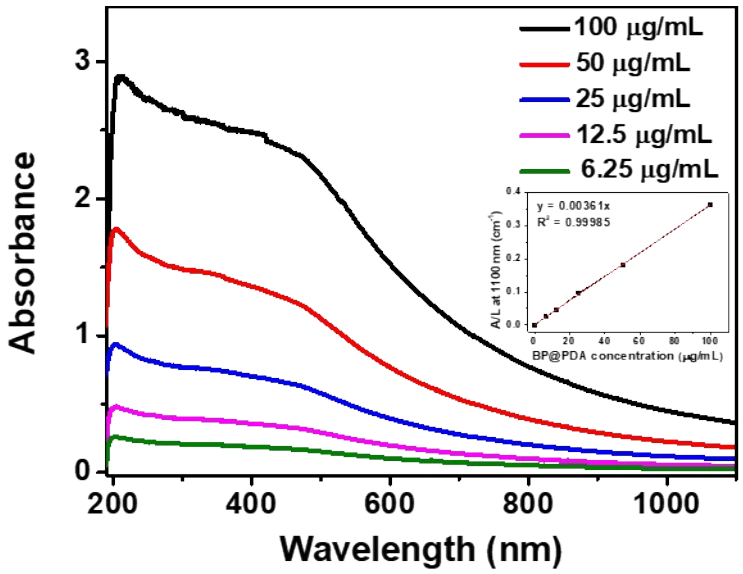

Figure S8. UV-vis-NIR absorption spectra of BP@PDA NSs at different concentrations.

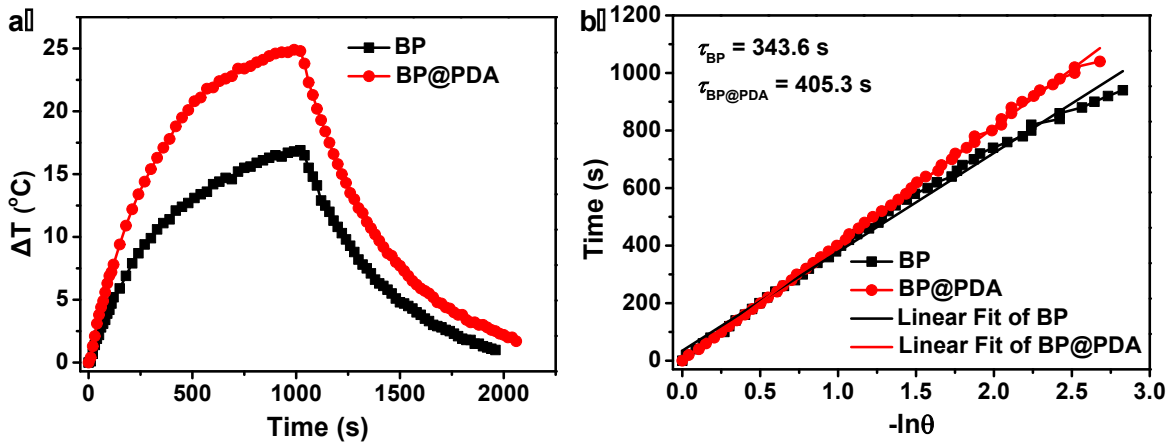

Figure S9. (a) Photothermal profiles of BP NSs before and after PDA coating with the same absorbance at 660 nm under laser irradiation (1.0 W/cm^2, 10 min). (b) The relationship between time and -lnθ obtained from the cooling process of BP NSs and BP@PDA NSs with the same absorbance at 660 nm.

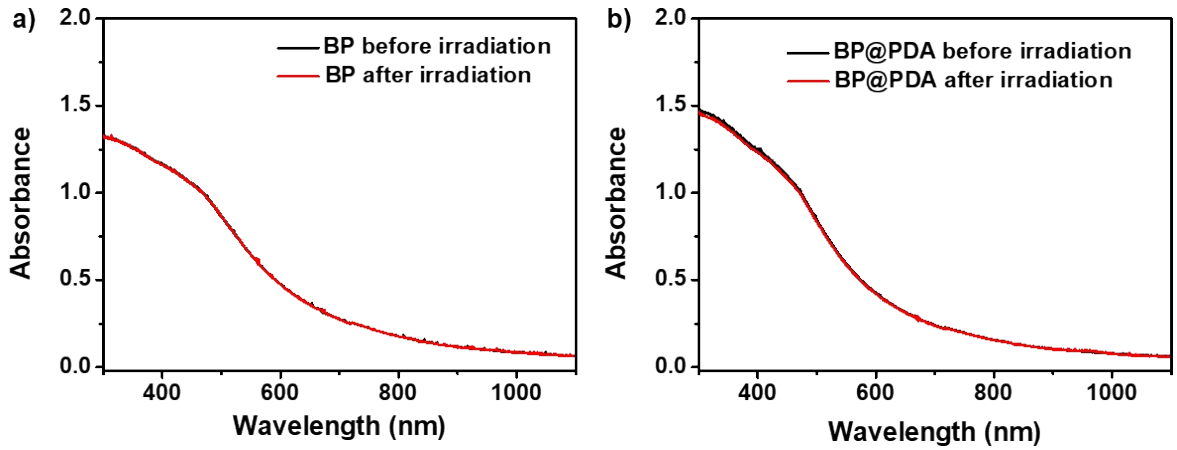

Figure S10. The UV-vis spectra of (a) BP and (b) BP@PDA before and after irradiation (660 nm, 0.5 W/cm^2, 10 min).

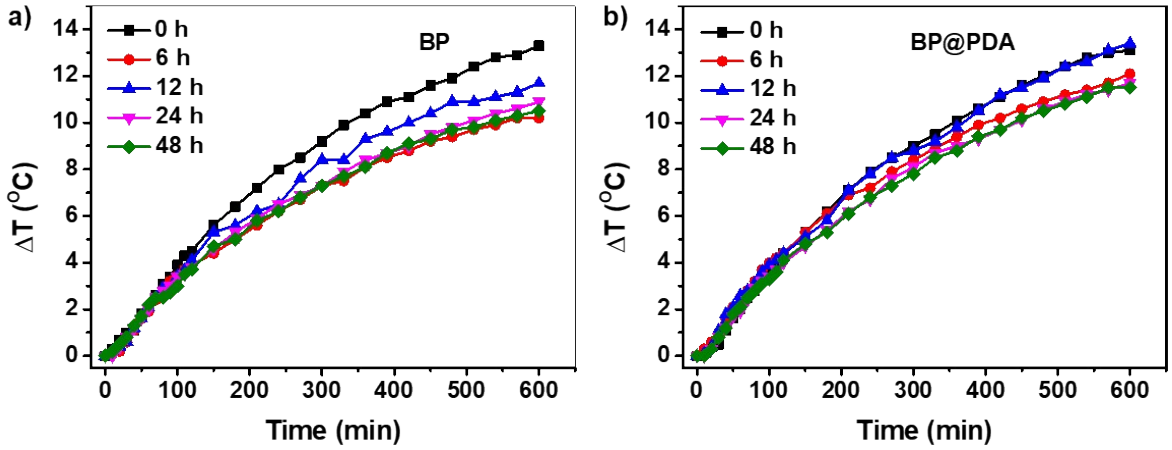

Figure S11. The photothermal properties of BP and BP@PDA dispersion at different time (660 nm, 0.5 W/cm^2, 10 min).

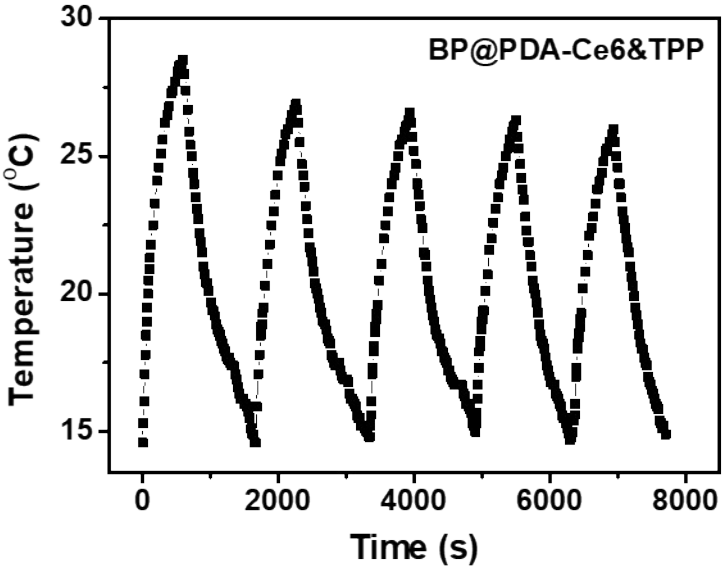

Figure S12. Photothermal stability of BP@PDA-Ce6&TPP NSs in five heating/cooling cycles (660 nm, 0.65 W/cm^2).

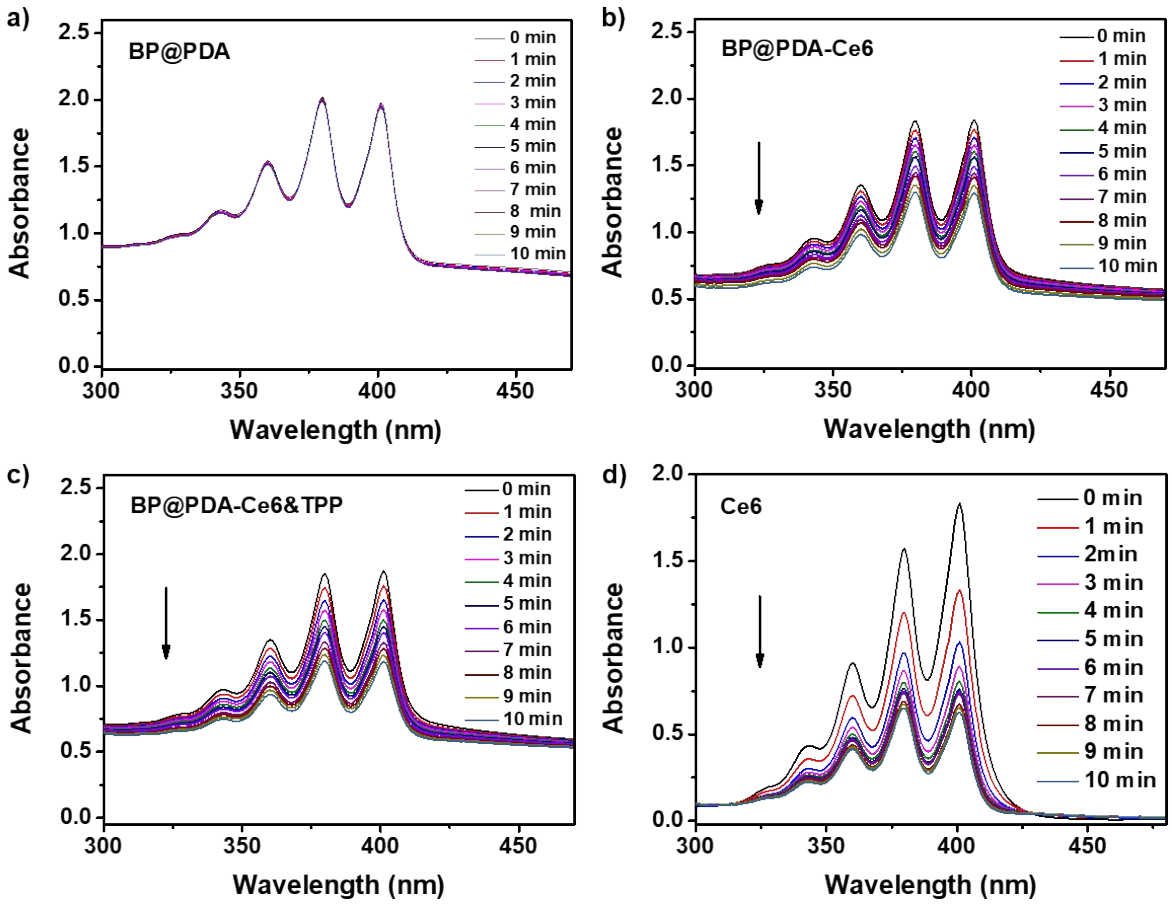

Figure S13. Detection of singlet oxygen generation using ABDA as the probe. Time-dependent absorption spectra of ABDA in (a) BP@PDA NSs, (b) BP@PDA-Ce6 NSs, (c) BP@PDA-Ce6&TPP NSs and (d) Ce6 solutions under 660 nm laser irradiation (0.10 W/cm^2).

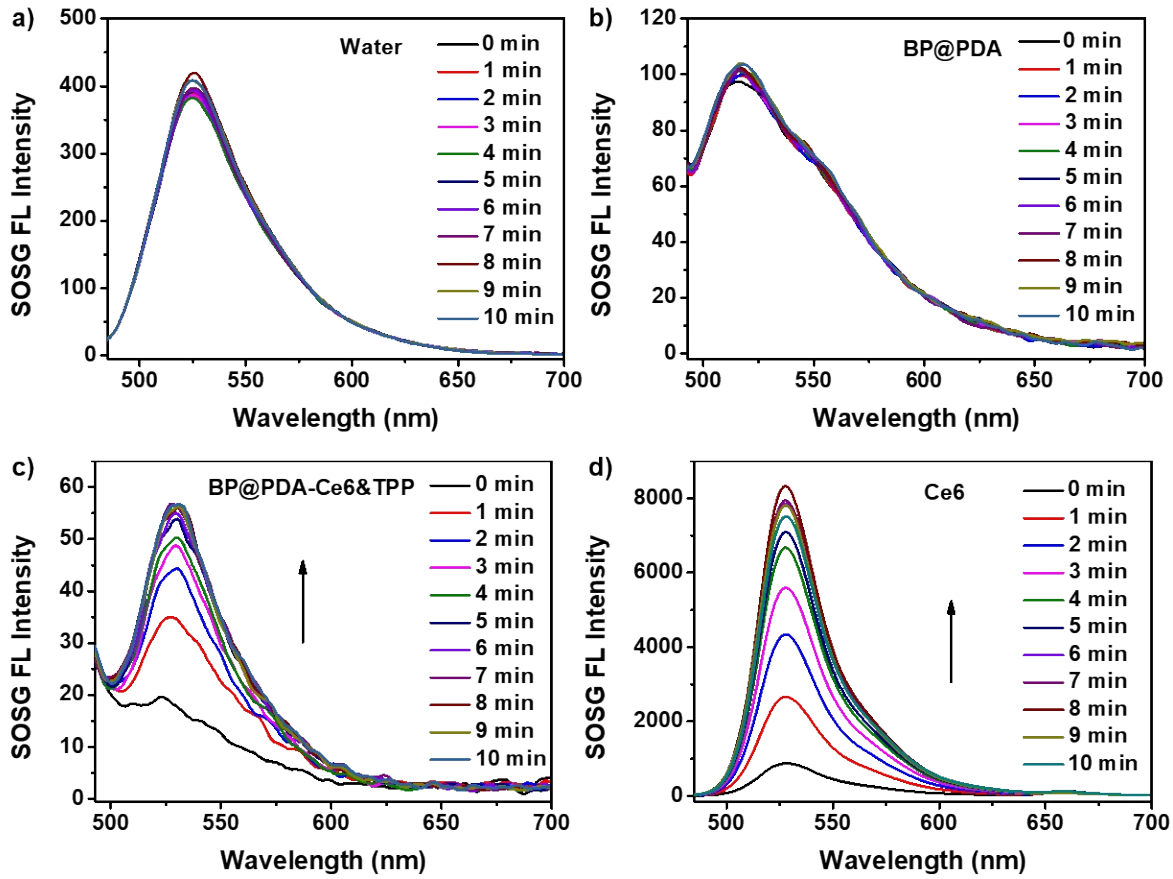

Figure S14. The detection of $^1O2$ by SOSG fluorescence after 660 nm laser irradiation (0.01 W/cm^2) in (a) water, (b) BP@PDA, (c) BP@PDA-Ce6&TPP and (d) Ce6.

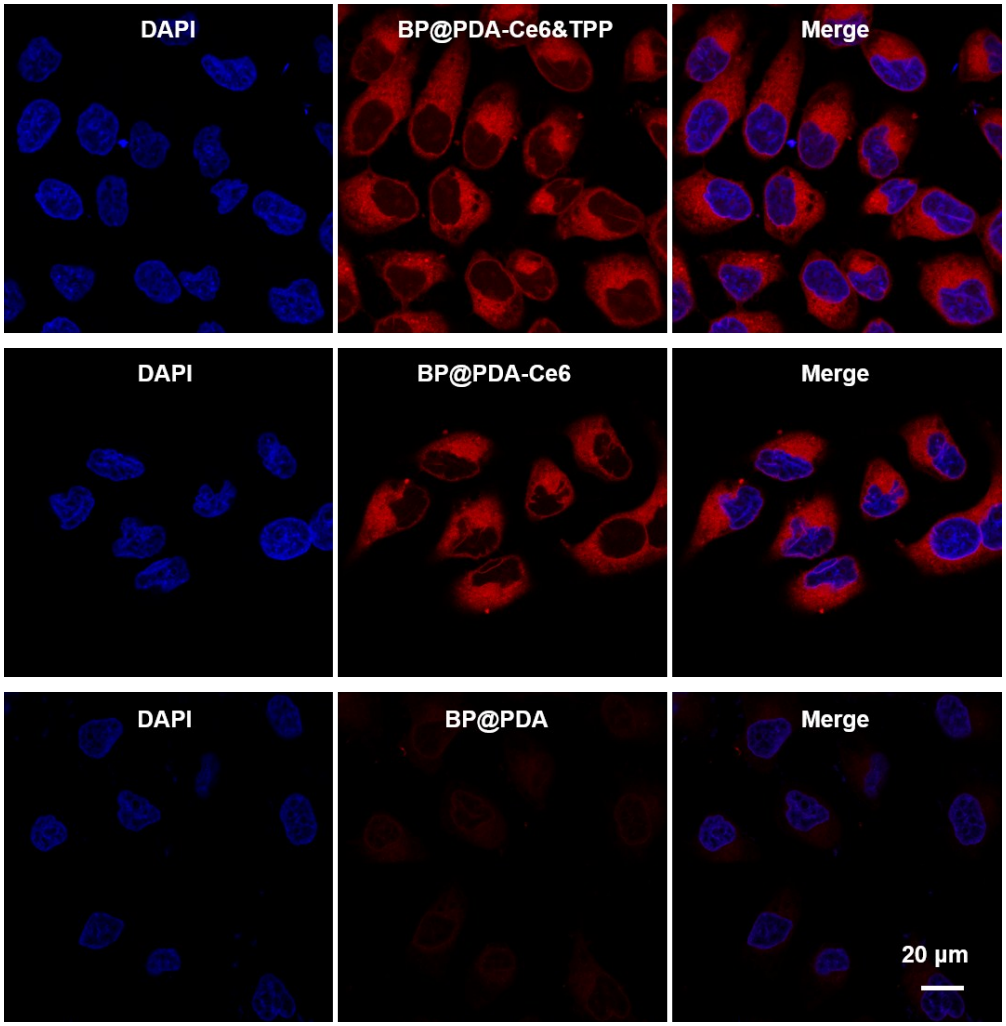

Figure S15. Fluorescence images of HeLa cells cultured with BP@PDA-Ce6&TPP NSs, BP@PDA-Ce6 NSs, and BP@PDA NSs.

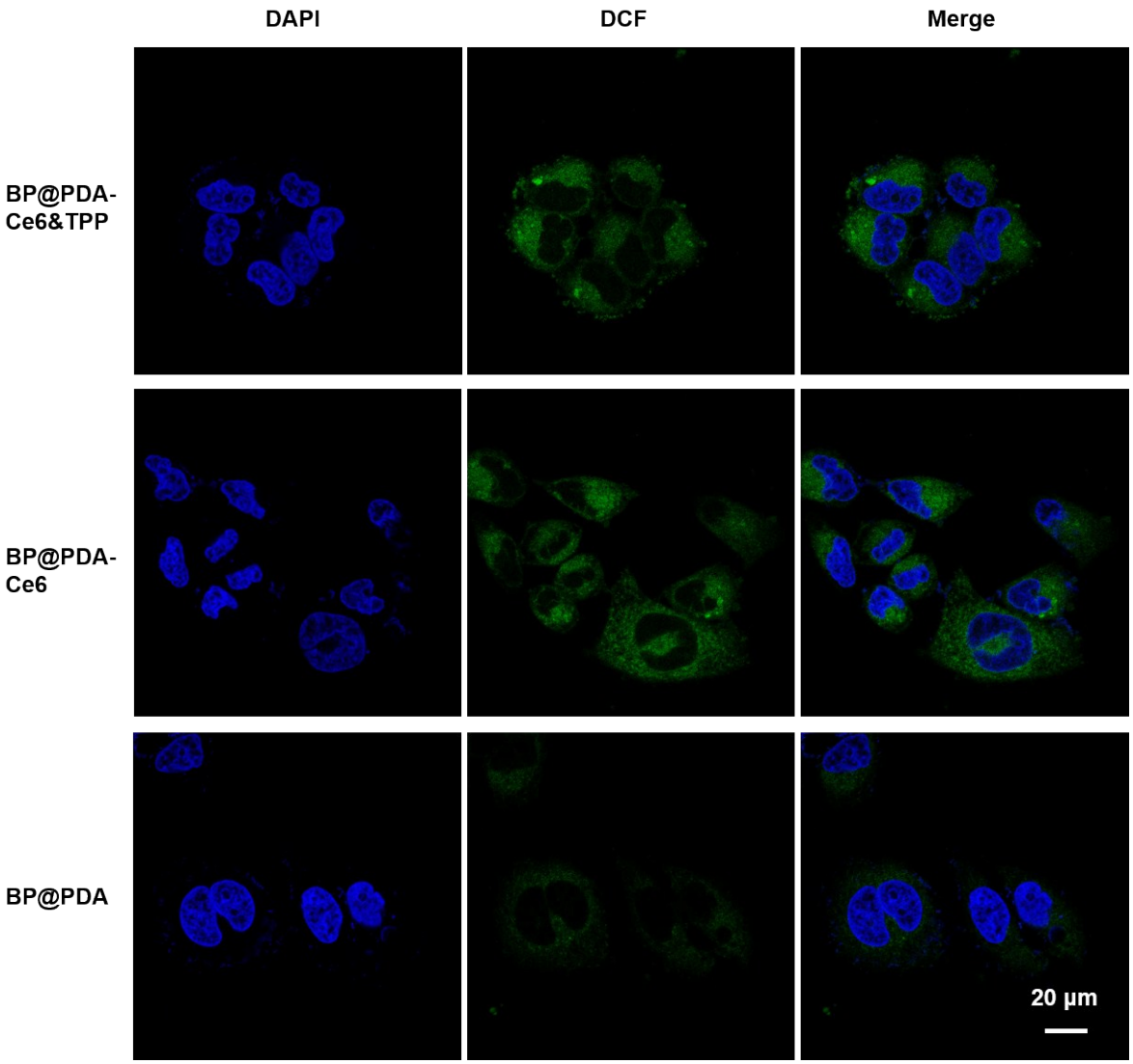

Figure S16. Intracellular ROS generation of HeLa cells cultured with BP@PDA-Ce6&TPP NSs, BP@PDA-Ce6 NSs, and BP@PDA NSs after laser irradiation (660 nm, 5 min, 0.5 W/cm^2).

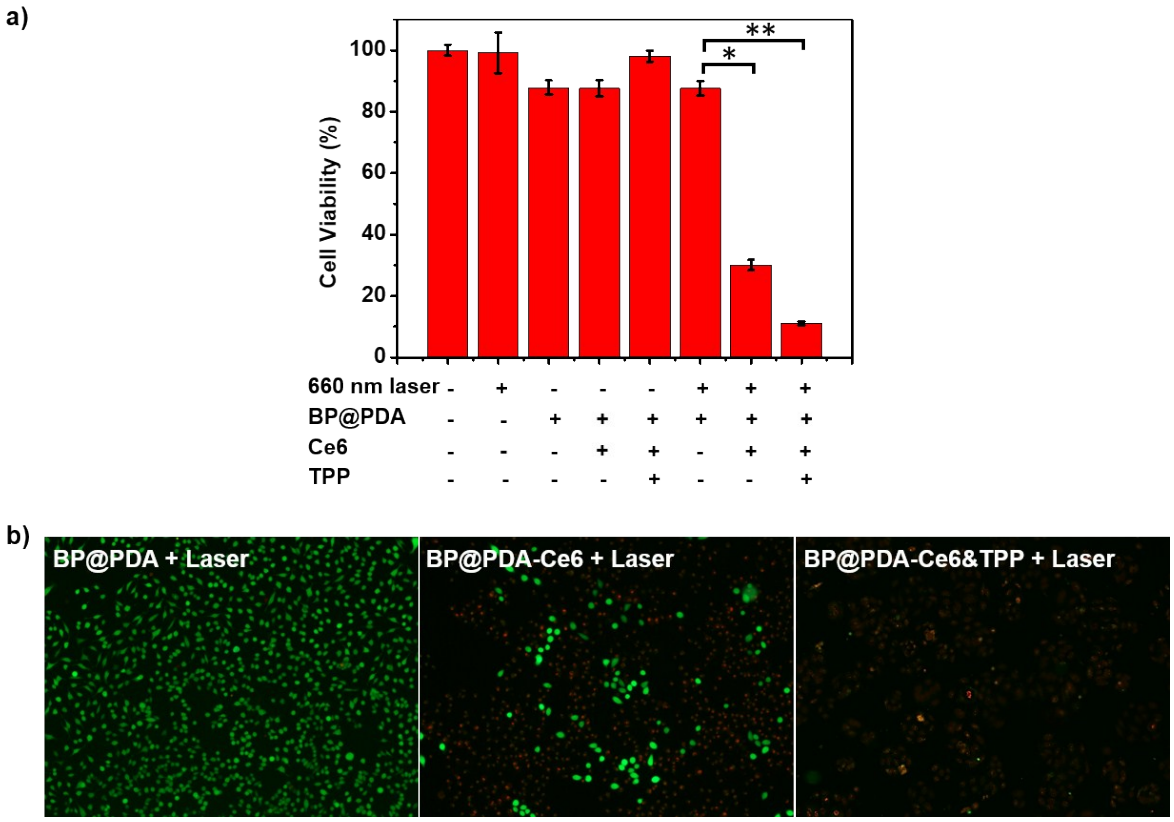

Figure S17. (a) MTT assay of HeLa cells with different treatments at BP@PDA concentration of 1 μg/mL (*P < 0.01, **P < 0.005). (b) Fluorescence images of HeLa cells co-stained with calcein AM (live cells, green) and PI (dead cells, red) upon the addition of BP@PDA NSs, BP@PDA-Ce6 NSs and BP@PDA-Ce6&TPP NSs (BP@PDA concentration of 10 μg/mL) after laser irradiation (660 nm, 0.5 W/cm^2, 5 min).

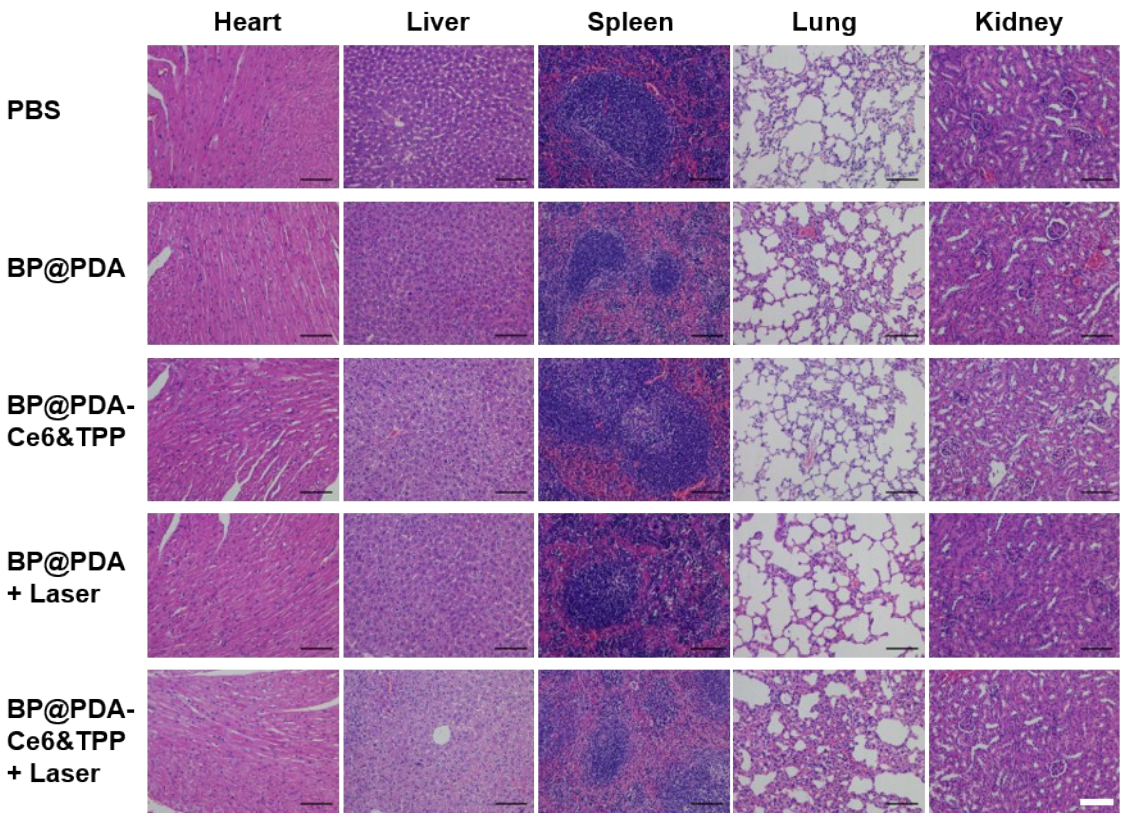

Figure S18. H&E stained histological images of major organs after treatment. Scale bar: 100 μm.