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Supporting Information

Binary Dimeric Prodrug Nanoparticles for Self-Boosted Drug Release and Synergistic Chemo-Photodynamic Therapy

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EXPERIMENTAL SECTION

Materials. 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC•HCl) was purchased from Shanghai Yuanye Biological Technology Co., Ltd.. 4-dimethylaminopyridine (DMAP) was purchased from Aladdin Co., Ltd.. Chloroform-d ($CDCl_3$) was purchased from Qingdao Tenglong Weibo Technology Co., Ltd.. Lyso-Tracker-Green was purchased from Shanghai Beyotime Biotechnology Co., Ltd.. Calcein-AM/propidium iodide staining assay kit, Tubulin-Tracker Red and Annexin V-FITC/PI apoptosis detection kit were purchased from Jiangsu KeyGEN Biotechnology Co., Ltd.. Ultrapure water was prepared from a Milli-Q system (Millipore, USA). Solvents for chemical synthesis were purified by distillation.

Characterizations. The morphologies of nanoparticles were observed by JEOL JEM-1011 electron microscope (acceleration voltage of 100 kV). The size, size distribution and ζ-potential of nanoparticles were measured by Malvern Zeta-sizer Nano. The measurement was carried out at 25 ℃ and the scattering angle was fixed at 90°. Analytical balance (XS105DU) and Rainin Pipettes from METTLER TOLEDO were used to quantify solid and liquid respectively. Ultraviolet-visible (UV-vis) absorption spectra and fluorescence spectra were recorded by Shimadzu UV-2450 PC UV-vis spectrophotometer and PerkinElmer LS55 luminescence spectrometer, respectively. The concentration of PTX-containing formulations was determined by high performance liquid chromatography (HPLC) (Shimadzu, CBM-20A). The mobile phase was a mixture of methanol, acetonitrile and water (v/v/v=11/11/3) at a flow rate of 1.0 mL/min. Confocal laser scanning microscopy (CLSM) images were taken by a Zeiss LSM 700 (Zurich, Switzerland). Flow cytometry analysis was performed by a flow cytometer (FlowSight, Amnis, Merck Millipore).

Synthesis of TPP_2-TK. ROS-cleavable thioketal linker (TK-COOH) and 5-(4-hydroxylphenyl)-10,15,20-triphenylporphyrin (TPP-OH) were synthesized according to the previous reported literature.[1, 2] In detail, 150 mg TPP-OH, 30 mg TK-COOH, 96.4 mg EDC·HCl and 3.2 mg DMAP were added sequentially into 10 mL

dichloromethane (CH_2Cl_2). The solution was stirred at room temperature for 1 h, and 50.2 mg EDC·HCl and 3.4 mg DMAP were added into the reaction mixture again. The reaction was continued for another 60 h and monitored by thin-layer chromatography (TLC). After rotating evaporation of organic solvent, the crude product was purified by silica gel column chromatography (CH_2Cl_2/CH_3OH, v/v = 20:1, as the eluent). ^{1}H NMR (500 MHz, $CDCl_3$) δ 8.83 (dt, *J* = 11.0, 5.1 Hz, 8H), 8.24 – 8.20 (m, 4H), 8.17 – 8.13 (m, 4H), 7.76 – 7.66 (m, 9H), 7.58 – 7.55 (m, 2H), 3.26 (t, *J* = 7.0 Hz, 2H), 3.17 (t, *J* = 6.9 Hz, 2H), 1.83 (s, 3H), -2.79 (s, 2H). MS (LTQ) m/z: calculated for TPP_2-TK ($C_{97}H_{72}N_8O_4S_2$) $[M+H]^+$ 1478.52, found 1478.6.

Preparation of Co-PT NPs. Briefly, a mixture of TPP_2-TK and PTX_2-TK in tetrahydrofuran (THF) were slowly dropped into deionized water under vigorous stirring. After the organic solvent was evaporated, the NPs were obtained. Unbound TPP_2-TK and PTX_2-TK were removed by centrifugation (3500 rpm, 5 min). Finally, the supernatant was dialyzed against deionized water for 72 h to remove residual THF (MWCO: 3.5 kDa). TPP_2-TK NPs and PTX_2-TK NPs were prepared in the same way. The content of TPP_2-TK and PTX_2-TK in NPs was determined by HPLC and UV-vis spectrophotometry, respectively.

***In vitro* stability of NPs.** To investigate *in vitro* stability, NPs were stored in an aqueous solution at 4 ℃ or dissolved in PBS (pH 7.4) containing 10% FBS or 5% glucose at 37 ℃. DLS was used to monitor the changes of size and polydispersity index (PDI) of NPs.

Intermolecular interactions investigation. Co-PT NPs were incubated with NaCl (10 mM), urea (100 mM) and Triton X-100 (100 mM) for 24 h. The changes of size and PDI were monitored by DLS.

ROS generation of Co-PT NPs upon light irradiation. The ROS generation ability of Co-PT NPs and TPP_2-TK NPs were evaluated using a ROS capturer, indocyanine green (ICG). Co-PT NPs and TPP_2-TK NPs (TPP: 10 μM) were mixed with ICG (7.5 μg/mL), and exposed to a 638nm laser lamp (100 mW/cm^2). The absorbance of ICG at

779 nm was measured every 1 min by UV–Vis spectrophotometer. Free ICG solution was used as a blank control. Another ROS trapping agent, vitamin C (VC, 4 mM) was added to capture the newly produced $^1O_2$, thus slowing down the rate of ICG absorption decrease. To test the photostability of Co-PT NPs and TPP_2-TK NPs, the same experiment was performed according to the designed protocol.

Light-activated drug release from PTX_2-TK in Co-PT NPs. To examine PTX release from Co-PT NPs under light irradiation, Co-PT NPs (2 mL, containing 100 μg PTX_2-TK and 300 μg TPP_2-TK) were illuminated by 638 nm laser (100 mW/cm^2). After certain time of irradiation, 200 μL of the dispersion was taken out and mixed with an equal volume of fresh acetonitrile. After centrifugation at 10,000 rpm for 10 min, the supernatant was analyzed by HPLC to detect the hydrolysis of PTX_2-TK.

Cell lines and cell culture. HeLa (human cervical carcinoma) cells were cultured in Dulbecco's modified Eagle's medium (DMEM, GIBCO) supplemented with 1% penicillin, 1% streptomycin, and 10% heat-inactivated fetal bovine serum (FBS, GIBCO) in a humidified incubator at 37 ℃ with 5% CO_2.

Cellular uptake of Co-PT NPs. HeLa cells were seeded in 6-well plates at a density of 5×10^4 cells per well and cultured overnight. The culture medium was subsequently replaced with fresh medium containing corresponding NPs (TPP: 10 μM). After incubation for 1 h or 6 h at 4 ℃ or 37 ℃, the cells were gently washed three times with PBS (pH 7.4), fixed in 4% paraformaldehyde for 10 min. Finally, the cells were stained with Hoechst for 5 min and imaged by CLSM. For lysosome co-localization, Lyso Tracker-Green probe was employed to stain lysosome for 30 min.

Intracellular ROS detection. Intracellular ROS generation was determined by DCFH-DA, a ROS-sensitive fluorescent probe. HeLa cells were cultured with Co-PT NPs (TPP: 20 μM) for 6 h, and then exposed to irradiation (638 nm, 100 mW/cm^2, 10 min) or incubated in the dark. After the medium was removed, FBS-free DMEM containing DCFH-DA (10 μM) was added and cultured for 30 min. Finally, the cells were carefully washed 3 times with PBS and observed as soon as possible by fluorescence microscope.

Besides, cells were pretreated with VC (1 mM) for 2 h to capture the generated ROS.

Cell viability assays. The *in vitro* cytotoxicity of NPs was evaluated using the 3-(4,5-dimethylthiazol-2-yl) -2,5-diphenyltetrazolium bromide (MTT) assay. First, HeLa cells were seeded into 96-well plates (5×10^3 cells/well) and incubated overnight. Next, the corresponding NPs dispersions diluted with cell culture media to designed concentration were added and incubated for 4 h. The cells were irradiated by a laser (638 nm, 100 mW/cm^2, 10 min) or incubated in the dark. After incubation for another 48 h, the drug-containing culture medium was replaced with 200 μL MTT (0.5 mg/mL) and incubated for 4 h. Finally, 150 μL DMSO was added to dissolve the formed violet formazan crystals. Finally, the plates were shaken for 3 min, and the absorbance of violet product at 490 nm was quantified by a microplate reader.

Calcein-AM/propidium iodide staining assay. To visually observe the cytotoxicity of NPs, calcein-AM/propidium iodide (PI) was used to distinguish live (green) and dead (red) cells. HeLa cells were treated with Taxol, PTX_2 -TK NPs, TPP_2-TK NPs and Co-PT NPs (TPP: 20 μM, PTX: 0.3 μM) for 4 h, and illuminated by a 638 nm laser (100 mW/cm^2, 10 min) or incubated in the dark. After further 48 h incubation, cells were co-stained with calcein-AM/PI for 30 min at room temperature and imaged by the fluorescence microscope.

Annexin-V FITC/PI apoptosis assay. The cell apoptosis induced by NPs was quantitatively evaluated by flow cytometry. HeLa cells were treated with various nanoformulations (TPP: 20 μM, PTX: 0.3 μM) with or without light irradiation (638 nm, 100 mW/cm^2, 10 min). Then, cells were incubated at 37 ℃ for additional 48 h. After washing and harvesting, HeLa cells were stained with Annexin V-FITC and PI for about 20 min in the dark. Finally, the quantitative analysis of apoptosis and necrosis was determined by flow cytometry.

The immunostaining of tubulin. To explore the anticancer mechanism of Co-PT NPs, microtubule network was stained and imaged by CLSM. HeLa cells were cultured with various formulations, including Taxol, PTX_2-TK NPs and Co-PT NPs (PTX: 0.3μM).

After 6 h of incubation, the cells of Co-PT NPs (L+) were subjected to light irradiation (638nm, 100 mW/cm^2, 10 min). After a further 24 h incubation, the cells were washed gently, fixed in 4% paraformaldehyde for 10 min, and washed again with 0.1% Triton X-100 three times to increase the cell membrane permeability. After staining with diluted Tubulin-Tracker Red in 0.1% Triton X-100 containing 3% BSA for 30 min in the dark, the stained cells were observed by CLSM.

SUPPLEMENTARY FIGURES

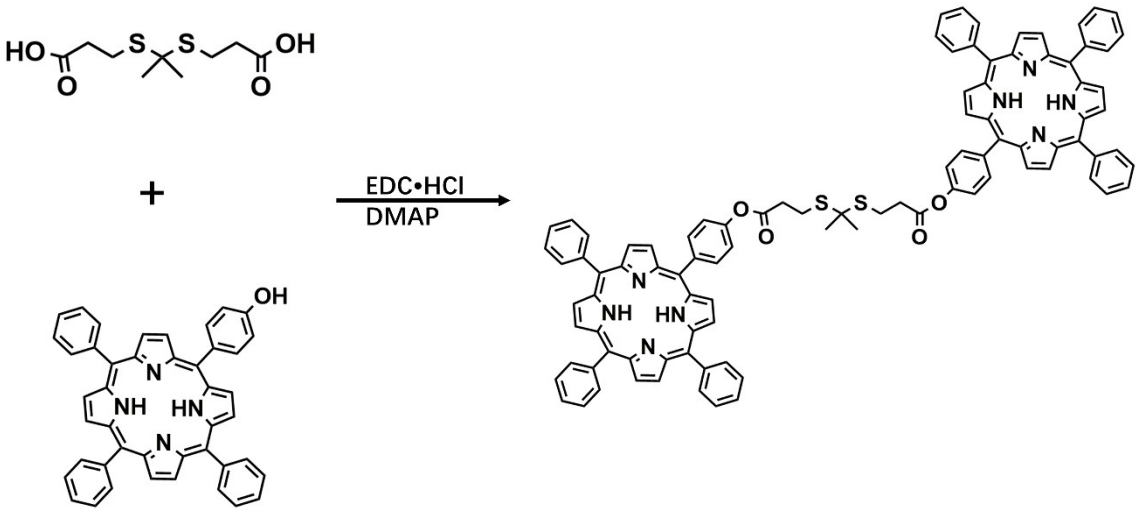

Fig. S1. Synthetic route of TPP_2-TK.

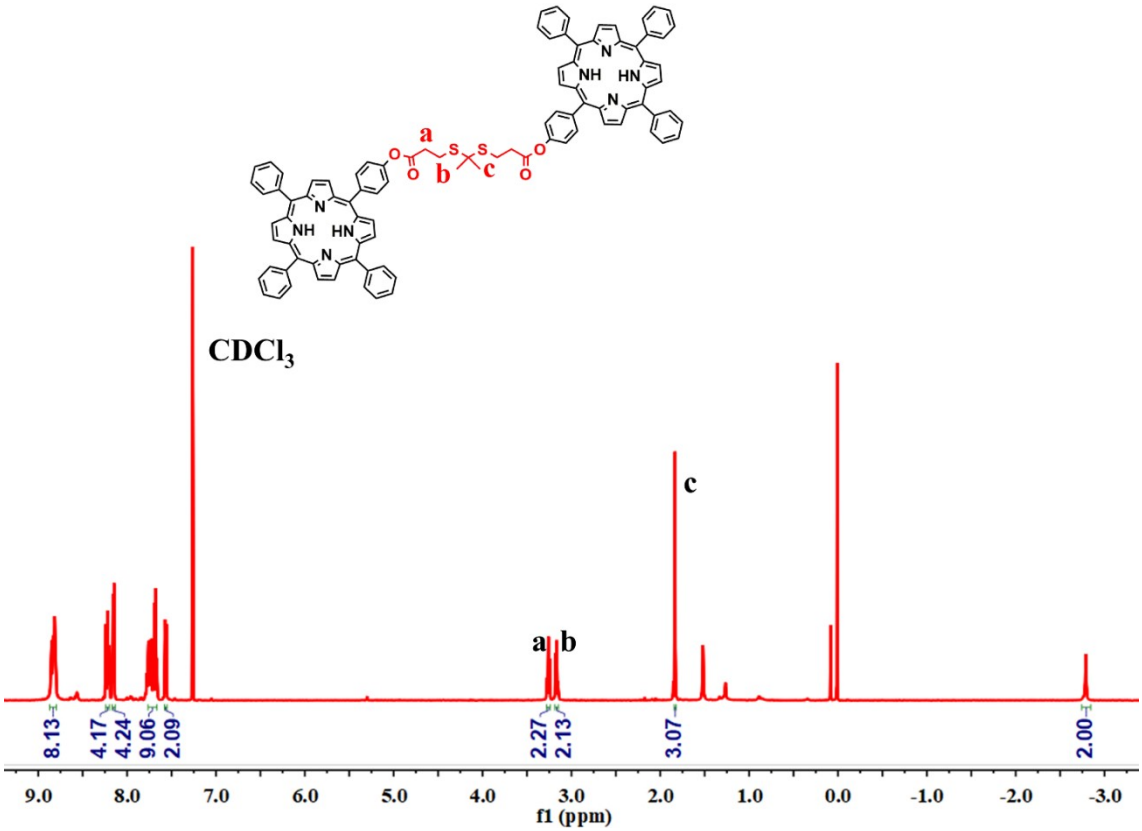

Fig. S2. ^1H NMR spectrum of TPP_2-TK in $CDCl_3$.

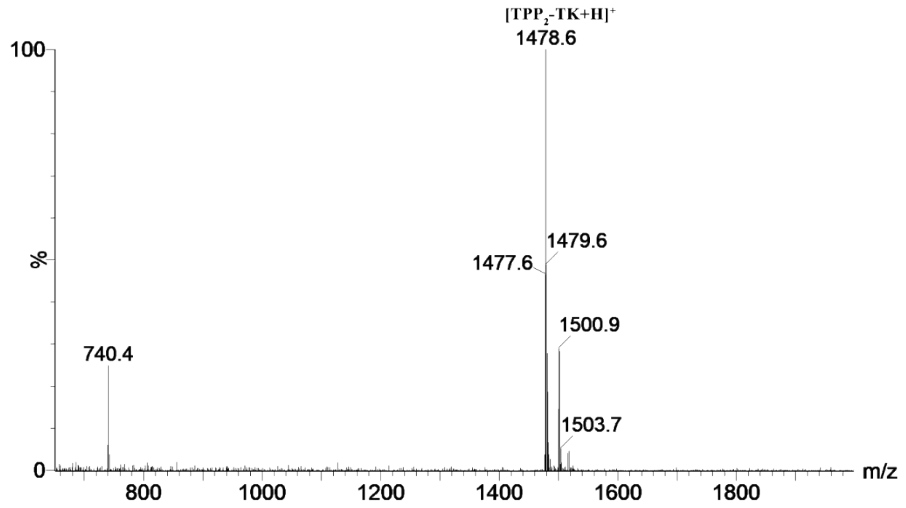

Fig. S3. ESI-MS spectrum of TPP_2-TK.

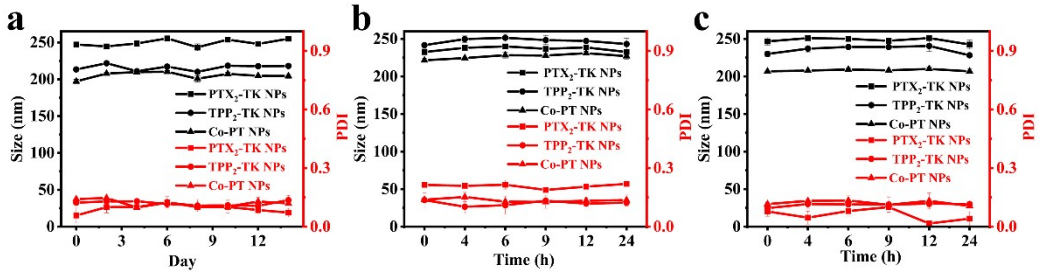

Fig. S4. Changes of size and PDI of all NPs in aqueous solution at 4 ℃ for two weeks (a), PBS containing 10% FBS for 24 h (b), and 5% glucose solution for 24 h (c).

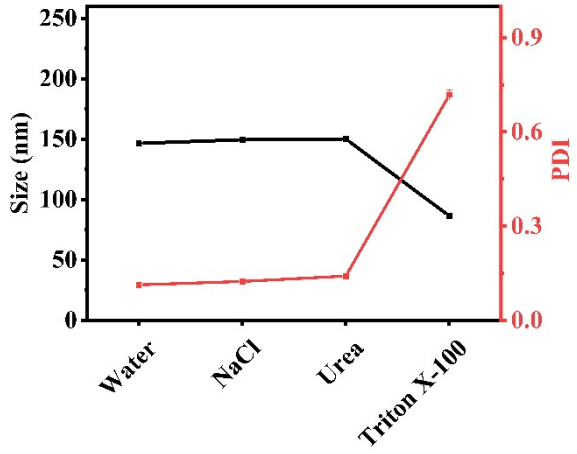

Fig. S5. Changes of size and PDI of Co-PT NPs in aqueous solution, NaCl, urea and Triton X-100 for 24 h.

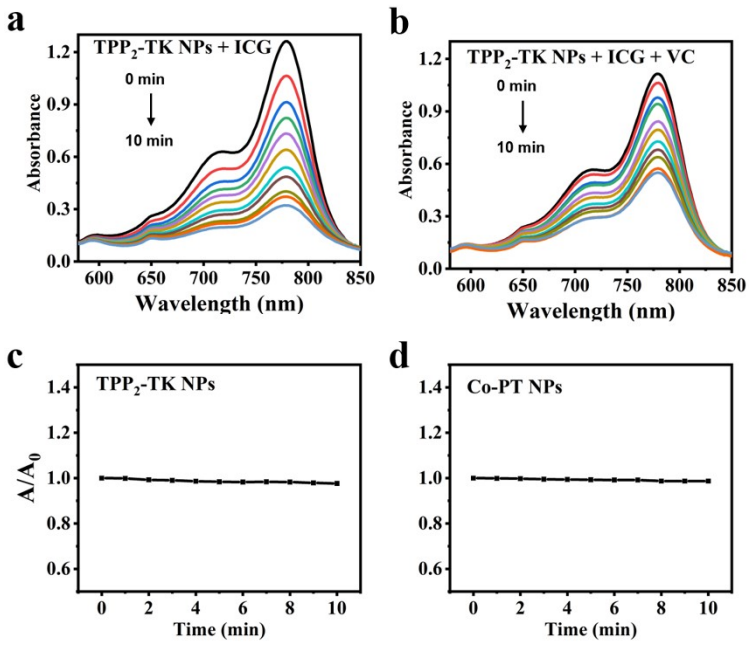

Fig. S6. Time dependent absorption spectra of TPP_2-TK NPs + ICG (a), TPP_2-TK NPs + ICG +VC (b), TPP_2-TK NPs (c) and Co-PT NPs (d) after laser irradiation (638 nm, 100 mW/cm^2, 10 min).

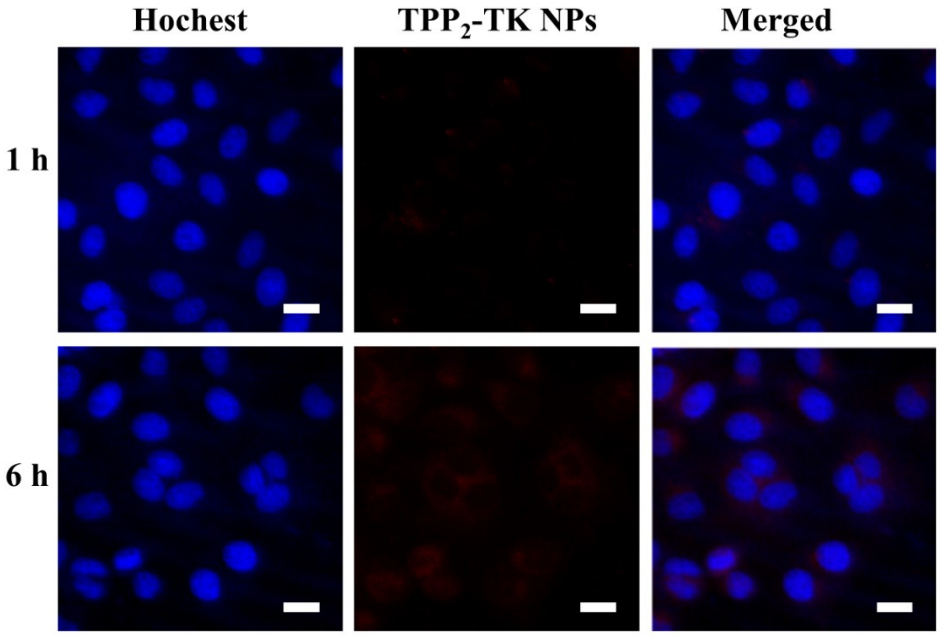

Fig. S7. CLSM images of HeLa cells treated with TPP_2-TK NPs (TPP: 10 μM) for 1 h and 6 h at 37 ℃. Scale bars, 20 μm.

References:

1. W. Zhang, W. Lin, X. Zheng, S. He and Z. Xie, *Chem. Mater.*, 2017, **29**, 1856-1863.

2. Q. Pei, X. Hu, X. Zheng, S. Liu, Y. Li, X. Jing and Z. Xie, *ACS Nano*, 2018, **12**,

1630-1641.