Supporting Information

Mitochondria-Targeting NO Gas Nanogenerator for Augmenting Mild Photothermal Therapy in the NIR-II Biowindow

Canchen Wu,‡ Danqi Wang,‡ Moupan Cen, Leyu Cao, Yue Ding, Jin Wang, Xiaolei Yuan, Yang Wang, Tingting Chen* and Yong Yao**

School of chemistry and chemical engineering, Nantong University, Nantong, Jiangsu 226019, China.

E-mail: ywang85@ntu.edu.cn, ttchen1980@126.com, yaoyong1986@ntu.edu.cn.

1. Experimental Section

1.1 Materials

Copper(II) 2,4-pentanedionate ($Cu(acac)_2$, 98%), thiobenzoic acid (94%), 1-Octadecene (ODE, 90%), Oleylamine (OAm, 90%), poly(ethylenimine) (PEI; branched, MW≈10000, 99%), (4-Carboxybutyl)triphenylphosphonium bromide (TPP, 98%), N-(3-Dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC, 97%) and N-hydroxysuccinimide (NHS, 98%) were obtained from Aladdin Chemical Reagent Co. Ltd. China. NO assay kit and 4-amino-5-methylamino-2,7-difluorofluorescein diacetate (DAF-FM DA) were purchased from the Beyotime Institute of Biotechnology. All chemical agents were of analytical grade and used directly without further purification.

1.2 Synthesis of CuS nanoplates.

65 mg $Cu(acac)_2$, 30 mL ODE and OAm were added into a three-neck flask and under magnetically stirring with a N_2 flow. Then, 100 μL of thiobenzoic acid was quickly injected into the solution and vigorously stirred for 30 min. The resultant solution was heated to 150 °C and refluxed for 1 h under N_2 protection. After cooling the solution to room temperature, CuS nanoplates precipitated upon the addition of ethanol, which were centrifuged and further washed with ethanol twice. The final product was dispersed in ethanol for further use.

1.3 Synthesis of PEI-functionalized CuS nanoplates (CuS-PEI).

The as-synthesised OAm capped CuS nanoplates (15mg) were dispersed in a mixed solution of ethanol (1mL) and hydrochloric acid (1mL, 1M). The resulting mixture

was sonication for 30 min to remove the surface OAm ligands. After the reaction, the precipitation was collected by centrifugation and washed with ethanol and deionized water, and redispersed in 9 mL of deionized water. Then, 0.1mg of PEI was poured in the solution under vigorous stirring. The water dispersion was then added to 10 mL of DEG, and the mixture was stirred at 105 ˚C for 1 h to remove water. Finally, the solution was transferred to the Teflon-lined autoclave and incubated at 160 ˚C for 2 h. The obtained PEI modified CuS nanoplates were collected by centrifugation.

1.4 Surface modification of CuS-PEI with TPP (CuS-PEI-TPP).

Briefly, 50 mg of TPP was dissolved in 10 mL of anhydrous DMSO. Then EDC (22mg) and NHS (32mg) were added to activate the carboxylic acid groups of TPP, and stirred for 5 h under room temperature. Then, the CuS-PEI dispersed in DMSO (5 mL) was added to the activated TPP solution, followed by further stirring for another 5 h. The TPP-conjugated CuS-PEI was separated via centrifugation and washed with DMSO and PBS solution with 3 times.

1.5 Preparation of CuS-PEI/NO-TPP nanoplates.

The as-prepared CuS-PEI-TPP nanoplates were suspended in 10 mL of anhydrous ethanol with 50 mg of sodium methoxide through sonication for 10 min. Afterwards, the resulting solution was poured into a high-pressure reactor that was flushed with 6 atm of argon (Ar) gas three times rapidly and then three times for 10 min each to eliminate oxygen inside the solution. Next, the solution was kept for 3 days with stirring under 6 atm of NO gas. The produced CuS-PEI/NO-TPP nanoplates were separated from the solution and washed twice with cold ethanol by centrifugation, and

rapidly dried under vacuum within 30 min and stored in a vacuum-packed bag at -20 °C.

1.6 Photothermal conversion experiments

For measuring the photothermal conversion performances of these CuS-PEI-TPP nanoplates, 980 nm NIR laser was delivered through a quartz cuvette containing aqueous dispersion (3 mL) of CuS-PEI-TPP nanoplates with different concentrations (0-200 μg/mL), and the light source was an external adjustable power (1 W/cm^2) 1064 and 808 nm semiconductor laser device (Changchun Femtosecond Technology Co. Ltd., China). The temperature was monitored by a thermometer and recorded once every 10 s. The temperature signals also recorded at different time intervals (0-5 min) were analyzed with FLIR tools systems.

The photothermal conversion efficiency (η) was calculated according to the eq. 1:

$$\eta = \frac{hS(T_{max} - T_{surr}) - Q_0}{I(1 - 10^{-A})} \quad (1)$$

Where h is the heat transfer coefficient, S is the surface area of the container, T_{max} is the steady state maximum temperature, T_{surr} is the ambient temperature of the surroundings, Q_0 is the baseline energy input by the solvent and the sample container without CuS-PEI nanoplates, I is the laser power, and A is the absorbance of CuS-PEI nanoplates solution at 808 nm or 1064 nm. The value of hS is calculated by eq. 2:

$$hS = \frac{m_d C_d}{\tau_s} \quad (2)$$

Where τ_s is the characteristic thermal time constant (Fig. 2d and 2e), the mass of the m_d and C_d are the mass and heat capacity of water, respectively. The heat energy

(Q_0) of the sample container and solvent without CuS-PEI nanoplates were measured independently using the eq. 3:

$$Q_0 = hS(T_{max} - T_{surr}) \quad (3)$$

1.7 Detection of NO release in the aqueous solution with a Griess kit.

The controlled NO release profiles were determined by typical Griess reagent kit that could detect the nitrite ions in solution by UV-vis spectrophotometer. In brief, the CuS-PEI/NO-TPP solution were irradiated by 1064 nm laser light with different power (0, 0.5, 1.0 W/cm^2). The induced released NO could change into nitrite following by reacting with Griess agent to a pinkish compound with signal at 540 nm.

1.8 Detection of intracellular NO release with the DAF-FM DA fluorescence probe.

4T1 cells (1×10^5) were seeded in a glass bottom cell culture dish and cultured overnight. Then 30 mL of CuS-PEI/NO-TPP was incubated with cells for 4 h at 37 °C. After replacing with fresh culture media, 10 mL of DAF-FM DA were loaded with cells under shaking medium for 20 min at 37 °C. Subsequently, the cells were irradiated (1064 nm, 1 W/cm^2) for 5 min followed by rinsing of culture medium three times. The DAF-FM DA fluorescence of each sample was observed immediately at 515 nm (excitation at 495 nm) by CLSM.

1.9 *In vitro* cytotoxicity study.

For studying the cytotoxicity of the CuS-PEI-TPP, HeLa and 4T1 cells were seeded in a 96-well plate at a density of 10^4 cells/well for 24 h at 37 °C in 5% CO_2. Then, the cells were treated with CuS-PEI-TPP and CuS-PEI/NO-TPP at a desired

concentration. After incubation for 24 h, cell viabilities were tested by standard 3-(4,5)-dimethylthiahiazo-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. To confirm that the PTT of CuS-PEI-TPP under laser irradiation, 4T1 cells were cultured in 96-well plate for 24 h. After that, the medium was replaced by 200 mL of cell medium with CuS-PEI-TPP and CuS-PEI/NO-TPP at the appropriate concentration. After being maintained for 12 h, the medium was replaced by 200 mL of fresh medium, and the plates were photo-irradiated by a laser lamp (λ=1064 nm, 1 W/cm^2) for 5 min. Cell viabilities were also tested by using the standard MTT assay.

1.10 Western blotting analysis of HSP-90.

4T1 cancer cells were treated/incubated with diverse conditions: (1) PBS; (2) CuS-PEI-TPP; (3) CuS-PEI/NO-TPP; (4) CuS-PEI/NO+1064 nm laser irradiation for 5 min; (5) CuS-PEI-TPP +1064 nm laser irradiation for 5 min; (6) CuS-PEI/NO-TPP + 1064 nm laser irradiation for 5 min. Afterward, cells were obtained for the analysis of western blotting. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) was set as a control of protein loading.

1.11 In vivo antitumor effect.

The Laboratory Animal Centre of Nantong University performed the animals' experiments with an approval from Nantong University. In order to understand the anticancer effect of CuS-PEI/NO-TPP *in vivo*, 100 μL 5×10^6 of 4T1 cells were inoculated by subcutaneous injection onto each back of mice. The mice were divided into seven groups (n = 4) randomly when the tumor on the right-hind back of mice grew into the volume of 80～90 mm^3. Then the mice were injected by intratumoral

injection everyday with PBS, CuS-PEI-TPP, CuS-PEI/NO-TPP at a CuS dose of 100 μg/mL. Mice in group 5 and 7 received the same injection as in group 3 and 4, group 6 were treated with CuS-PEI/NO, additionally; the tumor regions of the mice were irradiated with 1064 nm laser (a power density of 1 W/cm^2, an irradiation time of 5 min) through the skin surface within 2 h after injection. The tumor sizes were measured by a caliper every three days and calculated as the volume = (tumor length)×(tumor width)^2/2. The tumor inhibitory rates (TIR) of various treatments are calculated by the equation: TIR (%) = 100 × (mean tumor volume of the PBS group mean tumor volume of others)/(mean tumor volume of the PBS group).When the experiments were finished, the mice were killed by CO_2 asphyxiation and the tumors were collected and weighed.

1.12 Blood analysis and histology examination.

At the end of the anti-tumor research, 4T1 tumor-bearing mice were sacrificed. Main mouse viscera: heart, liver, spleen, lung, kidney and tumors were excised, fixed and embedded. For histological examination, organs from the treated group and control group were fixed in 4% formalin and conducted with paraffin embedded sections for H&E staining. All the blood parameters were measured in the Affiliated Hospital of Nantong University.

2. Figures

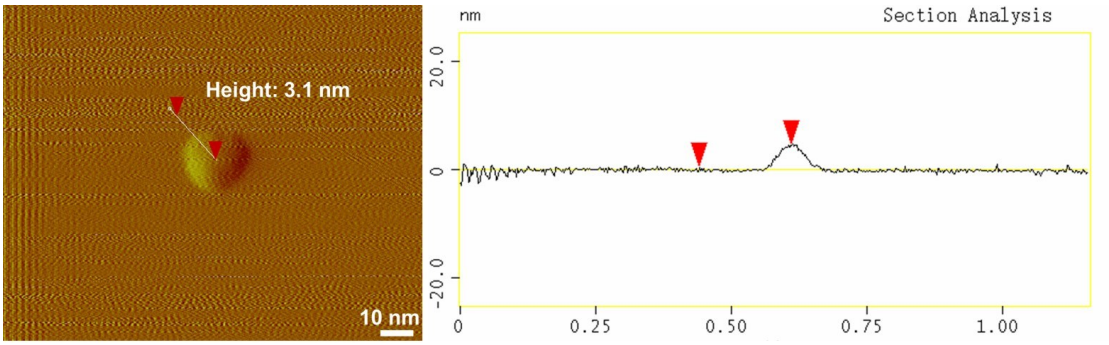

Fig. S1 AFM image and cross-section analysis of CuS-PEI-TPP nanoplate.

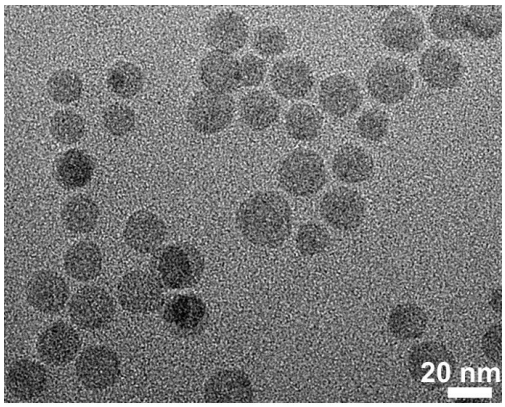

Fig. S2 TEM image of CuS-PEI/NO-TPP nanoplates.

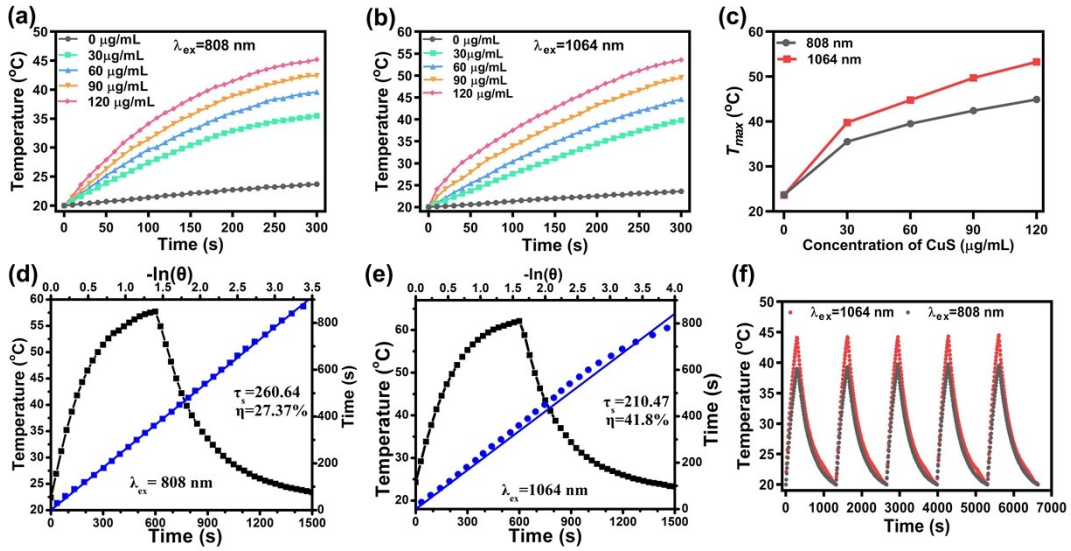

Fig. S3 Temperature change curves of CuS-PEI-TPP dispersions under different laser irradiation at a power density of 1.0 W/cm^2: (a) 808 nm, (b) 1064 nm. (c) Plot of temperature change vs CuS-PEI-TPP concentration. Calculation of the photothermal-conversion efficiency value of CuS-PEI-TPP at (d) 808 nm and (e) 1064 nm. (f) Temperature curves of CuS-PEI-TPP with concentrations

of 60 μg/mL for five laser on/off cycles under the 808 nm laser (black line) and the 1064 nm laser (red line) with 1.0 W/cm^2 for the 5 min irradiation, respectively.

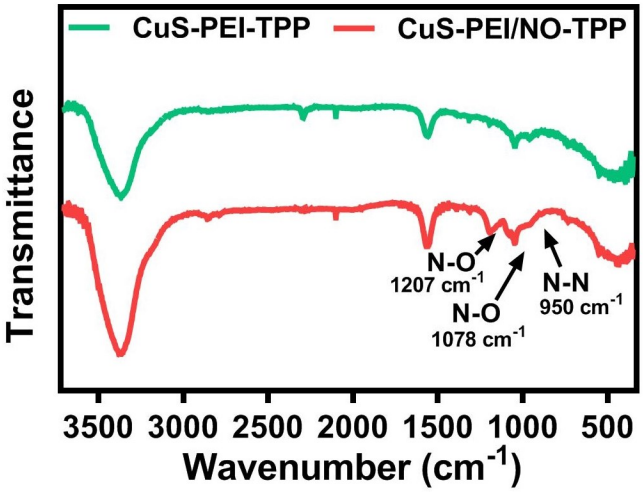

Fig. S4 Fourier-transform infrared (FT-IR) spectra of CuS-PEI-TPP and CuS-PEI/NO-TPP.

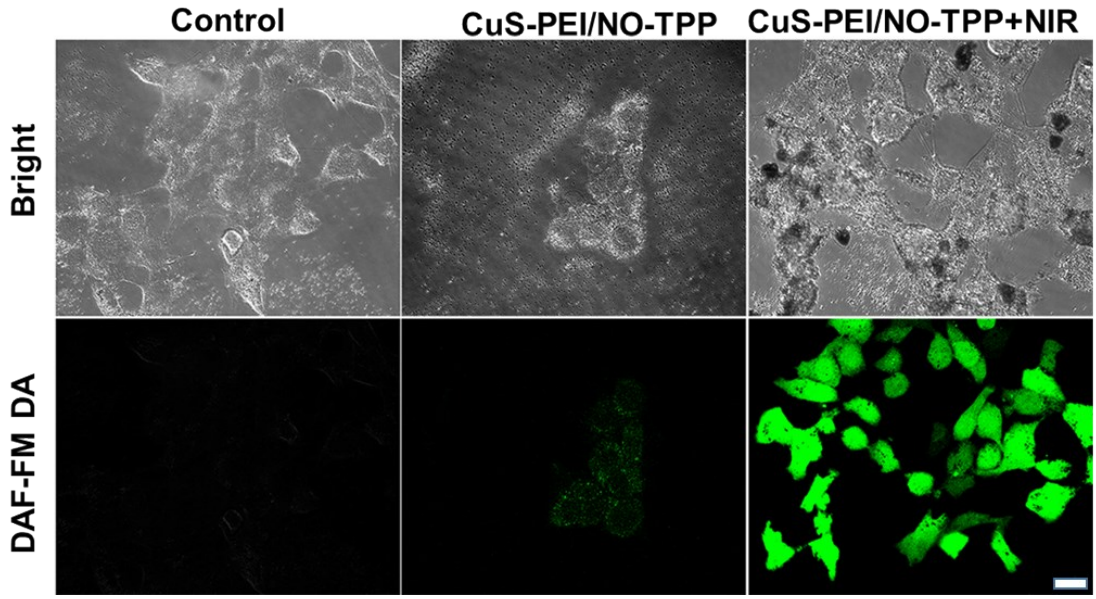

Fig. S5 Fluorescence images of 4T1 cells after incubation for 4 h with CuS-PEI/NO-TPP in the presence/absence of 1064 nm laser irradiation (1 W cm^{-2}, 5 min). The generation of NO was detected using DAF-FM DA. Scale bar = 20 mm.

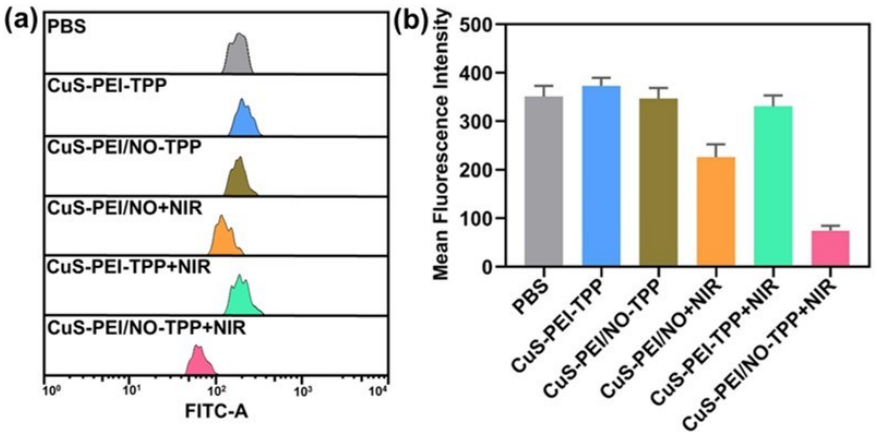

Fig. S6 Flow cytometry assays for detecting HSP90 expression level of 4T1 treated at different conditions.

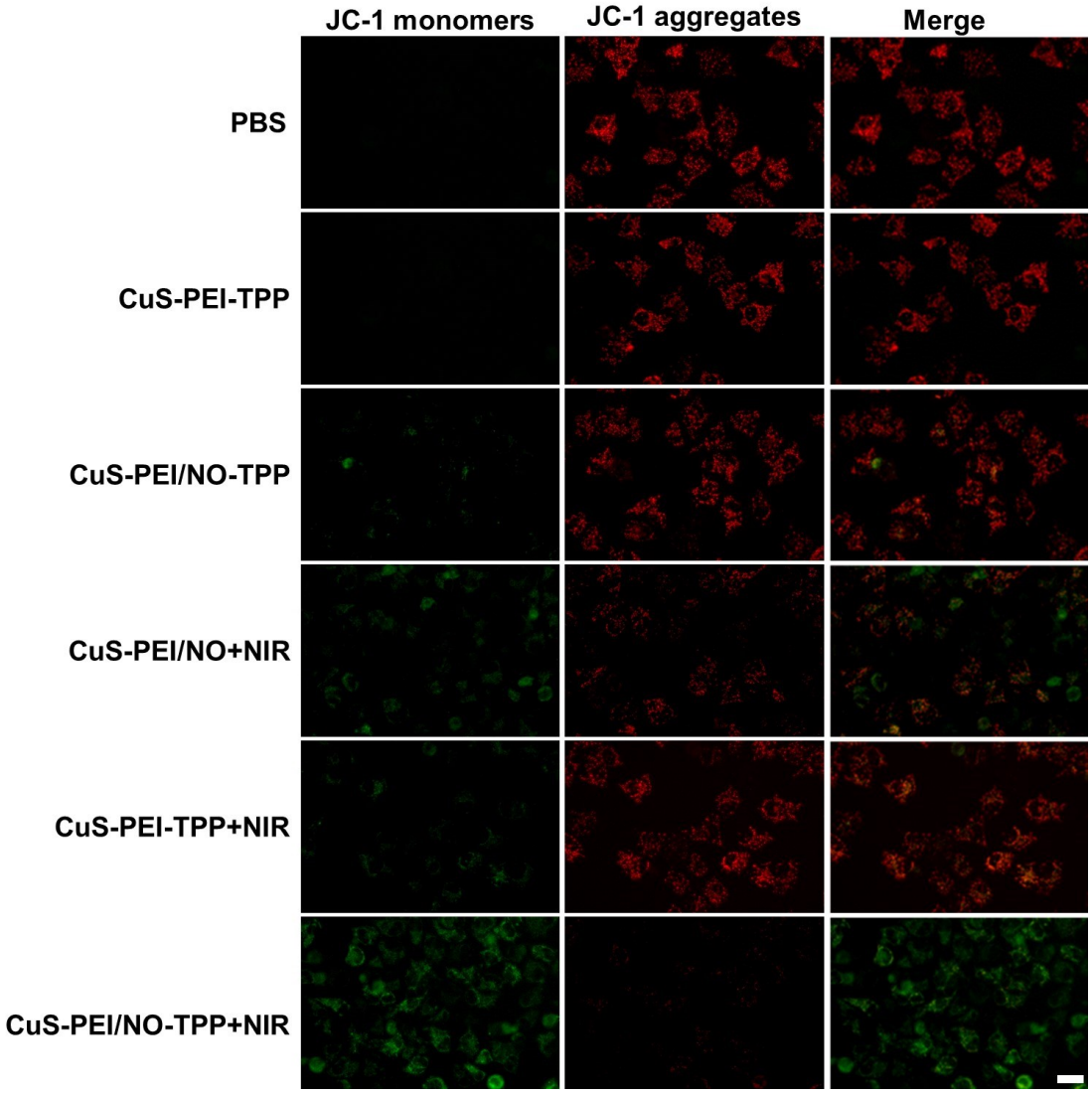

Fig. S7 Mitochondrial memberane potential (ΔΨm) of 4T1 cells after treated with PBS, CuS-PEI-TPP, CuS-PEI/NO-TPP, CuS-PEI/NO+NIR, CuS-PEI-TPP+NIR and CuS-PEI/NO-TPP + NIR. Scale bar indicated 20 μm.

JC-1 dye was used as an indicator of changes in mitochondrial membrane potential. When the mitochondrial membrane potential is high, JC-1 aggregates in the matrix as a polymer and generates red fluorescence. On the contrary, JC-1 forms a monomer when the potential is low and produces green fluorescence. The decrease of cell membrane potential is regarded as a sign of mitochondrial damage in terms of the proportional changes of JC-1 by the transition of red to green fluorescence.

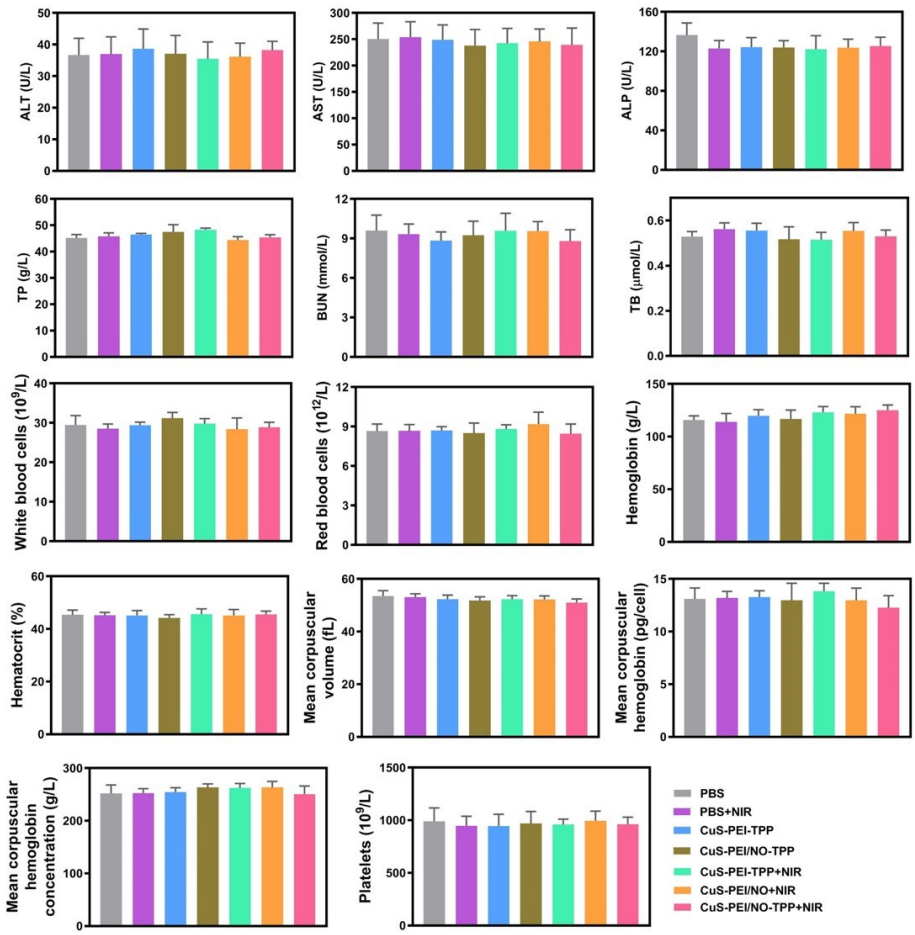

Fig. S8 Blood analysis data of mice 24 days after the different treatments. These findings did not indicate a trend of toxicity. ALT = alanine aminotransferase; AST = aspartate aminotransferase; ALP = alkaline phosphatase; TP = total bilirubin; BUN = blood urea nitrogen; TB = total bilirubin.

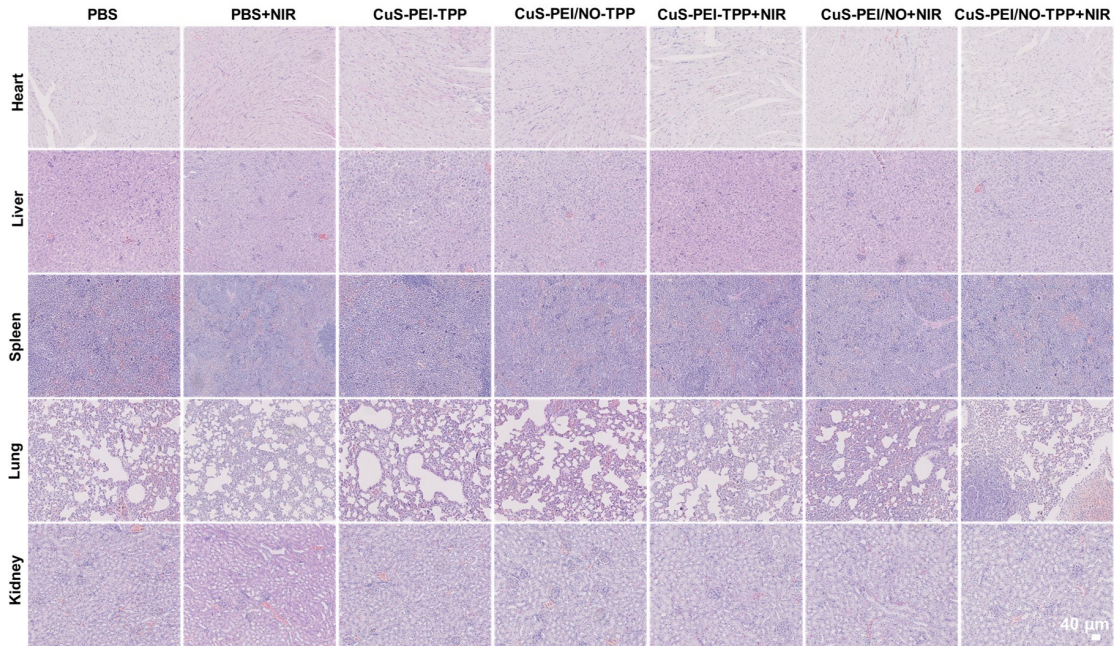

Fig. S9 H&E staining assay of normal organs obtained at the end of tumor therapeutic experiments. The organs were harvested from 4T1 tumor-bearing nude mice at the 24 day. Examined organs included lung, heart, kidney, spleen, and liver. No obvious organ damage was observed for the different groups.