Supplement Information

Stimuli-responsive ultra-small vanadate prodrug nanoparticles with NIR photothermal properties to precisely inhibit Na/K-ATPase for enhanced cancer therapy

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SUPPLEMENTAL MATERIALS AND METHODS

1. Materials and Methods

All chemicals were obtained from commercial sources and used without further purification unless otherwise noted. Bovine Serum Albumin (BSA), Tannic acid(TA), and Sodium orthovanadate were purchased from Aladdin Chemical *Co., Ltd.*(Shanghai, China). Tannic acid was purchased from Maclin Chemical *Co., Ltd.* (Shanghai, China). Modified Eagle's Medium (DMEM), RPMI-1640 medium, fetal bovine serum (FBS) penicillin/streptomycin (P/S) were purchased from (Gran Island, NY, USA), dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), calcein acetoxymethyl ester and propidium iodide (calcein-AM/PI), 4',6-diamidino-2-phenylindole (DAPI) and Micro Na/K-ATPase Assay Kit were purchased from Solarbio Science & Technology *Co. Ltd.* (Beijing, China)

2. Characterization

Transmission electron microscopy (TEM) images were taken from HT7700 (Hitachi) with an accelerating voltage of 120 kV. Zeta potential and dynamic light scattering measurements of hydrodynamic radii were made on a Malvern Zetasizer Nano-ZS (Malvern UK). X-ray photoelectron spectroscopy (XPS) was collected with a monochrome Al source from AXIS ULTRA DLD (Shimadzu Japan). SDS-PAGE was carried out by an Electrophoretic system (Bio-Rad USA). Inductively coupled plasma mass spectrometry (ICP-MS) was carried out on PerkinElmer NexION 300X (PerkinElmer USA). UV/Vis spectra were recorded on a NanoDrop™ One (Thermo Scientific USA). The MTT assay was conducted using a Microplate reader (SpectraMax, USA). The IR thermal images were taken from the FLIR E05 infrared imaging devices (FLIR USA). The photoacoustic imagines were taken from iThera Medical MSOT256 (iThera Medical Germany).

3. Synthesis

1) Pre-activation of sodium orthovanadate

200 mg sodium orthovanadate was dissolved in 10 mL H_2O, then the pH of the solution was adjusted to 10 by NaOH. The solution was boiled to dissolve all the crystals three times to ensure no crystals come out at room temperature. The activated sodium orthovanadate was stored at -20℃ for further use.

2) Synthesis of V(IV) NPs

To prepare V(IV) NPs, BSA (100 mg) and sodium orthovanadate (10 mg) dissolved in H_2O (7.5 ml) under vigorous stirring at room temperature. After stirring for another 30 min, the appropriate proportion of TA (2.5 ml) was dropwise added to the mixture. The color of the solution gradually turned from transparent to dark black. The reaction was continued for 30 min until no significant color change occurred, then the solutions were collected and dialysis (100 KDa) was to remove unreactive agents. Finally, the aqueous solution was filtered through a 0.22 μm polyvinylidene fluoride (PVDF) syringe. The prepared V(IV) NPs nanoparticles were stored at 4℃ for the following usage. The content of vanadium element in V(IV) NPs was measured by ICP-MS.

4. Electrophoretic Mobility Shift Assays

Free BSA and V(IV) NPs with equal BSA contents were loaded and separated on a 10% SDS-PAGE gel. The obtained gel was stained with coomassie brilliant blue for 30 min, then decolorate the extra dye by methanol (30%) and glacial acetic acid (30%) mixture for another 24 h to analyze the gel retardation phenomenon.

5. Release of vanadium

To monitor the vanadate release profile, V(IV) NPs was dispersed into 10 mM Hepes buffers at various conditions (pH=7.4, pH=5.5, and 1 mM H_2O_2), and the filtrates were collected from the solutions by ultrafiltration (100 KDa) to quantify remained vanadium.

6. Photothermal measurement

To evaluate the photothermal conversion of V(IV) NPs, the V(IV) NPs were dissolved in 1 mL H_2O with various concentrations (50, 100, and 200 $\mu g \cdot ml^{-1}$). Then the V(IV) NPs aqueous solution was irradiated by 808 nm laser (1 W cm^{-2}) for 10 mins. FLIR E05 infrared imaging devices with an accuracy of 0.1°C detected the temperature of the V(IV) NPs solutions perpendicularly to the laser path to avoid direct light irradiation on the probe. The temperature was recorded every 2 min.

7. Photothermal stability

V(IV) NPs aqueous solution (100 μg/mL) was irradiated by 808 nm laser at 1 W

cm^{-2} power density for 10 min. Then the power was turned off for natural cooling to room temperature. This cycle is repeated 5 times in total, and the infrared thermal camera was employed to continuously record the temperature change of the V(IV) NPs aqueous solution.

8. Photothermal conversion efficiency

To obtain the photothermal conversion efficiency (η) for V(IV) NPs solution, the heating curve of V(IV) NPs solutions was monitored. V(IV) NPs dispersion was recorded upon 808 nm laser irradiation (1 W cm^{-2}) for 10 min to achieve the maximum plateau of temperature, and the laser was turned off for natural cooling to room temperature. Thus, η can be calculated by referring to the following Equation (1)

$$\eta = \frac{hS(T_{Max} - T_{Surr}) - Q_{Dis}}{I A_{808}}$$

Where *h* is the heat transfer coefficient, S is the surface area of the container, T_{Max} is the temperature change of the V(IV) NPs solution at the maximum temperature, T_{surr} is the surrender temperature, I is the laser power, A_{808} is the absorbance of V(IV)NPs at 808 nm, Q_{Dis} is the heat associated with the light absorbance of the solvent, and η is the photothermal conversion efficiency. hS can be calculated referring to the following Equation (2)

$$\tau_s = \frac{mCp}{hs} \qquad \ldots\ldots (2)$$

Where τ_s is the time constant, m is the mass of the solution, and Cp is the heat capacity of the corresponding solvent. Finally, the photothermal conversion efficiency can be calculated from the method above.

9. Molecular docking of vanadate and Na/K-ATPase

The protein crystal structure was downloaded from the PDB database (α-submit of Na/K-ATPase, PDB number 3A3Y). Molecular docking was performed with a genetic algorithm (repeated 30 times). All the processes were carried out by AutoDock software. The result of the interactions and the binding pocket was analyzed by PyMol.

10. Cell culture

Murine breast cancer cells (4T1) were cultured in RPMI 1640 medium supplemented with 10% FBS and 1% penicillin-streptomycin at 37°C in a humidified

atmosphere of 5% CO_2.

11. *In vitro* Cytotoxicity

The 4T1 Cells were seeded into the 96-well plate at a density of 5×10^3 cells/well beforehand. After 24 h incubation, cells were treated with V(V) compounds (1 mM) and maintained at 37℃ or 45℃ for 10 min. For the test on the cytotoxicity of V(IV) NPs, V(IV) NPs solutions with different concentrations (0, 0.1, 0.25, 0.5, 1, 2 mM (vanadium)) were added to the culture medium. After 6 h, cells were irradiated with or without laser (808 nm) for 8 min. The treated cells were incubated for 24 h, then the standard MTT assay was employed to evaluate the performance of the mild hyperthermia effect. MTT reagent (10 μL of 5 mg/ml solution in PBS buffer) was added to each well and the plates were incubated for another 4 h. Measurements of absorbance were subsequently completed with a Bio-Rad plate reader (Petromax M3) at 570 nm (peak absorbance) and 650 nm (background absorbance).

12. Cellular Na/K-ATPase activity assay

To evaluate the Na/K ATPase activity, 4T1 cells were seeded and cultured in 6-well plates at a density of 4×10^5 cells/well for 24 h. The medium was replaced with a fresh cell culture medium containing V(IV) NPs (1 mM). After 6 h incubation in the dark, the cells were irradiation by 808 nm laser (1 W/cm^2), and the temperature was detected by FLIR E05. The treated cells were incubated for 24 h, the cells were collected by cell scraper and the Na/K ATPase activity was measured by Micro Na/K-ATPase Assay Kit.

13. Live/Dead Cell Staining

For the Live/Dead staining, 4T1 cells were treated with the V(IV) NPs incubation. The treated cells were then stained with a PBS buffer containing Calcein-AM and PI for 30 min in the cell-cultured container. After PBS wash, the cell samples were imaged on CLSM with the excitation at 488 nm and 532 nm.

14. Animal model

Female Balb/C mice (18-22 g) bought from Beijing Vital River Laboratory Animal Technology *Co. Ltd.* were used to establish breast cancer mouse models and raised in SPF animal rooms. The 4T1 cells (1×10^6 cells/100 μL in 1:1 (v/v) PBS and

Matrigel) were injected subcutaneously into the back of the mouse.

15. Hemolysis Assays

The hemolytic activities of V(IV) NPs (PBS, pH 7.4) were evaluated. Briefly, fresh blood was collected and washed with PBS to prepare RBC suspensions. Then, 400 μL of RBC suspension was mixed with the above samples at various concentrations, followed by incubation at room temperature for 2 h. RBCs treated with 1% Tweem-80 and PBS solution were set as the positive control and negative control, respectively. The rate of hemolysis in each group was measured by a microplate read.

16. Photoacoustic (PA) imaging

V(IV) NPs were injected in transparent piping, and the PA signal was recorded by MSOT in 680 nm to 850. The intensity was calculated by viewMSOT. For *in vivo* PA Imaging, V(IV) NPs with a dosage of 10 mg kg^{-1} were injected into 4T1-tumor-bearing nude mice *via* the tail vein. PA signal was recorded by MSOT at different time points (0, 2, 4, 8, and 24 h) and the intensity was calculated by viewMSOT.

17. *In vivo* therapeutic effects

When the tumors grew to 100 mm^3, the mice were randomly divided into four groups (I: PBS, II: PBS +L, III: V(IV) NPS, IV: V(IV) NPs+L). Each group contained 8 mice. The PBS and V(IV) NPs (10 mg kg^{-1}, 100 μL) were injected *via* tail vein injection, and the 808 nm laser was employed to irradiate the tumor region. The IR thermal images and real-time temperature of PBS, V(IV) NPs were recorded during the irradiation (808 nm 1W cm^{-2}). Then mild hyperthermia conditions were mantained by adjusting the power of the NIR light. Tumor site Na/K-ATPase active were measured after 48 h treatment. The tumor volumes and the body weights were measured every two days, and calculated by the formula: Volume = (Length) × $(\text{Tumor width})^2/2$. After 14 day-treatment, all mice were sacrificed, and tumors of mice in different treatment groups were fixed and sectioned for H&E and TdT-mediated dUTP nick-end Labeling (Tunel) staining.

18. Toxicity and system side effects

On the 14th day, blood was collected from each group by eyelid blood sampling. The blood samples were centrifuged at 3000 rpm for 15 min to collect serum, which

was further used for analysis the Na/K ATPase activity. The major organs (heart, liver, spleen, lung, and kidney) were collected for pathology analysis.

19. Statistical analysis

Data are presented as mean ± SD. Asterisks indicate significant differences (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ and n.s.: not significant) analyzed by unpaired Student's two-sided t-test.

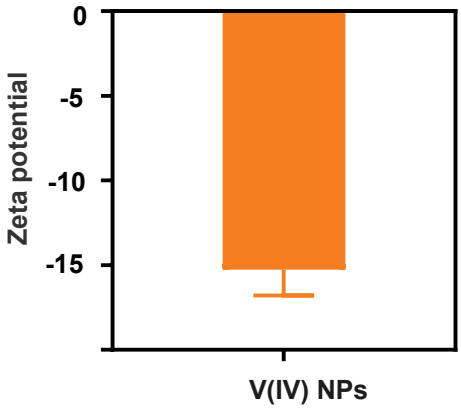


Figure S1. Zeta potential of V(IV) NPs.

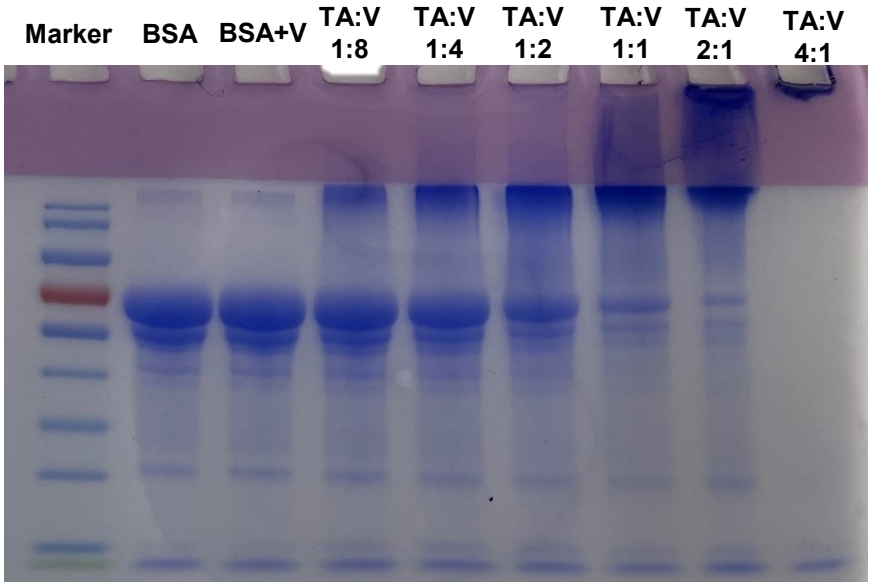
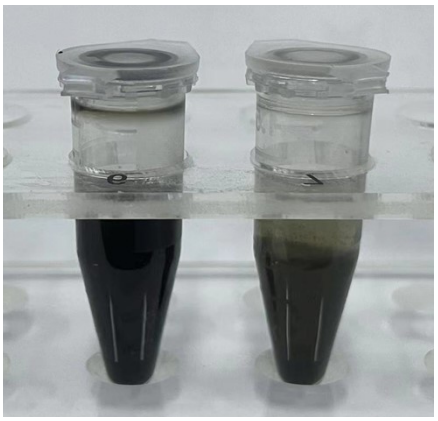


Figure S2. SDS-PAGE of V(IV) NPs with different ratios of TA-V.



BSA(+) **BSA(-)**

Figure S3. Synthesis of TA-V complex with (left) and without (right) BSA.

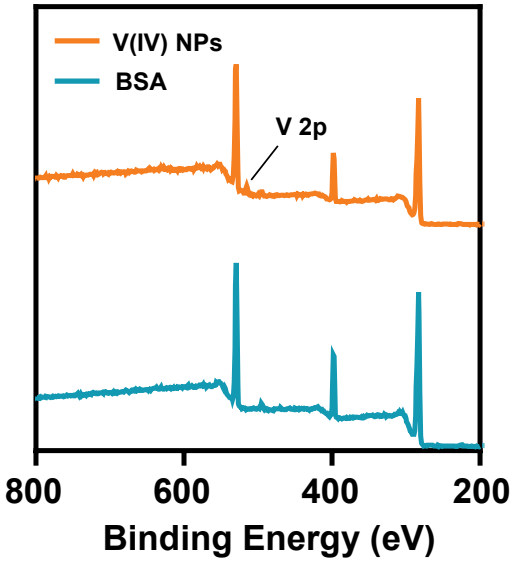


Figure S4. X-ray photoelectron spectroscopy of BSA and V(IV) NPs.

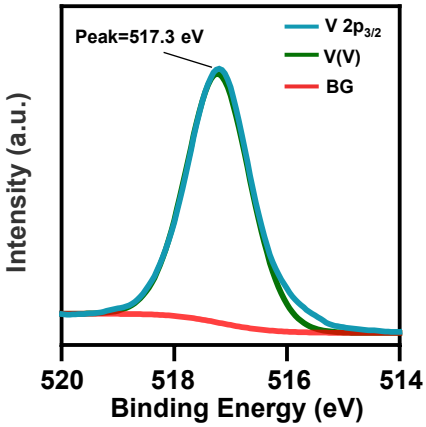


Figure S5. High-resolution X-ray photoelectron spectroscopy of V $2p_{3/2}$ of H_2O_2 treated V(IV) NPs. The fitting results of the spectra showed one characteristic peak at 517.3 eV, which was attributed to V(V).

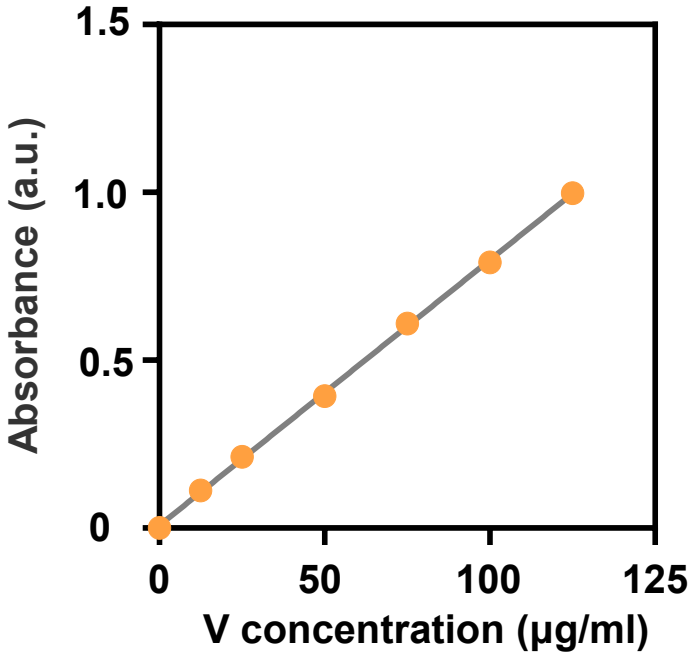


Figure S6. Proportional relationship with the vanadium concentration and absorbance of 808 nm.

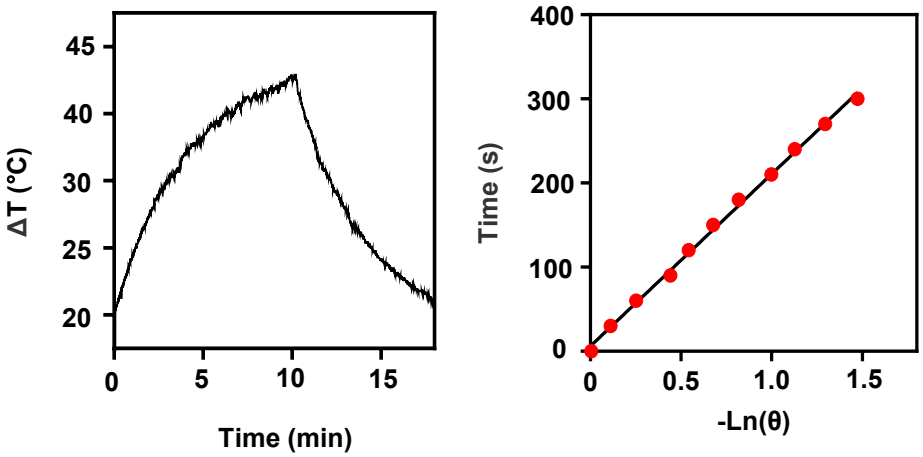


Figure S7. (A) Heating and cooling rate curve of V(IV) NPs. (B) Fitting curve of time vs. −ln(θ).

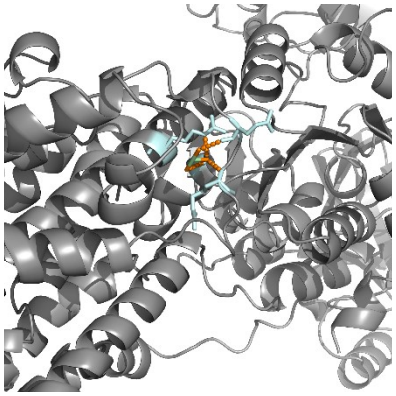


Figure S8. The results of molecular docking of overall structures of vanadate and α-submit of Na/K-ATPase (orange represents vanadium atom, green represents oxygen atoms, gray represents the unbinding amino acid and cyan represents binding amino acid residues, PDB number:3A3Y).

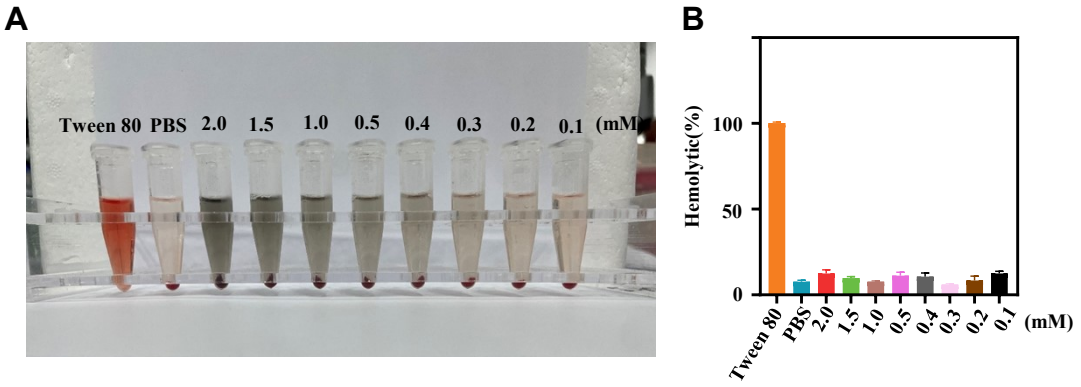


Figure S9. Hemolysis analysis of V(IV) NPs. (A) Photograph of V(IV) NPs treated RBCs. (B) Hemolytic percentages of different concentrations V(IV) NPs.

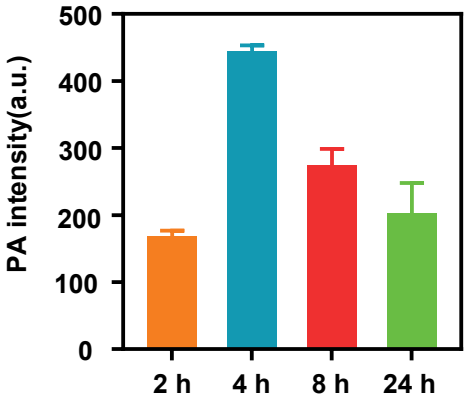


Figure S10. Photoacoustic signal intensity of tumors in mice taken at different time points post *i.v.* injection of V(IV) NPs. Data were expressed as mean ± standard deviation (SD)

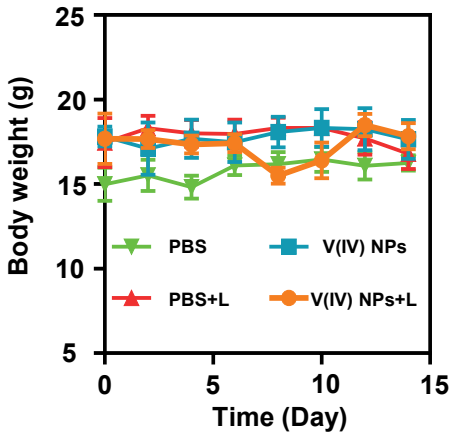


Figure S11. Body weight changes in different groups during the treatment.

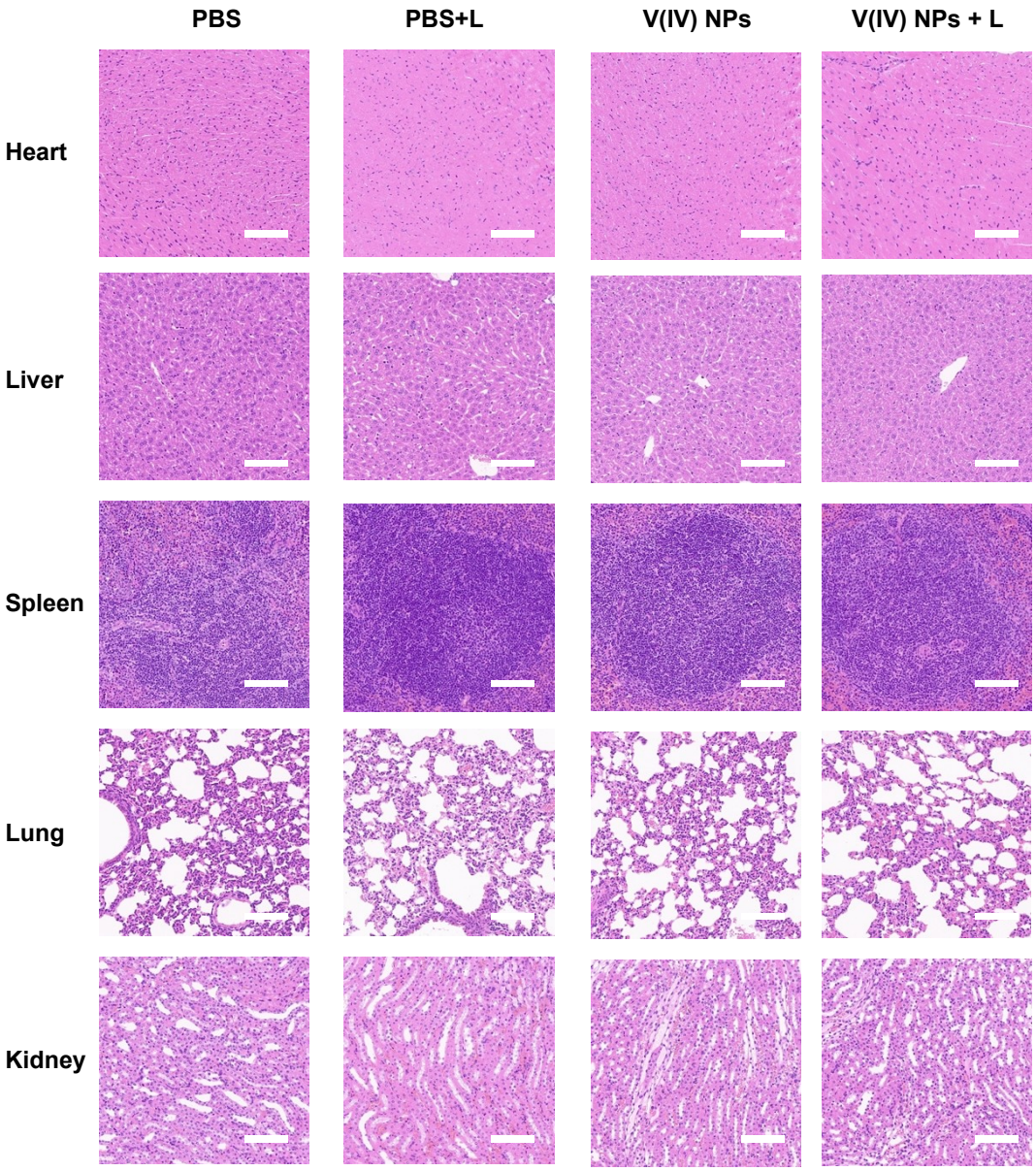


Figure S12. H&E images of major organs after different treatments. Scale bar = 100 μm.