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Supporting information

Prodrug-loaded semiconducting polymer hydrogels for deep-tissue sono-immunotherapy of orthotopic glioblastoma

Liyun Zhu[a,1], Xing Wang[a,1], Mengbin Ding[a], Ningyue Yu[a], Yijing Zhang[a], Hongwei Wu[a,*], Qin Zhang [c,*], Jiansheng Liu[b,*], Jingchao Li[a,*]

[a] State Key Laboratory for Modification of Chemical Fibers and Polymer Materials, Shanghai Engineering Research Center of Nano-Biomaterials and Regenerative Medicine, College of Chemistry, Chemical Engineering and Biotechnology, Donghua University, Shanghai 201620, China

[b] Department of Neurology, Zhongshan-Xuhui Hospital, Fudan University, Shanghai 200032, China

[c] Institute of Translational Medicine, Shanghai University, Shanghai 200444, China

[1] The authors contributed equally to this work.

*Corresponding authors:

wuhongwei@dhu.edu.cn, (H. Wu), sabrina_1985@shu.edu.cn,(Q. Zhang) drjianshengliu84@gmail.com (J. Liu), jcli@dhu.edu.cn (J. Li)

1. Materials

NLG919 was purchased from MedChemExpress (USA). Pluronic F127 (F127), methoxy-poly(ethylene glycol) amine (mPEG-NH_2, M_w = 2000), 1-ethyl-3-(3-di-methylaminopropyl) carbodiimide (EDC), N-hydroxysuccinimide (NHS), semiconducting polymer PFODBT and 4-dimethylaminopyridine (DMAP) were purchased from Sigma-Aldrich (USA). Sodium alginate (ALG) and anhydrous calcium chloride ($CaCl_2$) were supplied by Aladdin Reagent Co. Ltd. (Shanghai, China). Singlet oxygen sensor green (SOSG) and cell counting kit-8 (CCK-8) were obtained from Molecular Probes Lnc. (USA). Adenosine triphosphate (ATP) assay kit and high-mobility group box 1 protein (HMGB1) assay kit were provided by Beyotime Biotechnology Co. (Shanghai, China). All other chemicals, if not otherwise specified, were supplied by Sinopharm Chemical Reagent Co. (Shanghai, China). The TK linker was synthesized according to our previous reported method [1].

2. Characterization Methods

The morphology of the hydrogels was obtained using scanning electron microscopy (SEM, HITACHI, Japan). Fluorescence spectra were obtained by a fluorescence spectrophotometer (RF-6000, SHIMADZU, Japan) and UV absorption spectra were obtained using UV-vis spectrophotometer (TU-1810, Beijing, China). The release of NLG919 was determined by a LC-16 high performance liquid chromatography (HPLC, SHIMADZU, Japan).

The experiments based on confocal microscopy were performed using two channels, blue and green. The blue channel has an emission wavelength of 405 nm and an emission wavelength of 417-477 nm, while the green channel has an excitation wavelength of 488 nm and an emission wavelength of 500-550 nm.

3. Synthesis of mPEG-TK-COOH

28 mg (125 μmol) of COOH-TK-COOH linker, 24 mg (125 μmol) of EDC and 14.5 mg (125 μmol) of were dissolved in a mixed solution of dimethyl sulfoxide and MES buffer (1:1, v/v), stirring for 2 hour (h) at room temperature [1]. Next, mPEG-NH_2 (50 mg, 25 μmol) dissolved in MES (3 mL) was added dropwise to the above solution and stirred for another 72 h. The end products were obtained by dialysis (molecular weight cut-off = 1000 Da) and lyophilization. The product was evaluated by proton nuclear magnetic resonance spectroscopy (^{1}H NMR, Bruker, Ettlingen, Germany).

4. Synthesis of mPEG-TK-NLG919

mPEG-TK-COOH (100 mg, 45 μmol), EDC (18 mg, 90 μmol), DMAP (6 mg, 45 μmol) were dissolved in 2 mL of dichloromethane and stirred for 24 h at room temperature. NLG919 (13 mg, 45 μmol) dissolved in dichloromethane (1 mL) was slowly added to the above solution and stirred for another 24 h at room temperature. The organic solvents were evaporated under vacuum and distilled water was added to rehydrate the reaction products. Then, the products were purified by dialysis (MWCO = 1000) and lyophilized thereafter. ^{1}H NMR was used to verify the successful synthesis of the product.

5. Preparation of SPNs

Water-soluble SPNs were prepared using nanoprecipitation method. 0.5 mg of PFODBT and 10 mg of F127 were dissolved in tetrahydrofuran (THF, 1 mL) and deionized water (9 mL), respectively. Then, the PFODBT solution was added to the F127 aqueous solution under ultrasound. The mixed solution was then placed in the fume hood for 24 h to volatilize THF. The solution was then purified by ultrafiltration to obtain SPNs. Particle sizes and zeta potentials of nanoparticles were evaluated by a Zetasizer Nano ZS particle analyzer (Malvern Instruments, Malvern, UK).

6. Preparation of prodrug hydrogels

An ALG solution (5 mg/mL) containing pegylated NLG919 prodrug mPEG-TK-NLG919, SPNs was injected into $CaCl_2$ solution (Ca^{2+}, 1.8 mM) to obtain prodrug-loaded hydrogel APN. AP hydrogel without prodrug was prepared using similar methods. The gelling process after injection was video recorded. The surface topography of the hydrogel was observed by SEM.

7. $^1O_2$ generation measurements

To detect the efficiency of $^1O_2$ production in prodrug hydrogels, $^1O_2$ fluorescent probe (SOSG) was used. ALG solution containing SPNs (5 μg/mL) with or without mPEG-TK-NLG919 was injected into $CaCl_2$ solution (Ca^{2+}, 1.8 mM) and then with 3 μL SOSG solution (150 μM). SPNs without ALG were also evaluated. The fluorescence intensity of the mixed solution at 528 nm (F_0) was measured using a fluorescence spectrometer (excitation wavelength of 488 nm) before US irradiation. The solution was then irradiated with US (1.0 W/cm^2) for different times. The fluorescence intensity, defined as F, was measured after different times of US irradiation. The efficiency of $^1O_2$ production was measured by calculating the ratio of fluorescence intensity enhancement (F/F_0).

8. Evaluation of NLG919 release

To investigate the release of NLG919 under US irradiation, APN hydrogels were prepared. The release of NLG919 at different times was evaluated by measuring the NLG919 content in the supernatant using HPLC after different times of ultrasound irradiation. The mobile phase consisted of acetonitrile: distilled water (90:10, v/v) at a flow rate of 0.5 mL/min, the temperature of the column was set at 40 °C, and a UV detector was set at 300 nm.

9. In vitro cytotoxicity assessment

To evaluate the biocompatibility of hydrogels or SPNs, C6 glioma cells were seeded in 96-well plates at a cell density of 10^4/well. After 24 h of incubation for attachment, different concentrations of SPNs, AP, APN and Ca^{2+} solution (final concentration, 1.8 mM) were added to each well. Cell viability was then detected using CCK-8 kit after 24 h of incubation.

10. In vitro evaluation of the treatment effect of the hydrogel

To evaluate the therapeutic effect of APN hydrogel in vitro, C6 cells were seeded in 96-well plates at a cell density of 10^4/well and cultured in cell culture incubator for 24 h. AP or APN solution (PFODBT, 25 μg/mL) as well as Ca^{2+} solution was added to each well and incubated in incubator for 12 h. The cells were then irradiated by US (1.0 W/cm^2) for 2 min. After another incubation for 12 h, cell viability was measured using CCK-8 method.

11. Intracellular $^1O_2$ assay

Fluorescent probe DCHF-DA was used to detect the generation of intracellular ROS by AP and APN hydrogels. C6 cancer cells are seeded in 24-well plates at a cell density of 5 × 10^4/well and cultured in an incubator for 24 h. AP or APN solution (PFODBT concentration of 25 μg/mL) with Ca^{2+} solution was added and then cultured for 12 h. The culture medium was replaced with DCFH-DA (10 μM)-containing medium and then cells underwent 30 min incubation. US irradiation (1.0 W/cm^2) was performed for 2 min per well. Fluorescence images were acquired by inverted fluorescence microscopy (DMi8, Germany). The cell fluorescence after different treatments were semi-quantified using ImageJ software.

12. Detection of extracellular ATP in vitro

C6 cells were seeded at a density of 5 × 10^4/well in 24-well plates and cultured for 24 h. Cells were then treated with phosphate-buffered saline (PBS), AP or APN hydrogel solution and Ca^{2+}

solution for 12 h. Then, US (1.0 W/cm^2) was applied for 2 min, and then incubated for another 6 h. The cell culture medium was then centrifuged and the supernatant was aspirated. 20 μL of supernatant was added to 100 μL of ATP detection reagent. The ATP concentration was calculated by measuring the chemiluminescence value of the mixed solution by a microplate reader.

13. Evaluation of in vitro HMGB1 release

C6 cells seeded in 24-well plates were treated with PBS, AP, APN with or without US irradiation (1.0 W/cm^2). After culture for another 6 h, the cell culture medium was centrifuged and the supernatant was measured using ELISA kit (JYM0108Gu, jymbio, Wuhan, China) to detect the concentration of HMGB1.

14. Evaluation of intracellular calreticulin (CRT) expression

C6 cells were seeded in 24-well plates for 24 h and treated the same way as described above and immunofluorescence staining was used to evaluate the expression level of CRT.

15. Evaluation of tryptophan (Trp) metabolism at the cellular level

To evaluate the cellular Trp metabolism after different treatments, Trp and kynurenine (Kyn) were measured [2]. C6 cells were seeded in 24-well plates and interferon-γ (IFN-γ) was added to the culture medium for 24 h. Then, PBS, AP, and APN with or without US (1.0 W/cm^2) was applied to different cells, and then cells were cultured for another 24 h. The cell culture medium was collected for centrifugation and the supernatant was obtained to determine the concentration of Trp and Kyn using HPLC.

16. Establishment of mouse orthotopic glioma model

Mouse orthotopic glioma model was established using BALB/C mice (female, 4-6 weeks old) purchased from Shanghai Jie Si Jie Laboratory Animal Co., Ltd (China). Mice were anesthetized with

pentobarbital sodium, 5 μL of C6 cell suspension (1×10^6 cells) was stereotactically injected into the right striatum (anterior/posterior: +1.0 mm, medial/lateral: −2.0 mm, dorsal/ventral: −3.0 mm). The animal experiments were approved by the Animal Care and Treatment Committee of Donghua University. Hematoxylin and eosin (H&E) staining of brain tissue from randomly picked mice 14 days after implantation was performed to confirm the growth of glioma.

17. In vivo antitumor evaluation

14 days after implantation, the orthotopic C6 tumor-bearing mice were randomly divided into 6 groups, including PBS group, PBS + US group, AP group, AP + US group, APN group, and APN + US group. 5 μL of PBS solution or ALG solution (final concentration 5 mg/mL) containing SPNs (final concentration 800 μg/mL) with or without NLG919 prodrug was stereotactically injected into the right striatum. After 24 h of drug injection, the right brain was exposed to US treatment (1.0 W/cm^2) for 10 min. 7 days after treatment, mice were euthanized to obtain brain tissue. H&E staining of brain slice was used to evaluate therapeutic efficacy.

18. Evaluation of ICD in tumors

Different groups of C6-bearing tumor BALB/c mice were treated as described above. After one day of treatment, the BALB/c mice were euthanized and the brain tissue was obtained. For ATP detection, the glioma tissue was separated, weighted, and homogenized in PBS. Then the sample was centrifuged and the ATP concentration was determined by the ATP detection kit. For the evaluation of intratumoral HMGB1 and CRT expression, the brain slices were immunofluorescently stained and the fluorescence intensity of corresponding protein was semi-quantified by ImageJ software.

19. In vivo evaluation of intratumoral T cells

Different groups of C6 tumor-bearing BALB/c mice were treated as described above. 7 days after treatment, each group of BALB/c mice was euthanized and brain tissues were extracted. The brains were sectioned, and then immunofluorescently stained with fluorescence-labelled anti-CD4, anti-CD8 and anti-Foxp3 antibodies, respectively. The fluorescent images were analyzed using ImageJ software.

20. In vivo biosafety evaluation

The body weights of mice in different treatment groups were measured every 2 days for 20 days. Normal organs including heart, liver, spleen, kidney, and lung were extracted from PBS and APN + US treated mice for H&E staining.

21. Statistical analysis

One-way analysis of variance (ANOVA) statistical analysis was used to compare the difference between different groups. Significant differences were indicated as follows in figures: $p < 0.05$ (*), $p < 0.01$ (**), and $p < 0.001$ (***).

References

[1] J. Li, D. Cui, J. Huang, S. He, Z. Yang, Y. Zhang, Y. Luo, K. Pu, Organic semiconducting pro-nanostimulants for near-Infrared photoactivatable cancer immunotherapy, Angew. Chem., Int. Ed., 2019, 58(36), 12680-12687.

[2] N. Yu, M. Ding, F. Wang, J. Zhou, X. Shi, R. Cai, J. Li, Near-infrared photoactivatable semiconducting polymer nanocomplexes with bispecific metabolism interventions for enhanced cancer immunotherapy, Nano Today, 2022, 46, 101600.

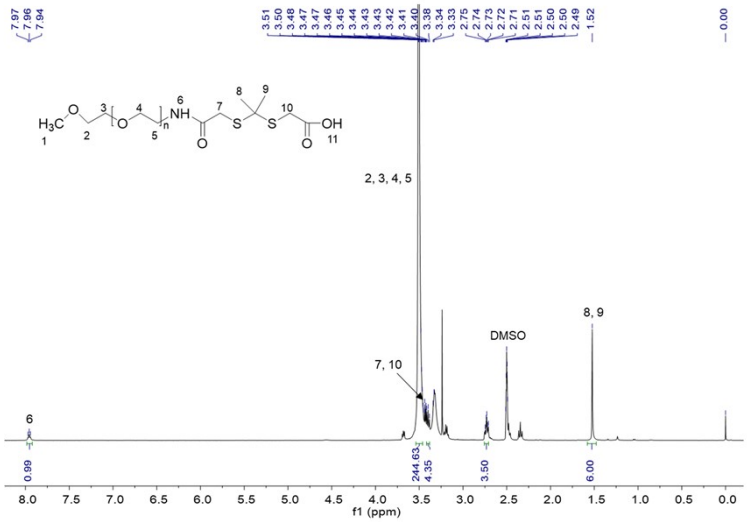

Figure S1. ^{1}H NMR spectrum of mPEG-TK-COOH in (Methyl sulfoxide)-d6.

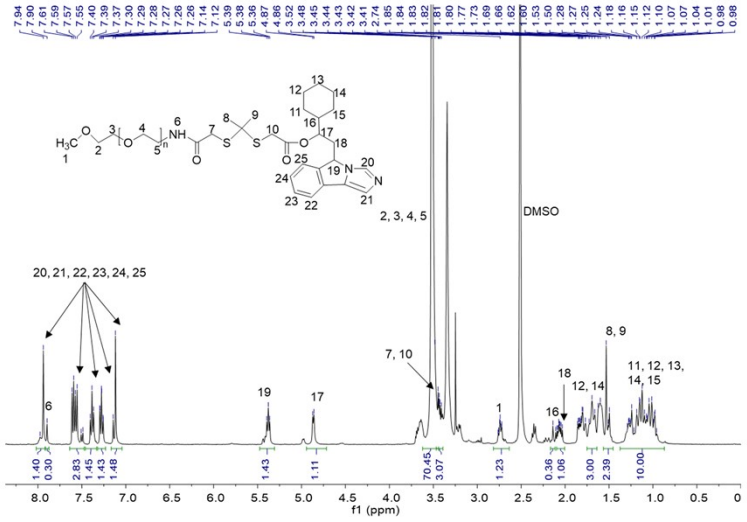

Figure S2. ^{1}H NMR spectrum of mPEG-TK-NLG919 in (Methyl sulfoxide)-d6.

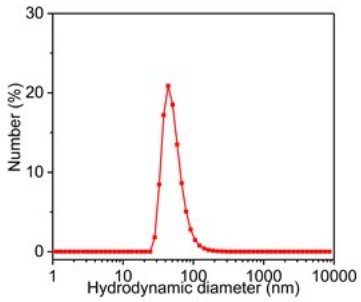

Figure S3. Hydrodynamic sizes of SPNs.

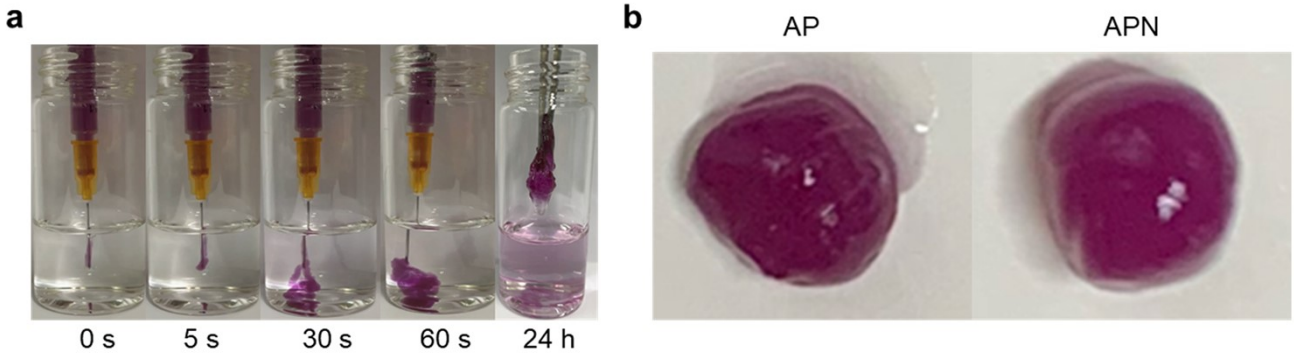

Figure S4. (a) The gel formation process of AP hydrogel and photo documentation. (c) AP and APN hydrogel image.

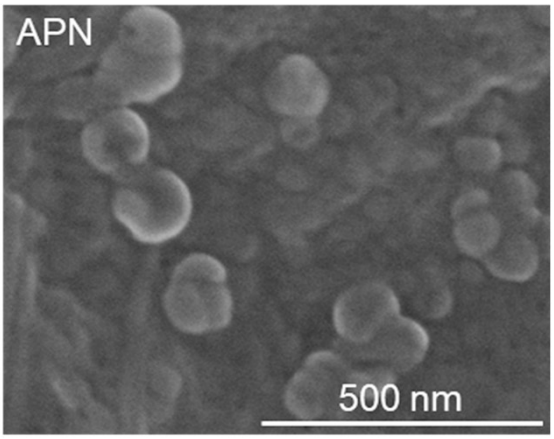

Figure S5. SEM images of APN hydrogels.

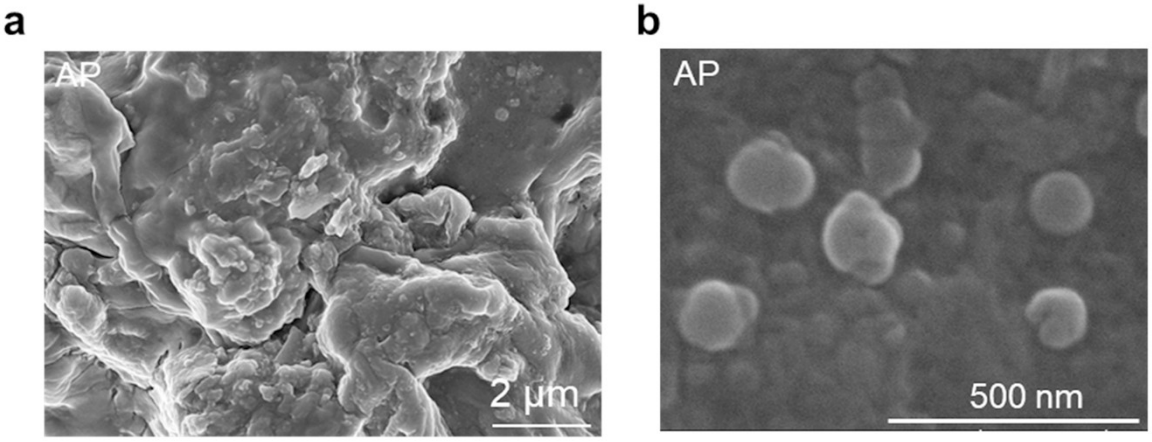

Figure S6. (a) SEM images of AP hydrogels. (b) Enlarged view of the nanoparticles in hydrogel.

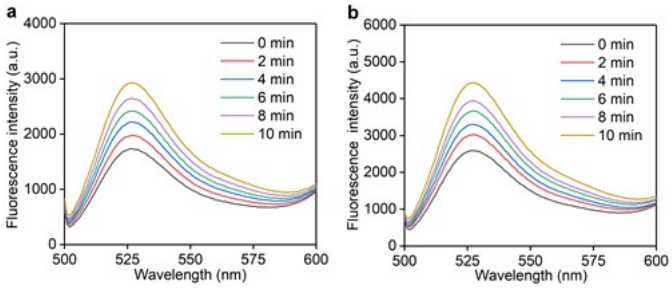

Figure S7. Fluorescence spectra of SOSG in solutions containing (a) AP hydrogels and (b) APN

hydrogels under Ultrasound irradiation (1 W/cm^2) for different time.

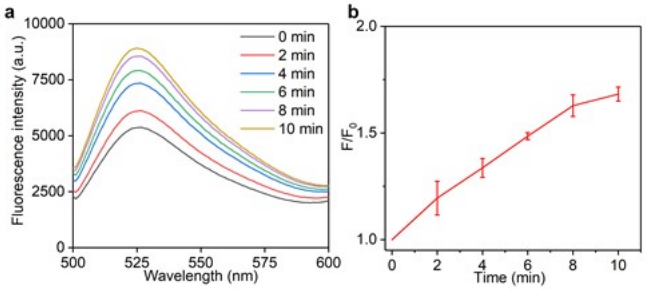

Figure S8. (a) Fluorescence spectra of SOSG in Ca^{2+} solutions containing SPNs nanoparticles under Ultrasound irradiation (1.0 W/cm^2) for different time. (b) SPNs nanoparticles $^1O_2$ generation as a function of ultrasound irradiation time.

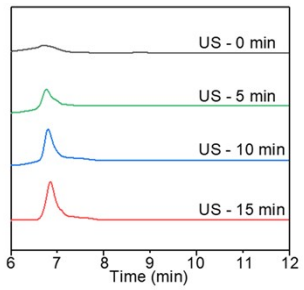

Figure S9. Release profile of NLG919 from APN hydrogel under US irradiation for different times.

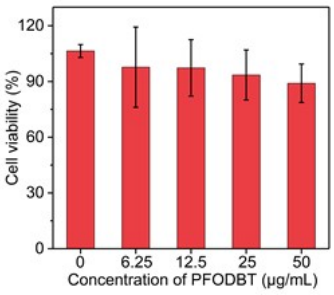

Figure S10. Cell viability of C6 cells after SPNs nanoparticles treatment.

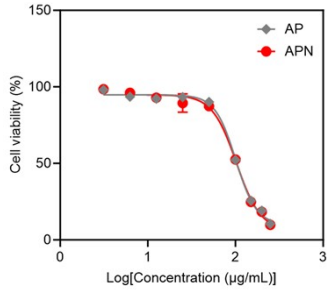

Figure S11. Cell viability of treatment with different concentrations of AP and APN hydrogel.

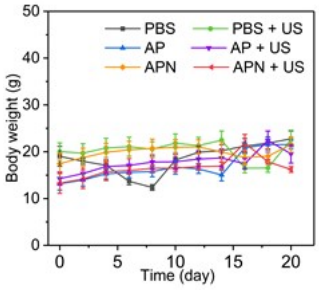

Figure S12. Body weight changes of C6 tumor-bearing BALB/c mice in different treatment groups were recorded.

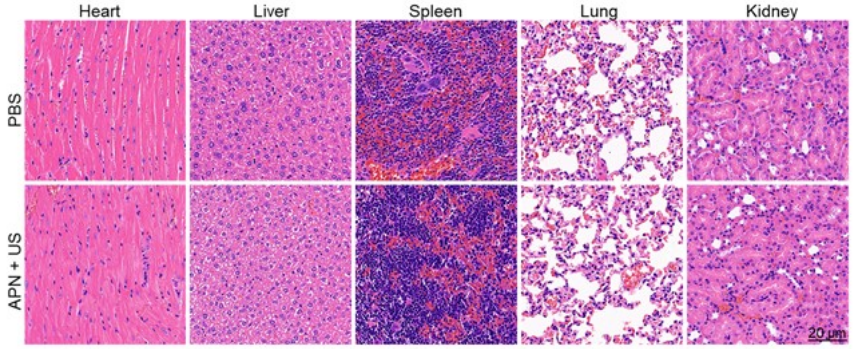

Figure S13. Heart, liver, spleen, lung and kidney H&E sections of C6 tumor-bearing BALB/c mice after different treatment.