Supporting Information

Injectable DNA-Architected Nano-Raspberry Depot-Mediated On-Demand Programmable Refilling and Release Drug Delivery

Ru-Siou Hsu,[a] Jen-Hung Fang,[a] Wei-Ting Shen,[a] Yu-Chen Sheu,[a] Cheng-Kuan Su,[b] Wen-Hsuan Chiang[c] and Shang-Hsiu Hu[a,*]

[a]*Department of Biomedical Engineering and Environmental Sciences, National Tsing Hua University, Hsinchu, 300 (Taiwan).*
[b]*Department of Chemistry, National Chung Hsing University, Taichung 402 (Taiwan).*
[c]*Department of Chemical Engineering, National Chung Hsing University, Taichung 402 (Taiwan).*

Email: shhu@mx.nthu.edu.tw

Multicellular spheroids chip fabrication

The multicellular spheroids chip was prepared by polydimethylsiloxane (PDMS) through soft lithography approach. Briefly, a negative photoresist (SU-8, MicroChem) was photolithographically patterned on silicon wafers to fabircate masters about 480 microwells for cell spheroids formation. The masters were then used as molds of the top cover and the bottom microchannel, on which a PDMS pre-polymer (Sylgard 184, Dow Corning) added into the crosslinking agent (10:1) with rapid mixing was then cured in a conventional oven at 65 °C for 3 h. The cured PDMS replicas were peeled off from the molds smoothly. A puncher with a 0.75 mm inner diameter was applied to form inlet and outlet holes on the top cover for the fluidic channel. The bottom microchannel was coated with poly(2-hydroxyethyl methacrylate) (pHEMA, BioReagent, powder, suitable for cell culture) which was dissolved in an ethanol solution (60 mg/mL in 95% ethanol). To improve the coating quality and cell formation efficiency, the bottom PDMS substrate was firstly oxygen plasma treatment treated to increase its hydrophilicity and then added 100 μL of pHEMA solution homogeneously. Afterward, the top cover and the bottom microchannel replicas were aligned and joined by oxygen plasma treatment, and then placed in a conventional oven at 65 °C for 24 h to achieve permanent bonding to obtain a complete multicellular spheroids chip.

Multicellular spheroids chip evaluation of donuts

5×10^6 cells/mL of RG2 cells were loaded into the multicellular spheroids chip. For each chip, 200 μL cell solution was injected, and until the loaded cells were settled down in the microwells, 400 μL fresh medium was applied with a high flow rate (100 μL/min) to remove the upstream cells. After the loading process, PE lines were used to fill the inlet and outlet holes and the chip was put into 10 cm dish with some wet cotton to preserve the humidity during incubation. RG2 spheroids were uniformly distributed inside each microwell as shown in the supporting information. After the formation of tumor spheroids, the drug-loading particles (10 μg/mL for each nanomaterial) were infused into the chip to estimate the therapeutic efficacy by the percent of the live cell spheroids before and after MF treatment. For the tumor penetration study, the chip treated by MF or control group were estimated by CLSM (Zeiss LSM 780), where quantum dots (QDs)-labelled particles showed in red, F-actin of cells represented in green, and nucleus (DAPI) stained in blue. Before quantifying the amounts of cell viability, Live/Dead kit was applied to figure out the cell activity. Before measurement, the cells were washed by PBS for three times to remove the free particles. To investigate the MF effect of pDox@donut on MTSs, RG2 tumor spheroids cultured with pDox@donut were subjected to MF for 5 min after 4 h of incubation, and then the pDox distribution in the spheroids was estimated after another 20 h of incubation.

***In vivo* flow cytometry analyses**

For the animal study, all surgical procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC), National Tsing Hua University, Hsinchu, Taiwan (IACUC protocol and approval number is 10704). Female C57BL/6 mice of 6 to 8 weeks old (purchased from National Laboratory Animal Center, NLAC, Taiwan) were adopted as the animal model of IHC staining. To form a subcutaneous tumor, 1×10^5 of ALTS1C1 cells in 100 μL was subcutaneously injected to mice. When the tumor volume reached 100 mm^3, 100 μL of saline solution containing 10 wt% of particles was injected at tumor via the intratumoral injection. The isolated tissues were digested by 0.1 mg/mL collagenase solution (Sigma, C0130), 1 μg/mL DNase

solution (Sigma, DN25), and 6.6 μg/mL dispase I solution (Sigma, D4818) in HBSS buffer (Sigma, H8264) for 90 minutes and then, fixed by 4% PFA for 30 minutes. The amount of cytotoxic T cells and helper T cells were analyzed by flow cytometry after the surface staining for 1 hour for primary antibody and 30 minutes for secondary antibody in the room temperature. The endothelial cells were identified using the rat-anti -CD31 antibody (BD Pharmingen, 550274, 1:200 dilution) and anti-rat-Alexa647 (Jackson, 112605167) to develop signal. The characterization of the T cell subsets was performed using fluorochrome-conjugated anti-mouse mAbs: anti-CD4 PE (BD, 553730), anti-CD3e FITC (BD, 553062), anti-CD8a APC (BD, 553035), and anti-CD45 PE-Cy7 (BD, 552848). Data were acquired using a BD FACSAria™ III flow cytometer (BD Biosciences) and analyzed with FlowJo software (Tree Star).

Immunohistochemistry (IHC) staining

For the immune response of animal study, all surgical procedures were performed in accordance with the protocol approved by the Institutional Animal Care and Use Committee (IACUC), National Tsing Hua University, Hsinchu, Taiwan (IACUC protocol and approval number is 10704). Female C57BL/6 mice of 6 to 8 weeks old (purchased from National Laboratory Animal Center, NLAC, Taiwan) were adopted as the animal model of IHC staining. To form a subcutaneous tumor, 1×10^5 of ALTS1C1 cells in 100 μL was subcutaneously injected to mice. When the tumor volume reached 100 mm^3, 100 μL of saline solution containing 10 wt% of particles was injected at tumor via the intratumoral injection. IHC staining was conducted using the protocol providing by Abcam plc. Briefly, the lungs were isolated and fixed in 4% PFA overnight and then, the tissue was embedded into optimal cutting temperature (OCT) compound (Sakura Finetek, 4583) at -80°C preparing for histologic section. After the frozen section, the samples were dehydrated and fixed in -20°C 100% methanol for 10 minutes. The fixed samples were washed by phosphate buffered saline (PBS) (Gibco, 10010023) to remove residual OCT compound. Next, the sections were incubated with the antibody solution which was diluted in 5% BSA solution at 4°C overnight with the appropriate primary antibodies, including

rat-anti-CD-4 (Abcam, ab25475, 1:1000 dilution), rabbit anti-CD8 (Abcam, ab217344, 1:1000 dilution), and rat-anti-CD31 (BD Pharmingen, 550274, 1:200 dilution). The secondary antibody was then introduced into the samples at room temperature for 1 hour, which included anti-rat-Alexa647 (Jackson, 112605167) anti-rabbit-Alexa647 (Abcam, ab150075), and anti-rat-Alexa488 (Jackson, 112545143). Finally, the sections were mounted in fluoroshield mounting medium (Abcam, ab104139) and gained the results by using the confocal microscope (Carl Zeiss, LSM800).

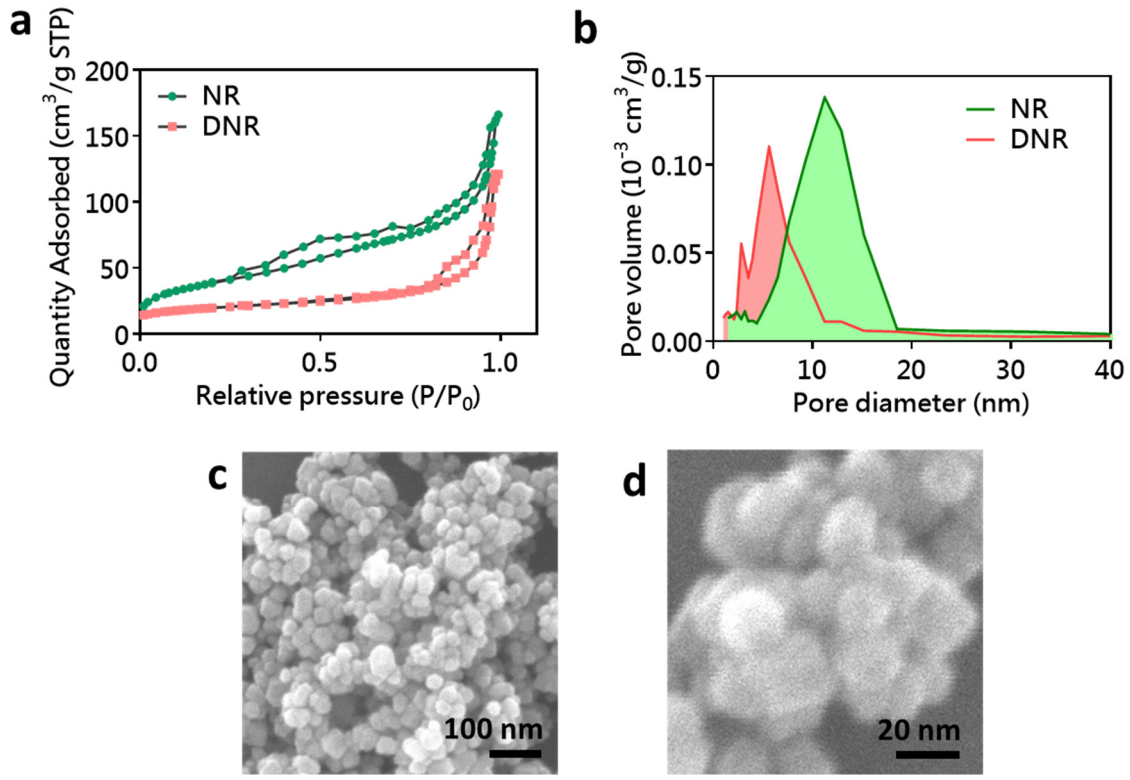

Figure S1. (a) N_2 adsorption–desorption isotherms of NR and DNR. (b) Pore diameter distribution of NR and DNR based on Barrett-Joyner-Halenda (BJH) analysis. (c, d) SEM images of NR fabricated for 24 h of hydrothermal treatment at 220 °C.

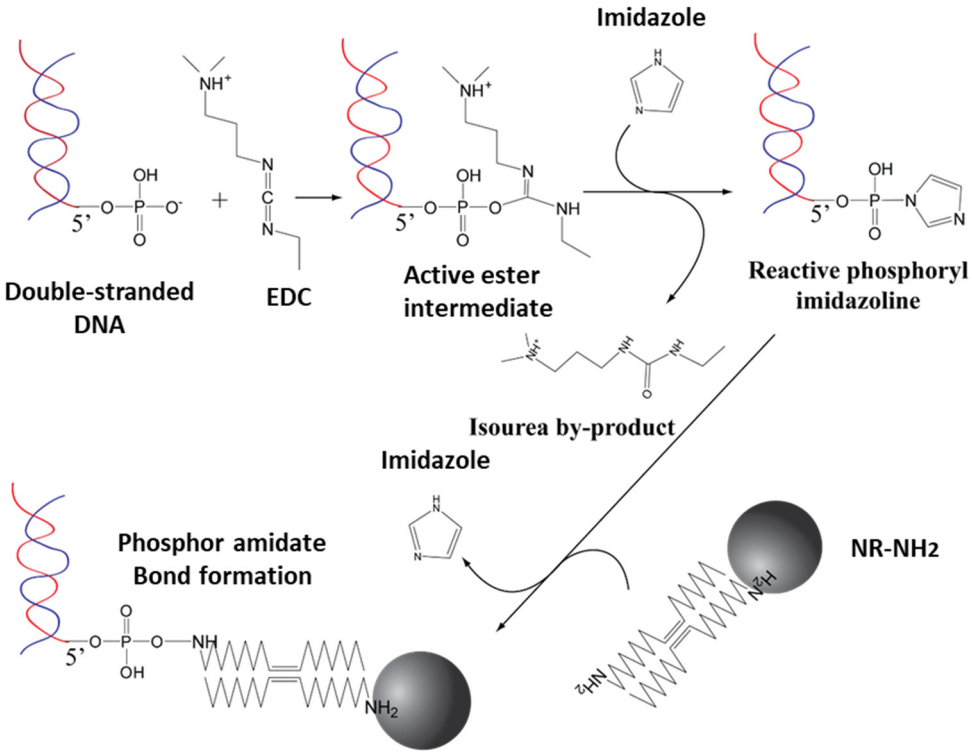

Figure S2. EDC reaction for OA-capping NR covalently conjugated by DNA through 5′-end specificity of oligonucleotide.

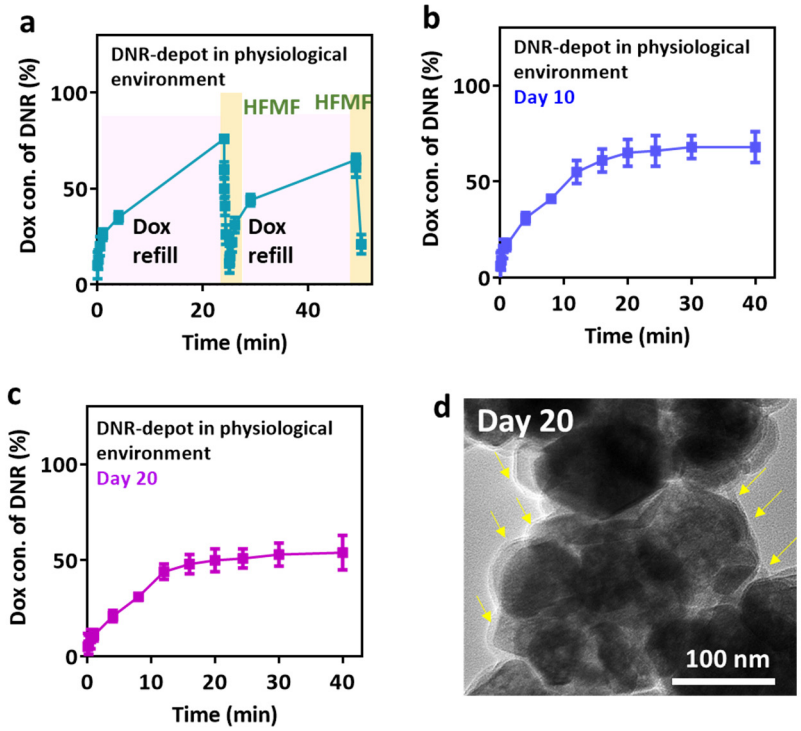

Figure S3. (a) Dox release and refilling of DNR-depot upon 2 min of HFMF treatment in physiological environment. Dox refilling to DNR-depot after depot was preserved in the physiological environment for (b) 10 and (c) 20 days. (d) TEM image of DNR after 20 days in physiological environment.

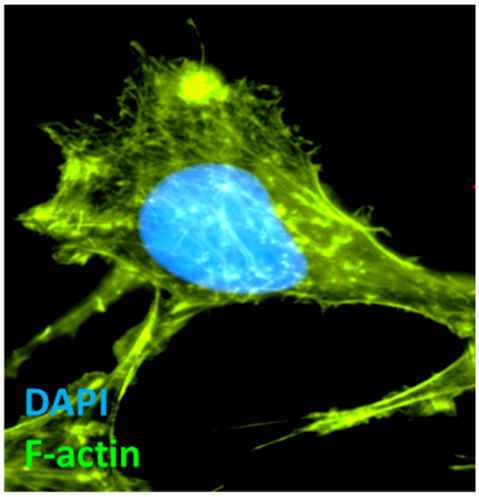

Figure S4. The control group of RG2 cells without treating any particles.

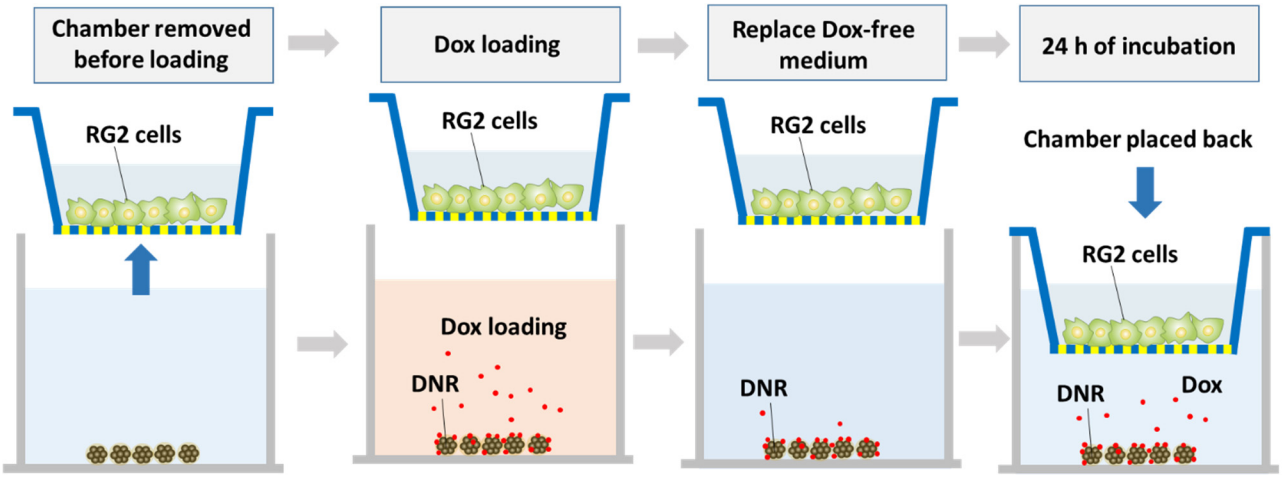

Figure S5. Dox refilling process to DNR in the transwell.

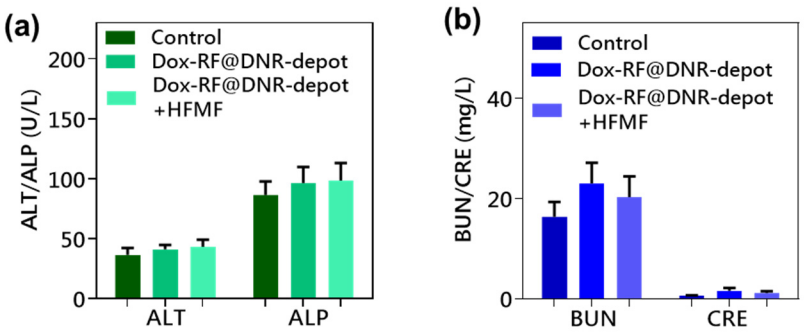

Figure S6. Liver and kidney functions of mice at 48 h post-Dox-refill (RF) to DNR-depot.

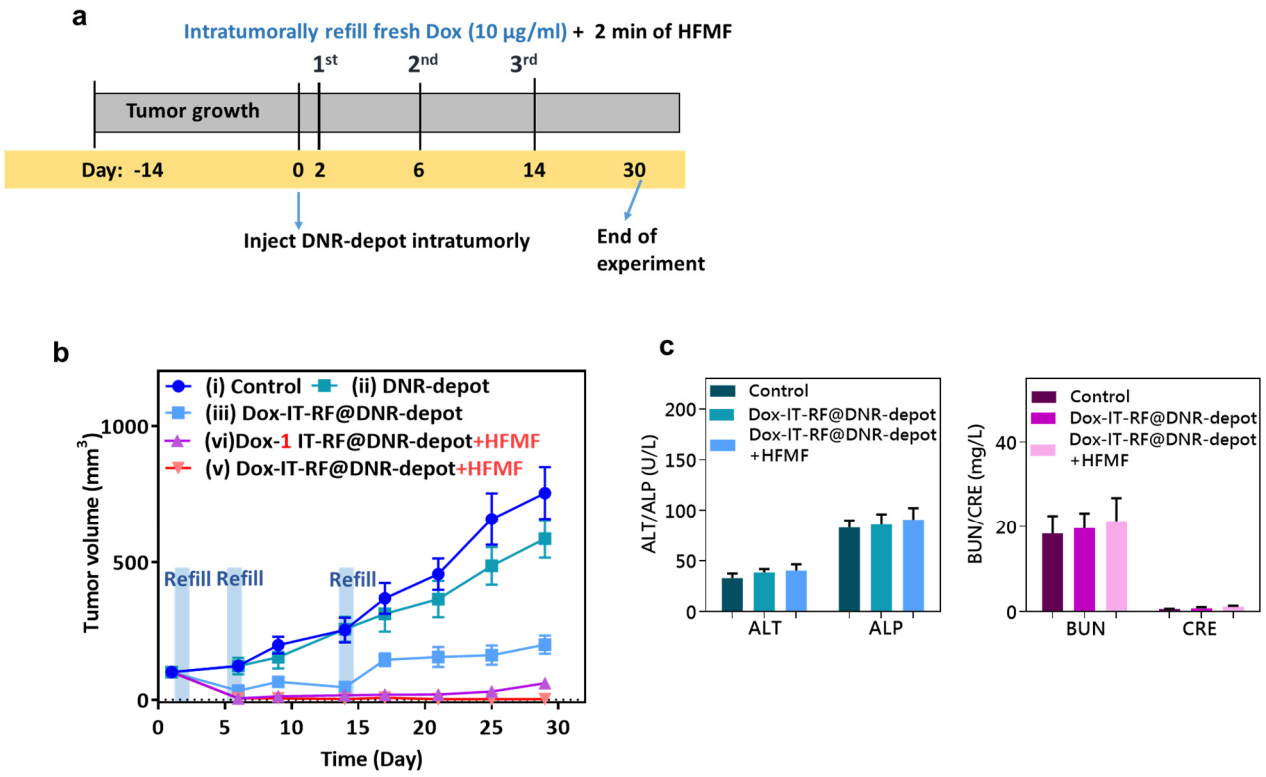

Figure S7. (a) Treatment schedule of implanting DNR-depot and intratumorally (IT) refilling Dox for tumor inhibition. (b) Tumor volumes implanting DNR-depot with intratumorally (IT) refilling Dox and HFMF treatment for 30 days. (c) Liver and kidney functions of mice at 48 h post-Dox-refill intratumorally (IT-RF) to DNR-depot.

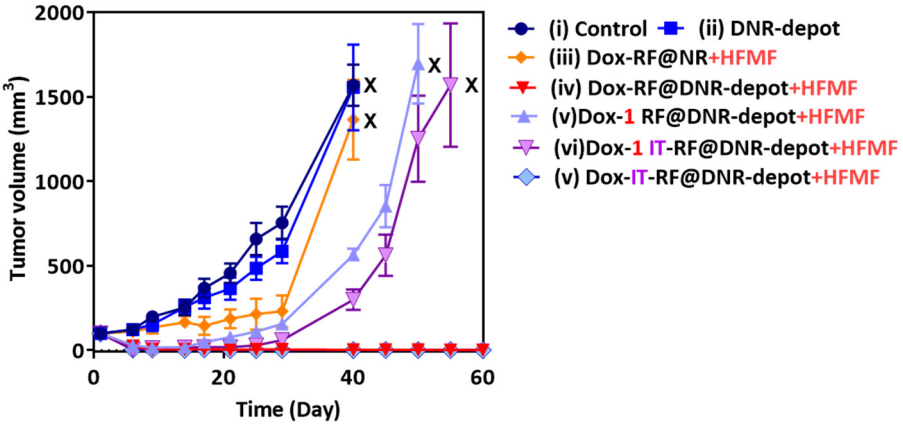

Figure S8. Tumor growth profiles of Dox-IT-RF@DNR-depot+HFMF and Dox-RF@DNR-depot+HFMF for 60 days (n=5, mean ± s.d.).

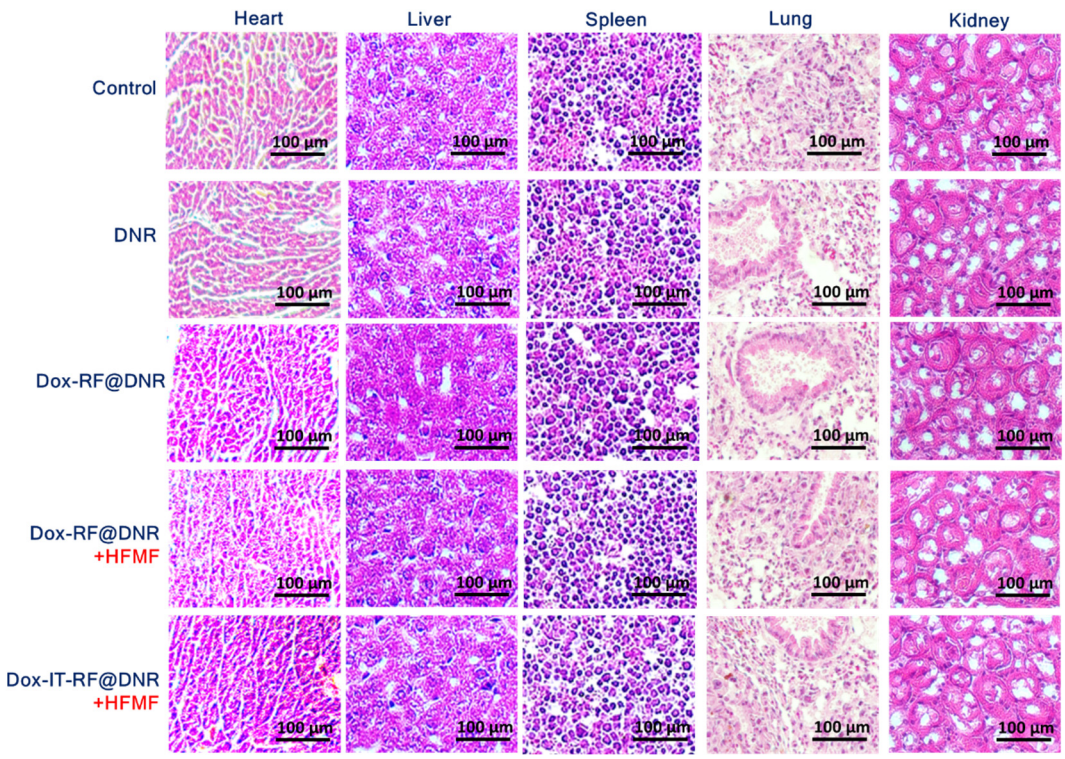

Figure S9. H&E staining of main organs of untreated and treated mice.