Supporting Information

Polyethylenimine Nanogels Incorporated with Ultrasmall Iron Oxide Nanoparticles and Doxorubicin for MR Imaging-Guided Chemotherapy of Tumors

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Part of experimental section

Materials and Reagents. Polyethylenimine (PEI, Mw = 2.5k), N, N′-methylenebisacrylamide (BIS) and Span® 80 were purchased from Sigma-Aldrich (St. Louis, MO). 1-Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC) and *N*-hydroxysuccinimide (NHS) were from J&K Scientific Ltd. (Shanghai, China). Triethylamine, acetic anhydride and toluene were from Aladdin Ltd. (Shanghai, China). All other chemicals and reagents were acquired from Sinopharm Chemical Reagent Co., Ltd. (Shanghai, China). Unless otherwise stated, all chemicals were used as received. Cell culture dishes were from Corning Incorporation (New York, NY). 4T1 cells were provided by Shanghai Institute of Biochemistry and Cell Biology, the Chinese Academy of Sciences (Shanghai, China). Dulbecco's modified Eagle medium (DMEM), penicillin, streptomycin and trypsin were from Hangzhou Jinuo Biomedical Technology Co., Ltd. (Hangzhou, China). Fetal bovine serum (FBS) was from Gibco Life Technologies Co. (Grand Island, NY). Water used in all experiments was purified using a MilliQ Plus185 water purification system (Millipore Bedford, MA) with a resistivity higher than 18.2 MΩ·cm.

Characterization Techniques. Thermogravimetric analysis (TGA) was performed using a TG209F1 Thermal Gravimetric Analyzer (NETZSCH, Selb/Bavaria, Germany). All samples were lyophilized before measurements. Each sample was heated from room temperature to 900 ℃ at the heating rate of 20 ℃/min under nitrogen atmosphere. Hydrodynamic size and zeta-potential were measured using a Malvern Nano-ZS model ZEN3600 zetasizer with a standard 633 nm laser (Worcestershire, UK). UV-vis spectra were recorded using a Perkin-Elmer Lambda 25 UV-vis spectrophotometer (Boston, MA). Samples were dispersed in water before analysis. Atomic force microscope (MFP-3D, Asylum Research, Santa Barbara, CA) was used to characterize the morphology of the materials according to protocols described in our previous work.[1] Leeman Prodigy inductively coupled plasma-optical emission spectroscopy (ICP-OES, Hudson, NH) was performed to analyze the Fe amount in the NGs. All samples were digested by *aqua regia* and diluted with water before measurements. The r_1 relativities of the ultrasmall Fe_3O_4 NPs and Fe_3O_4/PEI-Ac NGs were measured using the 0.5T NMI20-Analyst NMR Analyzing and Imaging system (Shanghai Niumag

Corporation, Shanghai, China). The parameters were set as follow: Q-IR sequence, point resolution = 156 mm × 156 mm, section thickness = 0.6 mm, TR = 6000 ms, TE =160 ms, number of excitations = 1. The r_1 relaxivity of the materials were calculated by linearly fitting the inverse relaxation time as a function of Fe concentration. The MR imaging of samples was performed using a clinical MR imaging system (3.0 T, United Imaging Medical Technology Co., Ltd., Shanghai, China). The detailed parameters were set as follows: point resolution = 256 mm × 256 mm, slice gap = 0.3 mm, TR/TE = 1959 ms/13.7 ms, and FOV = 80 × 110 mm.

Cell Biological Evaluation. 4T1 cancer cells (a mammary carcinoma cell line from the mammary gland tissue of a mouse) were continuously grown in the cell culture flask with DMEM supplemented with 10% FBS, 100 U mL^{-1} penicillin, and 100 U mL^{-1} streptomycin. The culture was maintained at 37 °C with 5% CO_2, and the medium was replaced every 3 days.

To check the therapeutic activity of the Fe_3O_4/PEI-Ac NGs/DOX complexes, cells were plated into a 96-well plate at a density of 1 × 10^4 cells per well in 0.1 mL DMEM to bring the cells to confluence. The next day, the medium in each well was replaced with fresh DMEM (100 μL) containing free DOX·HCl, Fe_3O_4/PEI-Ac NGs, or Fe_3O_4/PEI-Ac NGs /DOX complexes with different DOX concentrations (0, 2.5, 5, 10, 20, and 40 μg/mL, respectively). For the DOX-free Fe_3O_4/PEI-Ac NGs, the NG concentration was equal to that of the Fe_3O_4/PEI-Ac NGs/DOX complexes. Then the cells were incubated at 37 °C for 24 h. After treatment, cell morphology was observed by optical microscopy. Then, cell counting kit - 8 (CCK-8) assay was performed to quantify the viability of cells according to protocols reported in the literature.[1]

To check the cellular uptake of the Fe_3O_4/PEI-Ac NGs/DOX complexes, the cells were plated into a 15-mm diameter glass dish at a density of 15 × 10^4 cells per dish in 0.5 mL DMEM. The next day, the medium was replaced with fresh DMEM (200 μL) containing free DOX·HCl or Fe_3O_4/PEI-Ac NGs/DOX complexes at the same DOX concentration (10 μg/mL) and then the cells were incubated for 4 h at 37 °C. PBS was used as control. After that, the medium was dumped, the cells were fixed with glutaraldehyde (1%, v/v) for 15 min, washed with PBS, and treated with 4′,6-diamidino-2-phenylindole (DAPI, 0.1%, v/v) to stain the cell nuclei. After 30 min, the cells were

washed with PBS to remove excess DAPI.

To quantify the cellular uptake of the Fe_3O_4/PEI-Ac NGs/DOX complex, the cells were plated into a 12-well plate at a density of 15×10^4 cells per well in 0.5 mL of DMEM. The next day, the medium was replaced with fresh DMEM (0.5 mL) containing free DOX·HCl or Fe_3O_4/PEI-Ac NGs/DOX with different DOX concentrations (0, 1.25, 2.5, 5, 10, 20, and 40 μM, respectively) and then the cells were incubated at 37 °C for 4 h. After that, the cells were washed with PBS for 3 times, collected by centrifugation (1000 rpm, 5min) and resuspended in 1 mL of PBS. All the parallel groups of cells were collected for flow cytometry analysis using a BD FACS Calibur flow cytometer (Franklin, CA).

***In Vivo* Tumor MR Imaging.** All animal experiments were carried out after approval by the ethical committee for animal care of Donghua University and according to the policy of the National Ministry of Health. Female 4-6 weeks old BALB/c nude mice were purchased from Shanghai Slac Laboratory Animal Center (Shanghai, China). 4T1 cells were injected into the right hind leg of each nude mouse at a dose of 1.5×10^6 cells to build up the xenografted tumor model. When the tumor volume reached 0.5-1.2 cm^3 (about two weeks post-injection of cancer cells), each tumor mouse was intravenously injected with free ultrasmall Fe_3O_4 NPs or Fe_3O_4/PEI-Ac NGs/DOX (Fe mass = 150 μg, in 200 μL PBS). MR scanning was performed before injection and at different time points post-injection using the same clinical MR imaging system as described above with the same operation parameters.

To evaluate the Fe distribution in various organs and tumors of the nude mice at different time points post-injection of the Fe_3O_4/PEI-Ac NGs/DOX, ICP-OES analysis was carried out. After each mouse was intravenously injected with the Fe_3O_4/PEI-Ac NGs/DOX complexes (Fe mass = 150 μg, in 0.2 mL PBS), the mice were sacrificed at different time points post-injection (0.5, 1, 12, 24 and 48 h, respectively). The organs (heart, liver, spleen, lung, and kidney) and tumors was removed, weighed, cut into small pieces and digested by *aqua regia* for 24 h. The concentration of Fe in the organs and tumors was measured by ICP-OES. Mice injected with PBS (0.2 mL) were used as control. The results of three parallel nude mice were used for each group.

***In Vivo* Tumor Therapy.** The BALB/c nude mice were chosen to construct subcutaneous 4T1 tumor model according to protocols described above. The tumor-bearing nude mice were randomly divided into 4 groups (n = 5 for each group): PBS control group (200 μL PBS), free DOX·HCl group ([DOX] = 20 μg/mL, in 200 μL PBS), Fe_3O_4/PEI-Ac NGs/DOX group ([DOX] = 20 μg/mL, in 200 μL PBS), and drug-free Fe_3O_4/PEI-Ac NGs group with NG concentration equal to the drug-loaded NG group (in 200 μL PBS). All doses were intravenously injected through the tail vein for each mouse. The injection was given every three days after the 1st injection with a total of 5 doses. Tumor volume and mouse weight were measured every 3 days. Tumor volume and relative tumor volume were calculated using the following equations:

$$\text{Tumor volume (V)} = W^2 \times L/2 \quad (1)$$

where W and L represent the width and length of the tumor, respectively.

$$\text{Relative tumor volume} = V/V_0 \quad (2)$$

where V and V_0 represent the tumor volume after treatment and the tumor volume before treatment, respectively. At 30 days post treatment, representative mice were selected and sacrificed from each group, and their main organs and tumors were extracted and sliced for hematoxylin and eosin (H&E) staining according to standard protocols reported in the literature.[2]

Statistical Analysis. One-way ANOVA statistical analysis was performed to evaluate the significance of the experimental data. A value of 0.05 was selected as the significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Hydrodynamic size and zeta potential of PEI NGs, Fe_3O_4/PEI NGs and Fe_3O_4/PEI-Ac NGs.

Sample	Hydrodynamic size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
Fe_3O_4	27.5 ± 3.2	0.162 ± 0.058	-37.9 ± 1.35
PEI NGs	180.4 ± 5.5	0.234 ± 0.024	38.9 ± 0.62
Fe_3O_4/PEI NGs	251.4 ± 6.3	0.242 ± 0.003	29.3 ± 0.88
Fe_3O_4/PEI-Ac NGs	263.8 ± 3.8	0.334 ± 0.030	13.3 ± 1.15

Table S2. The drug loading content (DLC) and drug loading efficiency (DLE) of Fe_3O_4/PEI-Ac NGs/DOX.

Mass ratio (DOX/NGs)	Drug loading content (DLC)	Drug loading efficiency (DLE)
1:2	15.3%	22.9%
1:1	21.9%	43.9%
2:1	18.3%	51.4%

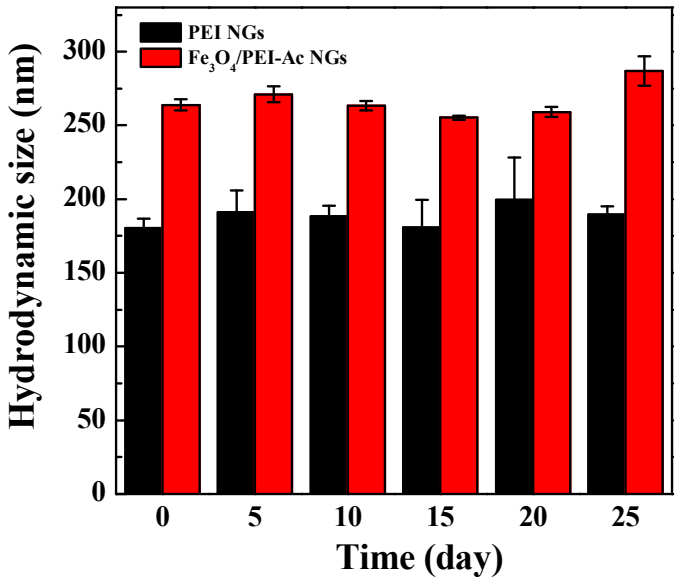

Figure S1. Hydrodynamic size of PEI NGs and Fe_3O_4/PEI-Ac NGs at different time periods.

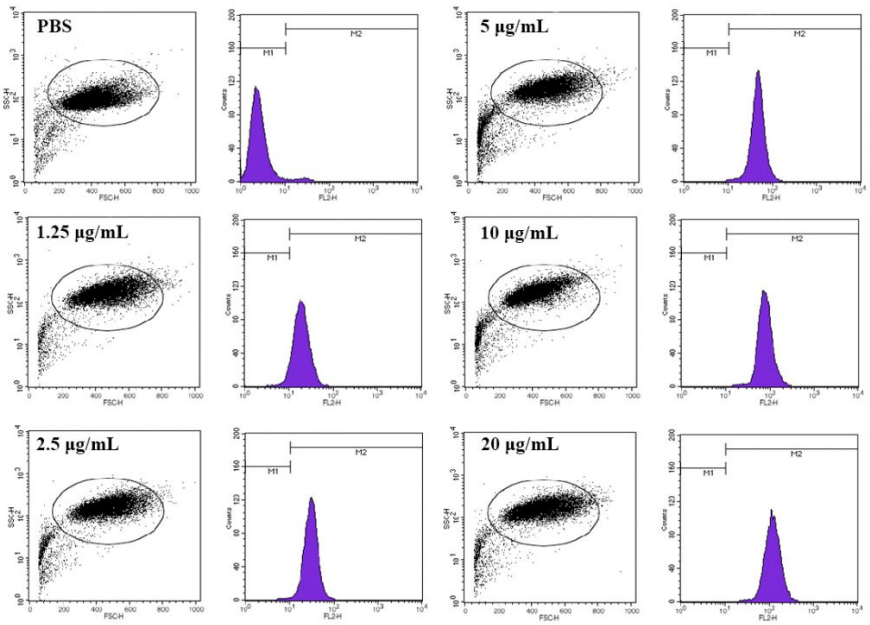

Figure S2. Flow cytometric analysis of 4T1 cells treated with Fe_3O_4/PEI-Ac NGs/DOX complexes at different DOX concentrations for 4 h. The 4T1 cells treated with PBS were used as control.

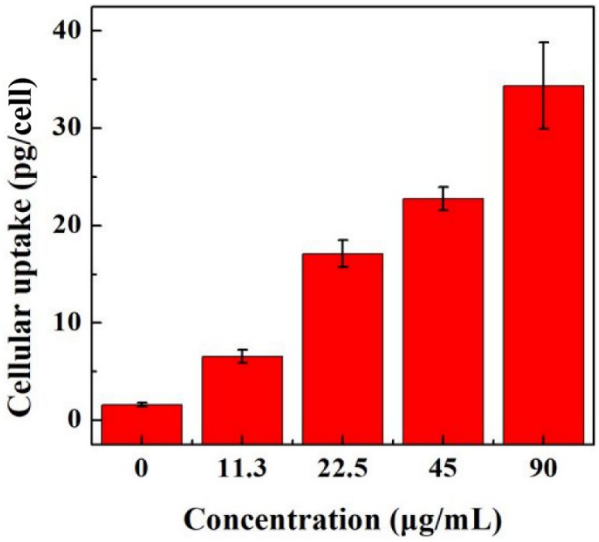

Figure S3. Fe uptake in the 4T1 cells treated with the Fe_3O_4/PEI-Ac NGs/DOX complexes at different DOX concentrations for 24 h.

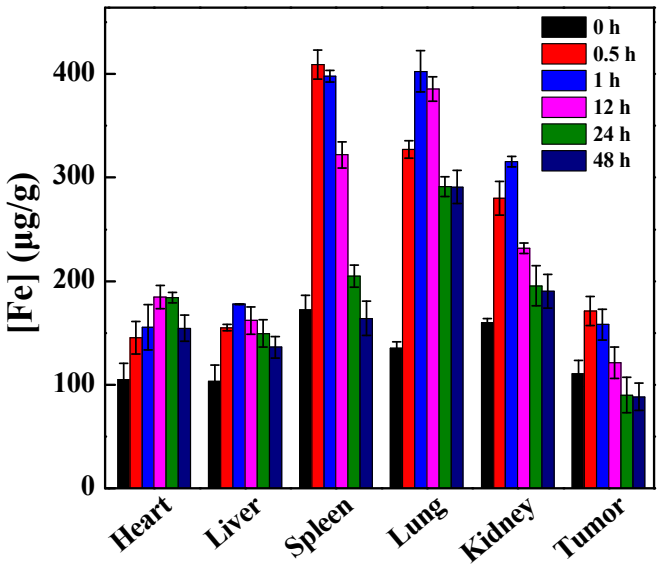

Figure S4. Fe biodistribution in major organs and tumors of the mice at different time points post-injection of the Fe_3O_4/PEI-Ac NGs/DOX complexes (Fe mass = 150 μg, in 0.2 mL PBS for each mouse).

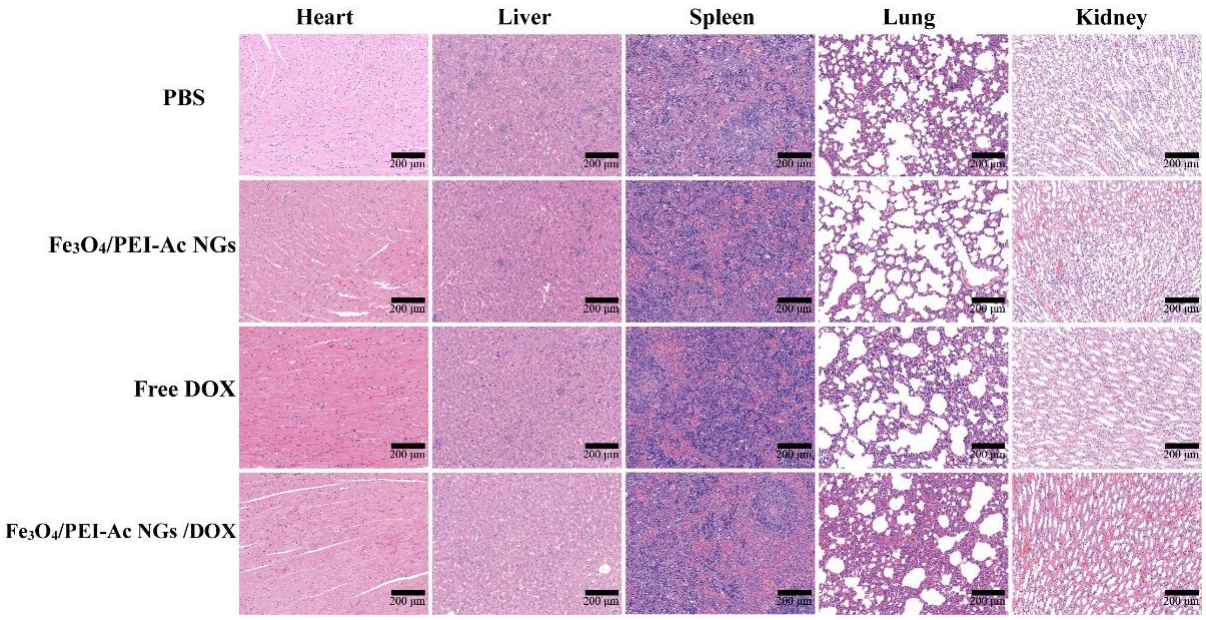

Figure S5. Representative H&E staining of the sections of the heart, liver, spleen, lung and kidney after each tumor-bearing mouse was treated with PBS, free DOX, DOX-free Fe_3O_4/PEI-Ac NGs, and Fe_3O_4/PEI-Ac NGs/DOX complexes for 30 days.

References

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(2) Sun, W., Thies, S., Zhang, J., Peng, C., Tang, G., Shen, M., Pich, A., and Shi, X. (2017) Gadolinium-Loaded Poly(N-vinylcaprolactam) Nanogels: Synthesis, Characterization, and Application for Enhanced Tumor MR Imaging. *ACS Appl. Mater. Interfaces 9*, 3411-3418.