Supporting Information

Multivalent nanoparticles for personalized theranostic based on tumor receptor distribution behavior

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1. Materials and methods

1.1. Materials

Raltitrexed (RTX) was supplied by meilunbio (Dalian, China). Folic acid (FA), FITC, EDC, NHS and acetic anhydride were obtained from J&K Chemical Ltd. Ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$), ferric chloride hydrate ($FeCl_3 \cdot 6H_2O$), citric acid, ammonia water (25 wt%), Polyethylenimine (branched, Mw =1800), MTT and other related reagents were provided by aladdin biochemical technology (Shanghai, China). KB cells, HeLa cells, A549 cells, H22 cells and HCT116 cells were provided by cobioer biological technology (Nanjing, China). RPMI 1640 medium, DMEM, and FBS were from Gibco. Deionized water used in all experiments was treated by a Millipore purification system.

1.2. Characterization

Malvern Nano-ZS90 instrument was used to conduct the DLS and zeta potential measurements. Transmission electron microscopy (Tecnai G2F20) was performed to observe the morphology and size of the NPs. UV-visible absorption spectroscopy was studied on SHIMADZU UV-2550 (Japan). The crystal structure is obtained by X-ray diffraction (XRD) (D/max 2550, Rigaku). The SQUID VSM (Quantum Design) was used to measure the superparamagnetic of the NPs. The Fe concentration was analyzed using a PerkinElmer Optima 8300 ICP-AES Spectrometer. T2 relaxometry was performed by a 1.2T HT/MRSI60-60KY NMR imaging system (Huan tong science and education equipment, China).

1.3. Preparation of Fe_3O_4-CA NPs

The Fe_3O_4-CA NPs were prepared according to a previously reported method.[36] Briefly, iron(II) chloride tetrahydrate (0.0037 mol) and iron chloride hexahydrate (0.0074 mol) were dissolved under inert gas in 40 mL of deionized water with constant mechanical stirring at 1000 rpm for 30min, then 20 mL of citric acid (10 mg/mL) aqueous solution was added and heated to 85 °C slowly. After stirring for half an hour, 6 mL of ammonia was dropped into the reaction solution within 10 min. After another 30 min at 85°C with vigorously stirring, a stable red-brown suspension was obtained. When cooled to 25°C, the obtained solution was dialyzed against a dialysis membrane (MWCO = 10 W) in deionized water (2 L) with water change every 6 hours for 2 days.

1.4. Synthesis of multivalent PEI-RTX$_n$ (PR$_n$) ligands

Different amounts of RTX (16.7 mg, 11.7 mg, 4 mg) were dissolved in DMSO, EDC and NHS were put into the above solutions with the mass ratio of (EDC: NHS: RTX =5:3:1) and stirred for 30 min to activate the carboxyl groups of RTX. Then, 1mL of PEI aqueous solution（20 mg/mL）was dropwise added into the above DMSO solution of RTX under stirring at 25℃.After one day of reaction at room temperature, the mixture was dialyzed against a dialysis membrane (MWCO = 2000) in deionized water (2 L) with water change every 12 hours for 2 days. Finally, the above solutions were lyophilized to obtain the PEI-RTX$_n$. The PEI-RTX$_n$ powders were accurately weighed and dissolved in moderate water, the RTX concentration was quantitatively determined by UV-Vis absorbance at 351 nm. The ligand valency n

was calculated by the molar ratio of RTX to PEI in each product (equation 1), also means the average number of RTX on per PEI polymer chain.

$$n = \frac{m_{RTX} * M_{PEI}}{(m_{total} - m_{RTX}) * M_{RTX}}$$

……equation (1)

1.5. Preparation of multivalent Fe-PR$_n$ (n = 2, 4, 8) NPs

Multivalent Fe-PR$_n$(n = 2, 4, 8) NPs were fabricated by surface modification of Fe_3O_4-CA NPs with PEI-RTX$_n$(n = 2, 4, 8) using an amidation reaction. The Fe_3O_4-CA NPs (1 mL, 4.9 mg) dispersed in water were mixed with EDC (0.52 mmol) and NHS (0.52 mmol), and stirring for 0.5 h to activate the carboxyl groups on the NPs. Then, PEI-RTX$_n$ (n = 2, 4, 8) contain equimolar RTX was dropped into the above activated NPs solutions. After 24 h reaction, the obtained solution were centrifuged, unreacted PEI-RTX$_n$ (n = 2, 4, 8) supernatants were collected and the quality of RTX was calculated by UV-vis, and from the total quantities minus the supernatant concentration of RTX, we obtain the RTX amount of the Fe-PR$_n$ (n = 2, 4, 8) NPs. The PBS (PH = 8) solution of FITC (1 mL, 0.2 mg) was dropwise added into 1 mL of multivalent Fe-PR$_n$ (n=2,4,8) NPs under stirring in dark at 25℃ for 24 h, then the mixture was centrifuged to remove the redundant FITC, and then the obtained FITC-Fe-PR$_n$ NPs were dispersed in 1 mL PBS.

1.6. Acetylation of multivalent Fe-PR$_n$ (n = 2, 4, 8) NPs

The surface amino groups on the formed Fe-PR_n NPs and FITC-Fe-PR_n NPs were further acetylated to reduce non-specific adsorption. Briefly, triethylamine (20 μL) was first added into 1 mL NPs solution and stirred for 0.5h. Then acetic anhydride (25 μL) was added dropwisely into the above solution, and stirred at 25℃. After 24 h of reaction, the resulting mixture was centrifuged and the resulting precipitate was dispersed into 1 ml of PBS.

1.7. In vitro stability tests and drug release

The NPs stability in the physiological environment against protein adsorption was tested at regular intervals. Briefly, 0.2 mL of NPs were suspended in RPMI-1640 (10% FBS) and stored in a shaker for 48 h at 37°C. The particle size was analyzed at 0, 6, 8, 12, 24, and 48 h by DLS, respectively. A dialysis method was performed to evaluate the in vitro release of RTX from the multivalent NPs. 0.8 mL of NPs, 0.2 mL Proteinase K Tris-HCl solution (0.66 mg/mL) was put into a dialysis bag (Mw = 6000 to 14000 Da), then immersed into PBS (PH = 7.4) at 37°C with soft shaking. At the fixed time points, the release medium (1 mL) was sucked out and then refilled by PBS (1 mL). Moreover, the release of the NPs without Proteinase K was measured as a control.

1.8. Cell Culture

Human oral squamous epithelium KB cell, Human cervical cancer Hela cell and Human lung epithelial Carcinoma A549 cell, were cultured in RMPI 1640 and DMEM respectively (both FA-deficient) with FBS (10%) and penicillin−streptomycin (1%) in cell incubator at 37℃, 5% CO_2.

1.9. In Vitro Cell Activity and Cellular Uptake

The cell viability of the Fe-PR_n (n = 2, 4, 8) and free RTX was assayed using a MTT assay. KB cells, HeLa cells, and A549 cells were incubated with the multivalent NPs and free RTX, with different RTX concentrations for 1 day. Cellular uptake of NPs and folate Competition Assays were evaluated using flow cytometry (BD LSRFortessa, BD Biosciences, USA) and fluorescence Microscope similar to our previous procedure.[28]

1.10. In Vivo Magnetic Resonance Imaging

KB and H22 tumor-bearing mice were used as a model for MRI using an Ingenia 3.0 T delivers high performance MRI (philips) with a mouse coil. 2D spin-echo T_2-weighted images were performed with slice thickness of 1.5 mm: TR=2191 ms; TE=68 ms; matrix=108*163 and FOV=5*10 cm. After the mice were injected with nanoparticles and PBS in the tail vein for 0.5h, the images were collected.

1.11. In Vivo Antitumor Efficacy and Biodistribution

Immunodeficient, 6- to 8-week-old BALB/C nude mice were purchased from Vital River Technology (Beijing, China), and the mice were in a folate-deficient diet (Trophic Animal Feed High-Tech Co., Ltd, China). The KB cells suspended in PBS (10^8 cells/ mL) was injected into the right hind limb of each mouse. After 2 weeks, the tumor volume reached to of 150-200 mm^3, and the BALB/C mice bearing a subcutaneous KB xenograft were randomly divided into five groups (10 mice per group) for different treatments. Once a week, animals received treatment of Fe-PR_n (n = 2, 4, 8) and free RTX containing equimolar concentrations of RTX and saline as a

control, and the RTX injection dose is 3mg/m^2. To measure the side effects of the drug, the body weight, the tumor volume and the survival time of the mice were recorded throughout the treatment. H22 tumor-bearing mice were also used as a model, and received an injection of Fe-PR_n (n = 2, 4, 8) once in three days. The other procedures are the same as above.

BALB/C mice bearing a subcutaneous KB xenograftwere were intravenous injected with a 0.2 mL solution containing equimolar concentrations of RTX and Fe element, and the mice with injection of PBS were used as controls. After 0.5 h, 2 h, 12 h, and 24 h postinjection, the animals were euthanized and samples of the major organ (liver, spleen, heart, lungs, kidneys and tumor) were taken and weighed. After tissue homogenization, all organs are immersed in aqua regia for dissolution for 48 h. The concentration of Fe in organs of different groups was analyzed by ICP-AES.

1.12. Blood and histopathological analysis

After four times treatments, the BALB/C mice were sacrificed to collect blood, tumor, and major organs. Blood samples in different treatment group were collected in anticoagulant tube and then the levels of TB, ALT and WB were examined using the beckmancoulter AU5800 Automatic biochemical analysis system. Isolated tumors and organs were immersed in tissue fixative for further hematoxylin and eosin staining (H&E).

1.13. Analysis of folate receptor expression by flow cytometry and fluorescence microscope

Cells were plated on a 6 well cell culture plate at a density of 3×10^5 cells/well. After 24h, cells were trypsinized and transferred into tubes for antibody staining. Cells were incubated on ice for 30 min with 100 μL of FACS buffer (1% BSA in PBS) containing 1 μg of either purified mouse IgG or FBP antibody (Abcam, ab3361). Cells were washed with PBS, and resuspended in 100 μL of FACS buffer containing 1 μg of Alexa Fluor 488 labeled goat anti-mouse antibody (Abcam, ab150113), then incubated on ice for 30 min. Flow cytometric analysis was carried out on a flow cytometer (BD) with 488 Laser. The cells were fixed (5 min) and then incubated in 1%BSA / 0.3M glycine in 0.1% PBS-Tween for 1h. The cells were then incubated with the antibody (ab3361, 10μg/ml) overnight at +4°C. The secondary antibody (green, ab150113) was used at 1μg/ml for 1h. DAPI was used to stain the cell nuclei (blue).

1.14. In vivo blood circulation behavior of Fe-PR_n

The pharmacokinetic studies were carried on Kunming mice (male, 6–8 weeks), after intravenous injection of Fe-PR_n (10mg Fe/kg), At specific time points after the injection (0.1, 0.5, 2, 4, 12, 24, 48h), 20uL blood was extracted from the tail and then dissolved in 0.6 mL lysis buffer. After centrifugation at 1200 rpm for 5 min, the supernatant is aspirated and then added to 0.6 mL HCl (0.1M) to decompose magnetic NPs. The blood concentrations of Fe content in serum at different time points were measured by ICP-AES and presented by unit of the percentage of injected dose per gram tissue (%ID/g). With time as the horizontal axis and %ID/g as the vertical axis, the curve is in accordance with the two-compartment model in pharmacokinetics. By

fitting the concentration-time function of the two-compartment model, the distribution half-life (t1/2 α), the elimination half-life (t1/2 β) and the area under the drug-time curve(AUC) can be calculated.

Table S1. The percentage of PEI and RTX conjugation on NPs with different ligand valency.

sample name	PEI : Fe_3O_4-CA(w : w)	RTX : Fe_3O_4-CA (w : w)
Fe-PR_2	0.85	0.212
Fe-PR_4	0.47	0.217
Fe-PR_8	0.21	0.223

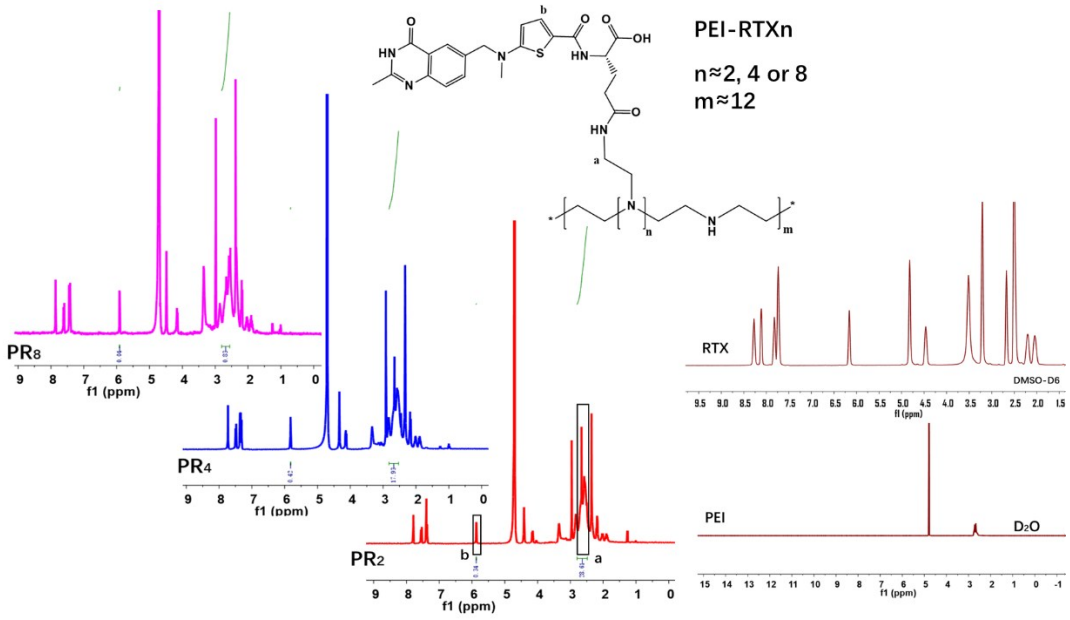

Figure S1. 400 MHz ^{1}H NMR spectra of RTX in DMSO-D6, PEI and PR_n (n = 2, 4, 8) in D_2O. δ: 7.85 (s, 1H, quinazolione-H), 7.65 (d, 1H, quinazolione-H), 7.45(m, 2H, quinazolione-H, thiophene-H), 5.9 (d, 1H, thiophene-H), 4.48 (s, 2H, -CH_2), 4.15 (m,

1H, -CH-), 3.0 (s, 3H, -NCH_3), 2.8~2.55 (m, 2H, -CH_2-), 2.05 (m, 5H, -CH_2COOH, -CH_3), 1.9 (m, 1H, -CH_2-).

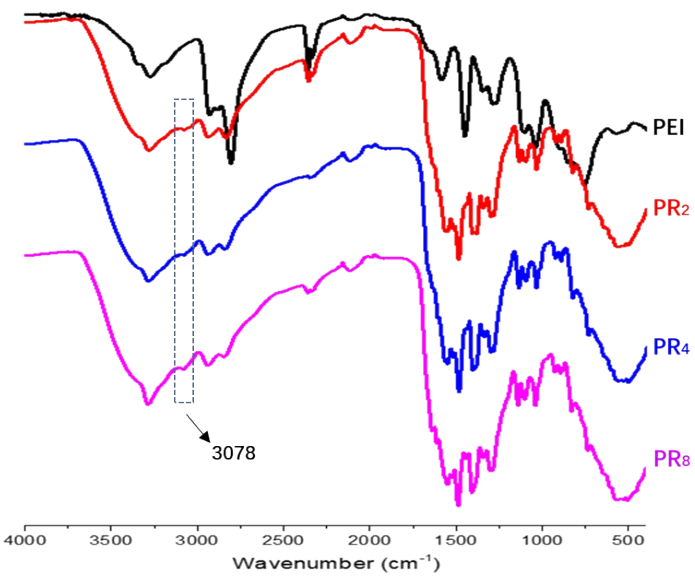

Figure S2. FTIR spectra of PEI and PR_n (n = 2, 4, 8).

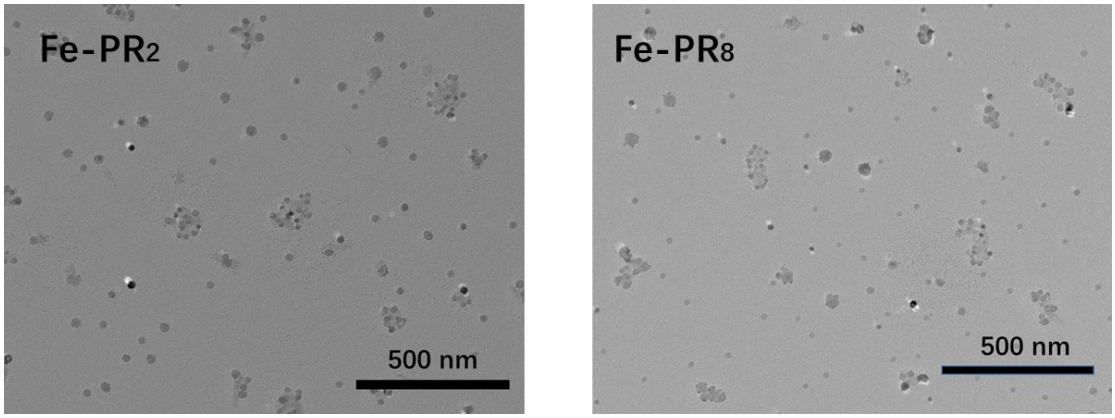

Figure S3. TEM of Fe-PR_2 and Fe-PR_8.

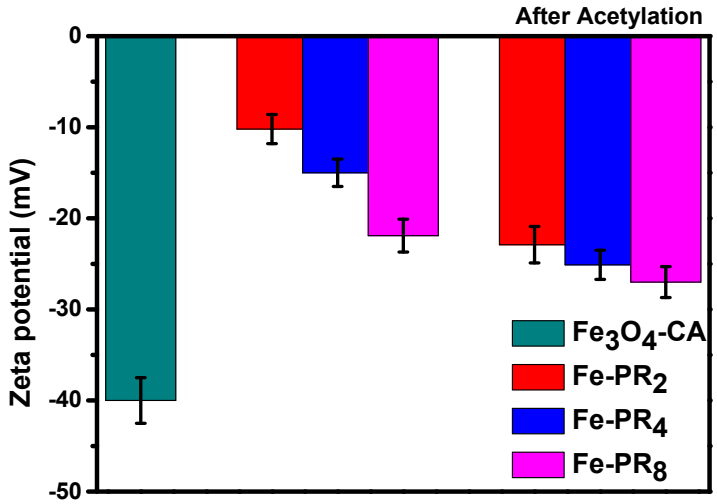

Figure S4. Zeta potential of Fe_3O_4-CA and Fe-PR_n.

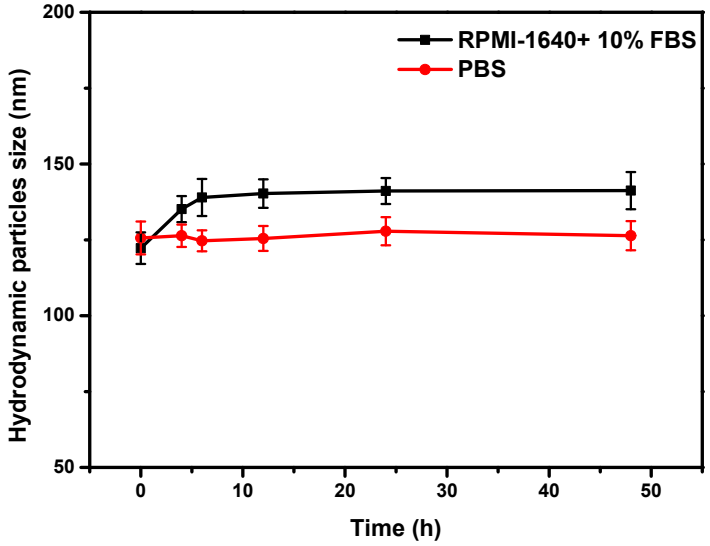

Figure S5. In vitro particle size stability of the Fe-PR_4 in PBS, RPMI 1640 with 10% FBS at 37 °C. Data were presented as mean ± SD (n = 3).

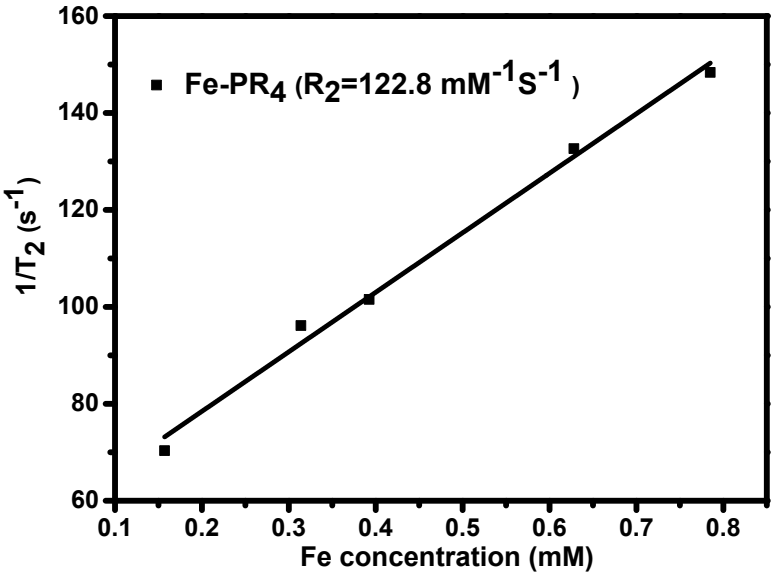

Figure S6. Linear fitting of $1/T_2$ of Fe-PR_4 as a function of Fe concentration.

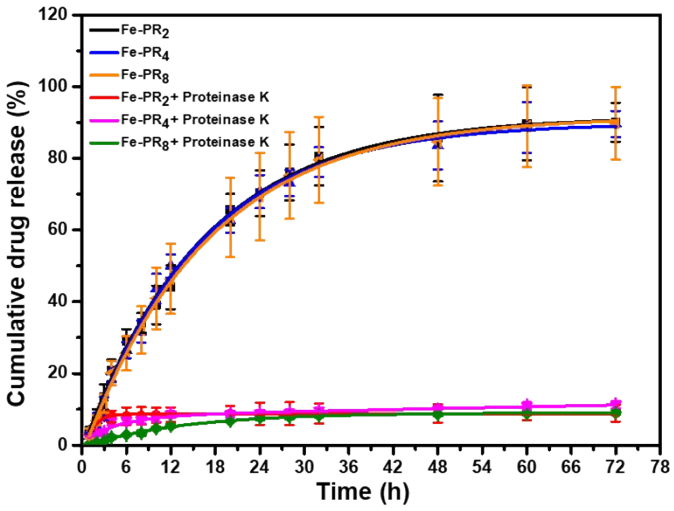

Figure S7. In vitro RTX drug release profiles of the Fe-PR_n. Data are presented as mean ± s.d. (n = 3).

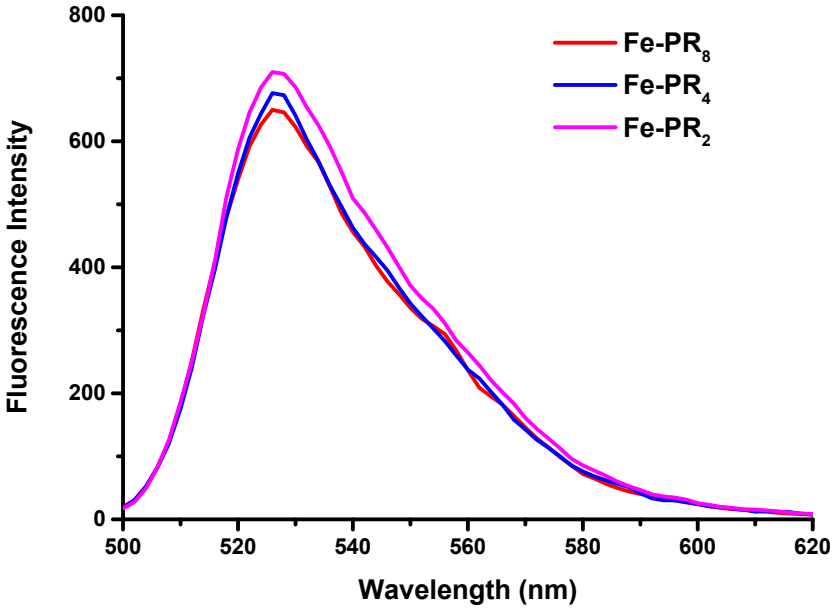

Figure S8. Fluorescence intensities of Fe-PR_n after labled with FITC.

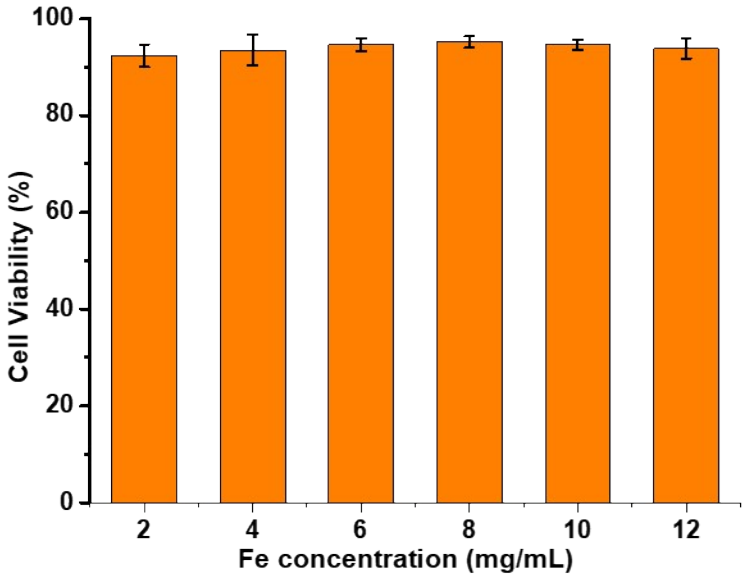

Figure S9. The cytotoxicity of Fe_3O_4-CA against KB cells

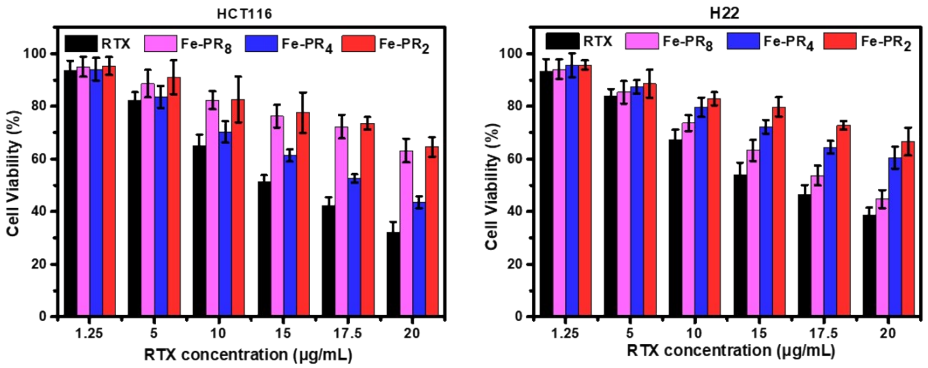

Figure S10. The cytotoxicity of free RTX, and Fe-PR$_n$ (n = 2, 4, 8) against HCT116 cells and HeLa cells.

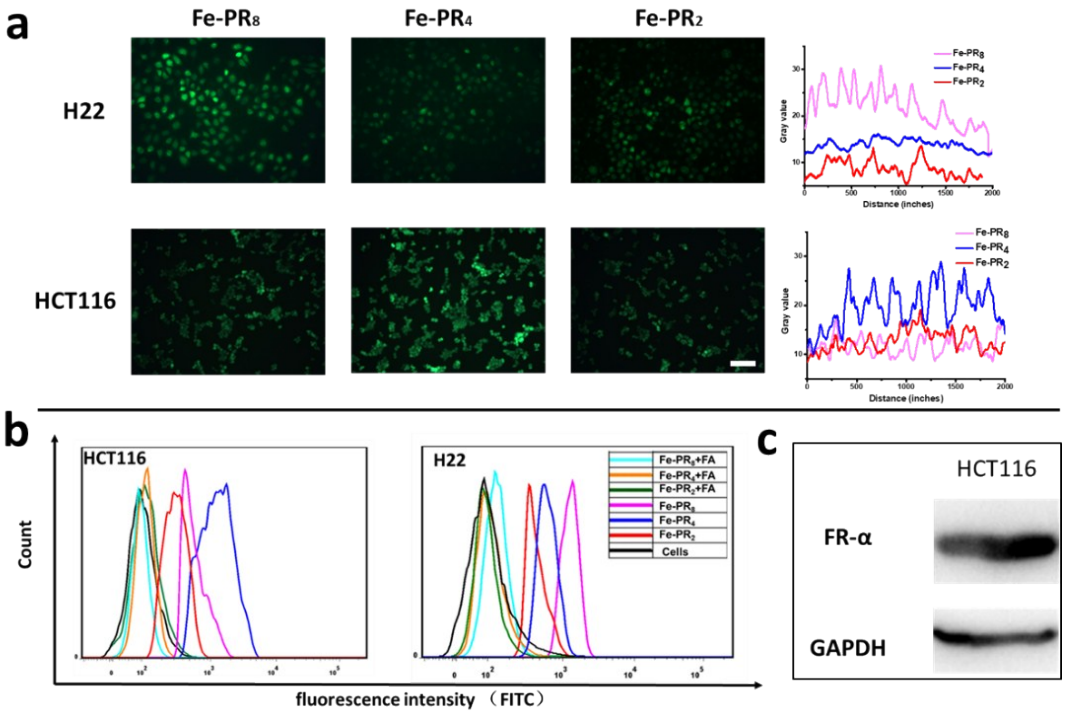

Figure S11. (a) Fluorescent images showing the selectively uptake of FITC-labeled Fe-PR$_n$ NPs (green) after co-incubation with H22 and HCT116 cells for 4 h and the corresponding gray value of each image is also presented. Scale bars, 100 μm. (b) Flow cytometer tests of mean FITC intensity in Fe-PR$_n$ treated (with and without FA)

H22 and HCT116 cells for 4 h. (c)Western blot analysis of folate receptor in HCT116 cell.

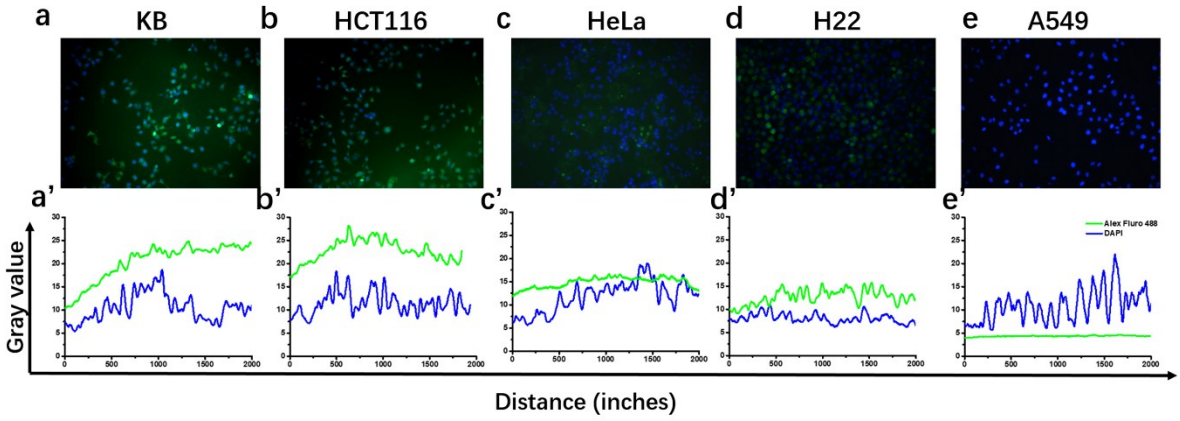

Figure S12. Folate receptor expression in cultured cells. (a) KB, (b) HCT116, (c) Hela, (d) H22 and (e) A549 cells. Alexa Fluro 488 (green), DAPI (blue). Scale bars, 100 μm.

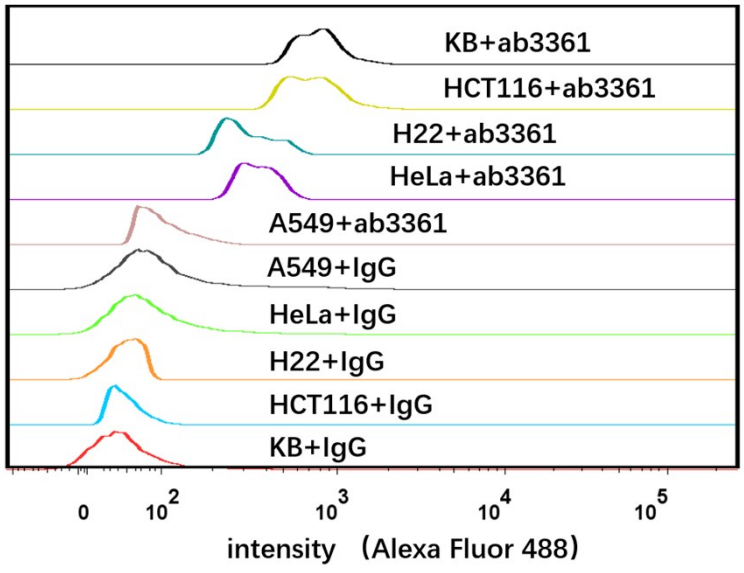

Figure S13. Flow cytometry was used to characterize the folate receptor expression in KB, HCT116, Hela, H22 and A549 cells. Cells were stained with either mouse anti-

folate receptor antibody (ab3361) or mouse IgG2b k isotype control, followed by FITC-labeled goat–anti-mouse secondary antibody.

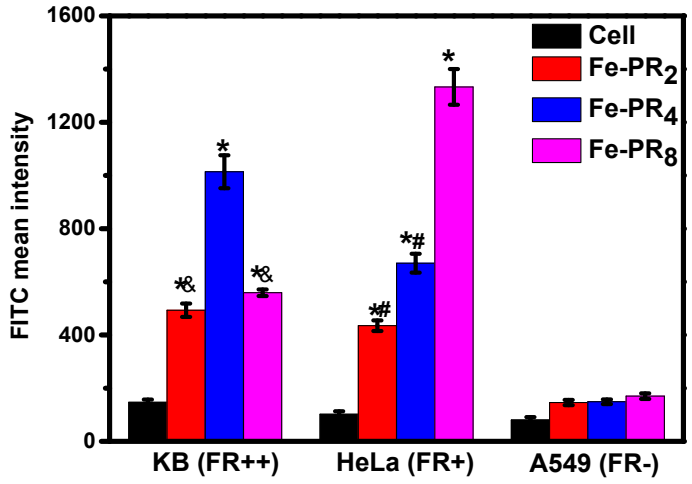

Figure S14. Quantitative analysis of the fluorescence intensity of FITC in Fe-PR_n treated KB, HeLa and A549 cells for 4 h. Data represent three separate experiments and are presented as the mean ± SD *P < 0.05 vs the Control group; # P < 0.05 vs the Fe-PR_8 group; & P < 0.05 vs the Fe-PR_4 group.

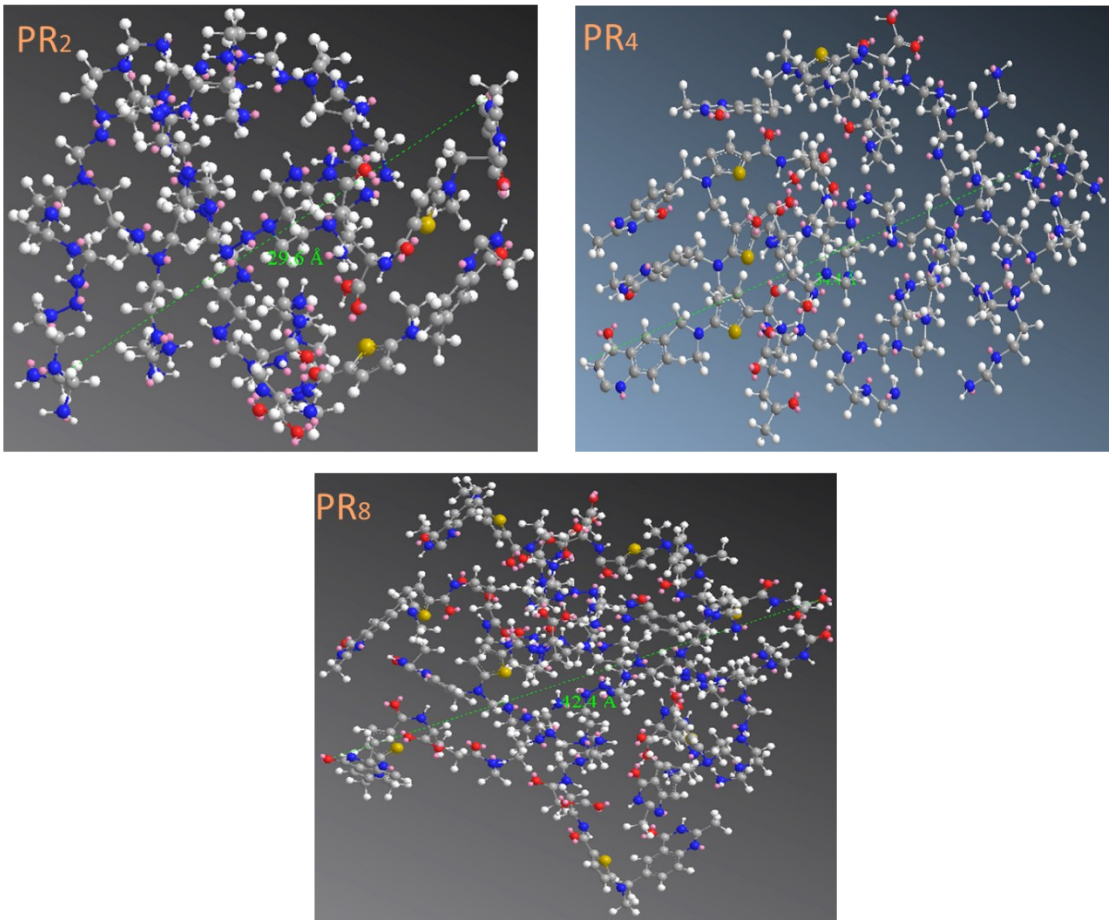

Figure S15. Simulation calculation on the surface ligand cluster PR_n by chem3D (minimize energy with MM2).

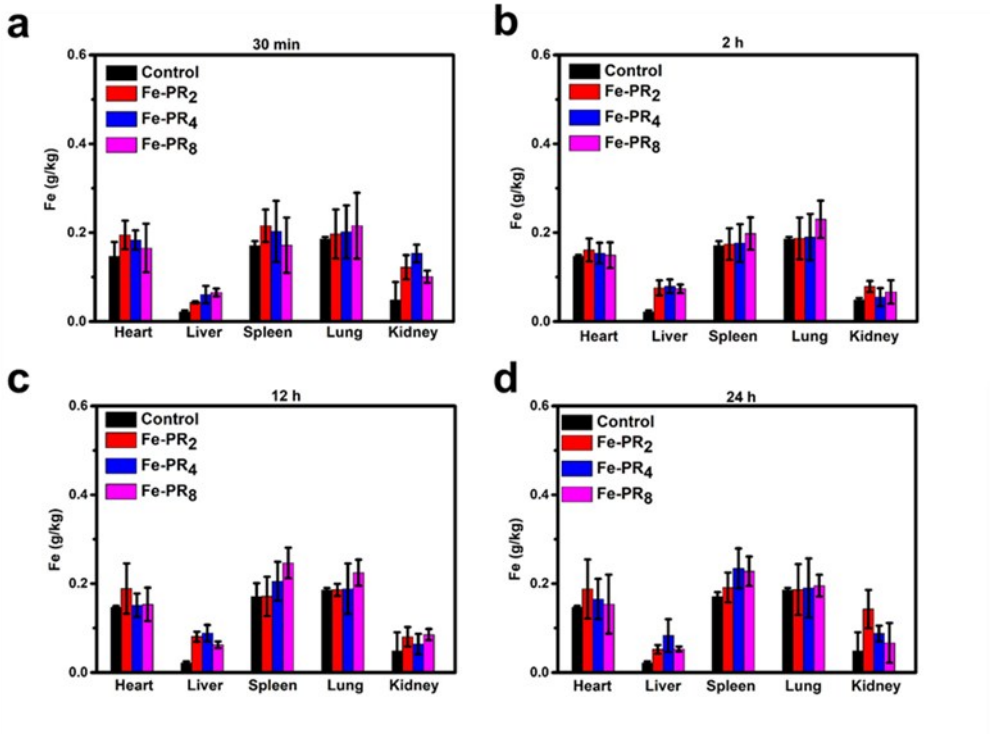

Figure S16. Biodistribution of the major organs of the KB tumor-bearing mice including heart, liver, spleen, lung, and kidney at 30 min, 2 h, 12 h and 24 h post-intravenous injection of Fe-PR_n. Data represent three separate experiments and are presented as the mean ± SD

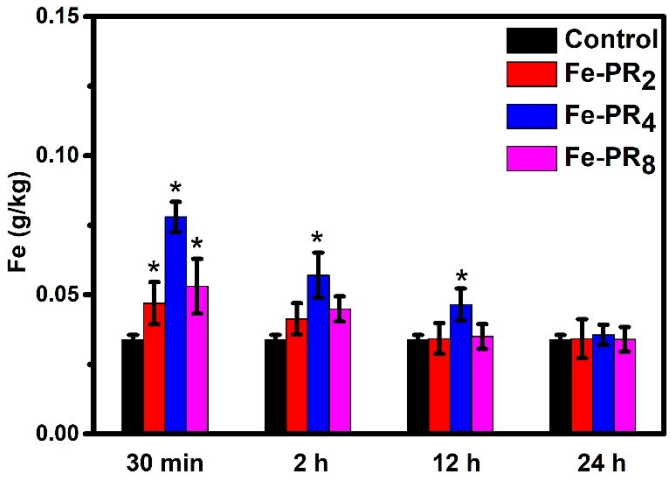

Figure S17. Fe element biodistribution in the tumor of the KB tumor-bearing mice at 30 min, 2 h, 12 h and 24 h post-intravenous injection of Fe-PR_n. Data represent three separate experiments and are presented as the mean ± SD

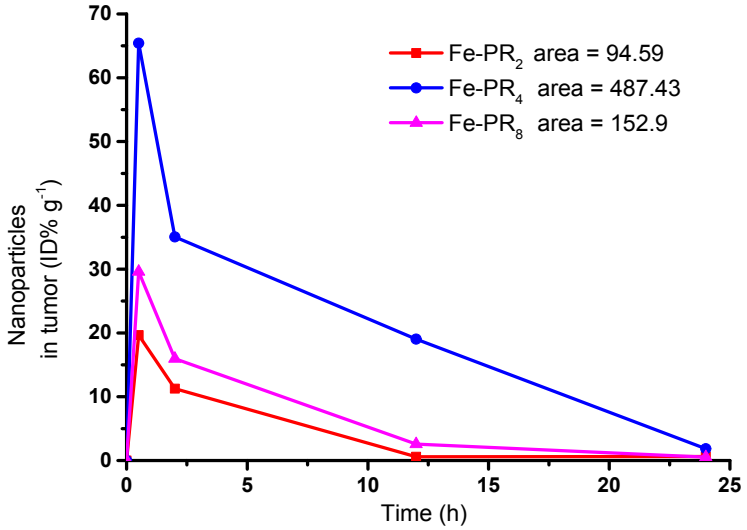

Figure S18. The linear trapezoidal method was used to calculate the area-under-the-curve (AUC_{Tumour}) of the plot of Fe-PR_n nanoparticle concentration in the KB tumour tissue as a function of time (0, 0.5h, 2h, 12h, 24h).

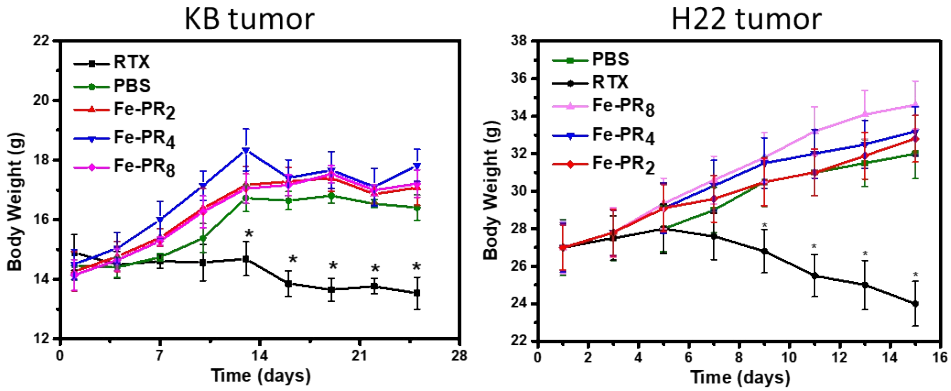

Figure S19. Body weight from mice in each group over 25 days in KB tumor and 15 days in H22 tumor. Data represent three separate experiments and are presented as the mean ± SD *P < 0.05 vs the PBS group.

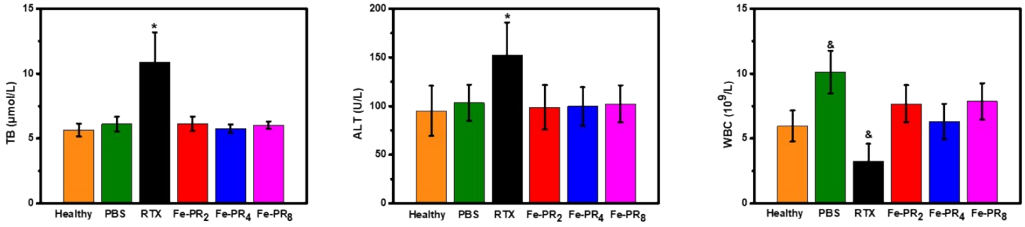

Figure S20. TB, ALT and WBC levels in the blood of KB tumor-bearing mice harvested at the 25-d endpoint.

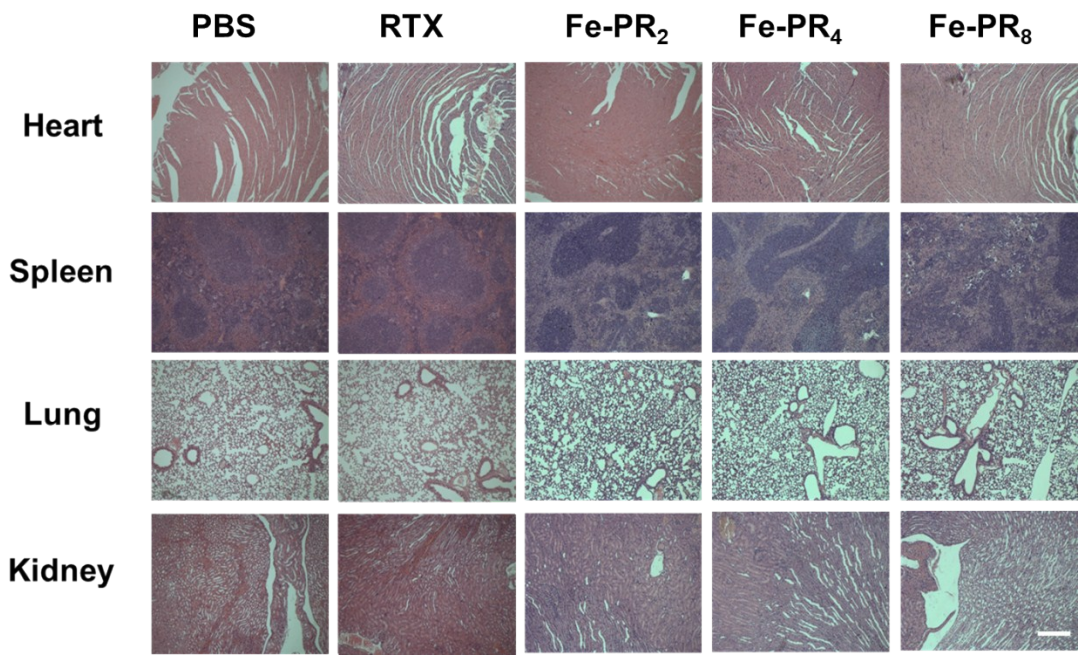

Figure S21. Effect of chemotherapy on the histopathology of organs from KB tumor-bearing mice from each group. Images were obtained at 100× magnification with standard HE staining. Scale bars, 200 μm.

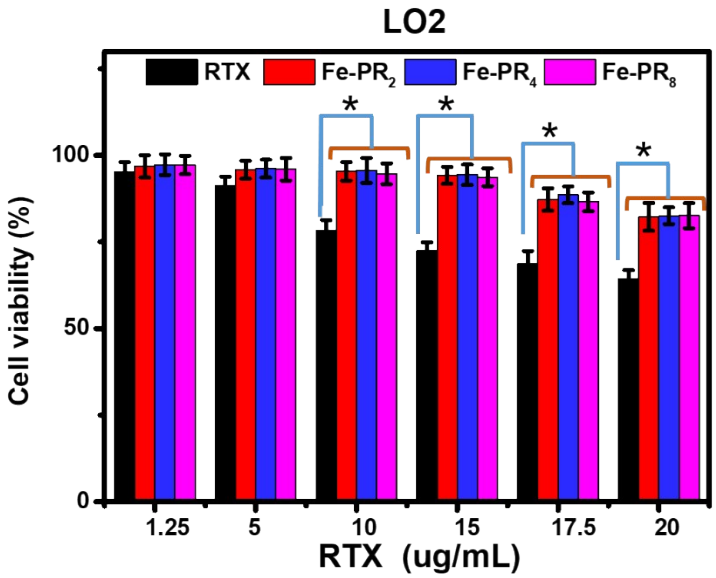

Figure S22. The cytotoxicity of Fe-PR_n against human liver LO2 cells. The experiment was performed three times in parallel and are presented as the mean ± SD *P < 0.05 vs the free RTX group.

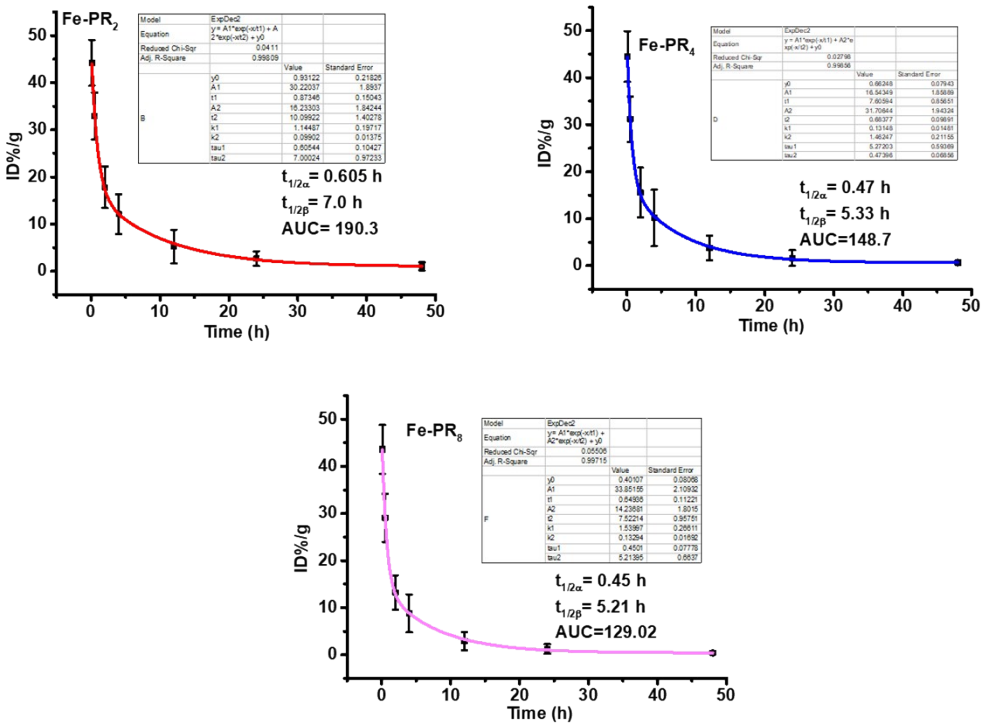

Figure S23. Pharmacokinetic studies of Fe-PR_n.