Electronic Supplementary Material (ESI) for Materials Horizons.

Supporting Information

Ferros-Cysteine-Phosphotungstic Nanoagent with Neutral pH Fenton Reaction Activity for Enhanced Cancer Chemodynamic Therapy

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1.Experimental section

Materials and reagents. Phosphotungstic acid(99%), L-cysteine(99%) and L-lysine(98%) were purchased from Adamas Reagent Co.,Ltd. Anhydrous iron(Ⅱ) chloride (99.5%), methyl orange and coumarin were purchased from Shanghai Macklin Biochemical Co., Ltd., China. Sodium hydroxide were purchased from Sinopharm chemical Reagent Beijing Co.,Ltd., China. All reagents were of analytical grade and used directly without further purification. The ultrapure water used during the experiments was prepared by ELGA PURELAB classic water purification system.

Synthesis of FcPWNPs, amorphous Fe nanoparticles (AFeNPs) and Fe_3O_4 NPs. The Ferrous-cysteine-PWO nanoparticles were synthesized via a well controlled one-step co-precipitation method. First, 0.1mmol of $Fecl_2$ and 0.2 mmol of Cysteine (Cys) were dissolved in 20mL of oxygen-free ultrapure water and continuous stirring in a 100mL flask to obtain ferrous cysteine chelate. Then, 15mL of 0.058mmol/L phosphtungstic acid solution (adjust pH to 7.1 by NaOH previously) was added into ferrous cysteine solution (7ml/h))with stirring. The resulting light yellow nanoparticles were collected by centrifugation and washed with water for three times.

The ferrous-asparagine-PWO and ferrous-lysine-PWO nanostructures were synthesized as the same process mentioned above, only the pH value of phosphotungstate solution needed to change into pH=6.8 (for asn) and pH=7.4 (for lys).

The AFeNPs were synthesized via the hubble-bubble reduction process. 0.36 mmol ammonium iron citrate, 18 mmol PVP and 1 g F-127 were dissolved in 30 ml deionized water (DI water) with stirring and heated to 70 °C under argon protection for 1 h. Then, 6 ml solution of 7.5 mmol sodium borohydride was added dropwise into the reaction system. The resulting AFeNPs were collected by magnetic separation and washed with ethanol for three times.

The Fe_3O_4 NPs were synthesized via previous report. Iron-oleate complex were first

synthesized: 5 mmol FeCl3·6H2O and 15 mmol sodium acetate were added into a mixture solution of hexane (17.5 ml), ethanol (10.0 ml), DI water (7.5 ml). Then the solution was heated to 70 °C and kept for four hours with stirring. The preparation of Fe3O4NPs: 5mmol iron-oleate complex was added into oleic acid (0.71g) and 1-octadecene (100g) at room temperature. Then the mixture solution was heated to 300℃ with constant rate of 3.3℃/min, and kept for 30min under continuous stirring. After the solution was cooled to room temperature, 50ml acetone and ethanol was added to precipitate the nanocrystals. The obtained nanoparticles were collected by centrifugation.

Materials characterization. Transmission electron microscope (TEM) images and element distribution were obtained with Thermo Fisher Scientific Tecnai G2 F30. Scanning electron microscope (SEM) images were obtained with HITACHI S-480. Element concentration was measured by Agilent Technologies 5100 inductively coupled plasma optical emission Spectrometry (ICP-OES). Fourier transform infrared spectroscopy (FT-IR) spectra were measured by a BRUKER TENSOR Ⅱ using a KBR pellet. X-ray powder diffraction (XRD) was measured on Rigaku Ultima Ⅳ at Cu Ka (λ=0. 0.154056 nm) in the 2θ range of 10°-80° with a scanning rate of 10°/min. The confocal laser scanning microscopy (CLSM) images were obtained by NIKON A1 R (NIKON, Japan).

Stability of FcPWNPs. 1ml of FcPWNPs solution with W concentration of 2mg/ml was sealed into a dialysate bag with the cutoff molecular weight of 1000Da. The dialysate bags were then put into 3 beakers with 500ml phosphate buffer solution at pH=7.4, pH=6.5 and pH=5.4 respectively with a moderate stir. 5ml of buffer was collected at the time on 5min, 10min, 20min, 30min, 1h, 2h, 4h, 6h, 8h, 12h, 24h and 48h respectively for Fe and W concentration analysis by using ICP-OES and another 5ml of fresh buffer was added. The release rate (r) of iron and tungsten ion was calculated as the following equation:

$$r = \frac{20c_n + \sum_{i=1}^{n-1} c_i}{m_{initial}} \times 100\%$$

ci is the concentration of Fe in the ith collection, and the minitial is the initial amount of W (2mg).

Study of •OH generation in acidic and neutral condition. The generation of •OH was measured by fluorospectrophotometry method based on the fluorescence intensity of highly fluorescent 7-hydroxycoumarin which is generated by the selectively oxidize of coumarin (Shanghai Macklin Biochemical Co., Ltd., China) by the •OH. In brief, the fluorescence intensity of coumarin solution (4ml, 1mmol/L in cuvette) with the excitation at λ=345nm and the emission at λ=453nm at different pH (pH=7.4, pH=6.5 and pH=5.4 (in phosphate buffered solution)) with or without H_2O_2 (400μmol/L) were measured before and after adding 40μg FcPWNPs (in 30μl oxygen-free water). To further confirm the ability of oxidation, a colorimetric method was measured which based on the degradation of methyl orange (MO, Shanghai Macklin Biochemical Co., Ltd., China) after •OH capture. The absorbance at λ=464nm of MO solution (10mg/L, 4ml in cuvette) at ph=7.4, ph=6.5 and ph=5.4 (adjusted by 0.1 mol/L Hcl) with or without H_2O_2 (400 μmol/L) was measured before and after the addition of 80 μg FcPWNPs (in 30μl oxygen-free water). To further confirm the generation of •OH, ESR spectroscopy was used with the •OH spin trap agent 5, 5-Dimethyl-1-pyrroline N-oxide (DMPO). With 50 μl of DMPO solution (in phosphate buffered solution, 100mmol/L) in an aluminium foil coated eppendorf tube, 20 μl H_2O_2 at pH =7.4, 1μg FcPWNPs at pH = 7.4, 20μl H_2O_2 (5 mM) + 1 μg FcPWNPs at pH = 7.4, 20 μl H_2O_2 + 1 μg FcPWNPs at pH = 6.5 and 20 μl H_2O_2 + 1 μg FcPWNPs at pH = 5.4. Then, the mixture was transferred into a quartz capillary by a capillarity, X-band EPR spectra were then measured on a Bruker EMX-8/2.7 spectrometer at room temperature with the parameters as follow: microwave frequency=9.872 GHz, microwave power=6.375 mW, modulation frequency=100.00 kHz and modulation amplitud =1.00 G.

Cell cultrure. 4T1, C6, U87, Hela cancer cell lines and LO2, 293t, PC12 normal cell lines were cultured in high-glucose DMEM (GENOM Biomedical technology co. LTD., Hangzhou, China.) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 units/ml) and streptomycin (100mg/ml). All the cell lines were obtained from Shanghai Institute of cells, Chinese Academy of Science, and cultured at 37°C in humidified atmosphere along with 5% of CO_2.

Confocol fluorescence imaging. Seeding 3×10^4 cells of 4T1 in 1ml DMEM (pH=7.4) in the CLSM culture vessel and stewing over night to ensure the cells adhere firmly. Co-incubating the cells with 1ml DCFH-DA (10 μmol/L in FBS-free DMEM) at 37°C in 5% CO2, then replacing the medium by DMEM of pH=7.4 and pH=6.5 after 20mins incubation with the following groups: 100μM H_2O_2 at pH=7.4, 40 ppm Fe of FcPWNPs at pH=7.4, 100μM H_2O_2 and 40 ppm Fe at pH =7.4 and 100 μM H_2O_2 and 40 ppm Fe of FcPWNPs at pH=6.5. After co-incubation with different time (15mins and 1.5 h for H_2O_2+FcPWNPs group at pH=6.5 and pH=7.4, 15 min, 1 h and 1.5 h for other groups), the cells were washed with PBS for three times. Subsequently, using the confocal laser scanning microscopy (A1 R, NIKON, Japan) to examine the relative quantity of intracellular ROS by inspecting the fluorescence of DCFH (λ_{ex}=488 nm, λ_{em}=525 nm). The co-localization experiment was measured by incubating 4T1 cells with FITC stained FcPWNPs for 6 and 8 hours, the cells were washed with PBS for three times. After 1ml of DAPI solution was added and incubated for another 10min, the cells were washed with PBS for three times. Then use the CLSM to examine the endocytosis of FcPWNPs.

Cytotoxicity measurement of FcPWNPs and H_2O_2. Cytotoxicities of FcPWNPs were conducted on 4T1, C6, U87, Hela cancer cell lines and LO2, 293t, PC12 normal cell lines. These cells were respectively seeded (5×10^3 cells in 100 μl of homologous culture medium per well) in sextuplicate in 96-well microplates, and permitted to adhere overnight. Subsequently, the culture medium was replaced by fresh culture medium including FcPWNPs at Fe concentration of 5, 10, 20, 40.80, 160 and 320 ppm, respectively. Co-incubation for 24 h, the culture medium were then replaced by FBS-free medium containing 0.6 mg/ml

3-[4,5-dimethylthiazol-2-yl-]-2,5-diphenyltetrazolium bromide (MTT). Lastly, 100 μl of dimethyl sulfoxide (DMSO) were used to replace the MTT solution after co-incubation for 4 h. Then a microplate reader (Bio-TekELx800, USA) is used to text, cell proliferation was examined by comparing the absorbance at λ = 490 nm to the control. The result showed that FcPWNPs did not restrain these cells growth in the range of concentrations used. The cytotoxicity of H_2O_2 to 4T1 cells in 24 h after incubation with the concentration of 12.5, 25, 50, 100, 200, 400, 500 and 600 μM was measured in the same way as mentioned above

Cancer cell inhibitory effect of CDT in vitro. 4T1 cells were seeded (5×10^3 cells in 100μl of DMEM per well) in sextuplicate in 96-well microplates, and allowed to adhere overnight. To simulate the slightly acidic (pH = 6.5-6.9) extracellular microenvironment in solid tumor, the DMEM (pH = 7.4) was acidized to pH = 6.5 by adding 12 mM hydrochloric acid, which shows negligible influence on the cell growth. The culture medium was then replaced by fresh medium (pH = 7.4 and pH = 6.5) containing 50, 100 and 200 μM H_2O_2 with FcPWNPs at Fe concentration of 25, 100 and 200 μg ml^{-1}, respectively. The cells were incubated at 37 °C in 5% CO2. After 24 h of incubation, the culture media were replaced by FBS-free medium containing 0.6 mg/ml 3-[4,5-dimethylthiazol-2-yl-]-2,5 -diphenyltetrazolium bromide (MTT), which can be reduced to the insoluble purple formazan by the viable cells. After 4h of further co-incubation, the MTT solution was replaced by 100 μl of dimethyl sulfoxide (DMSO) to solubilize the formazan. Cell proliferation was determined by monitoring the absorbance at λ =490 nm using a microplate reader (Bio-TekELx800, USA) and expressed as the percentage of untreated control cells.

Selective cancer therapy property of FcPWNPs. C6, U87, Hela cancer cell lines and 239t, Hela normal cell lines were seeded (5×10^3 cells in 100μl of DMEM per well) in sextuplicate in 96-well microplates, and allowed to adhere overnight. The culture medium is replaced by fresh medium containing 100μM H_2O_2 (cancer cells) and 10μM H_2O_2 (normal cells) and FcPWNPs with Fe concentration of 40ppm. After 24 h of incubation, the culture media were replaced by FBS-free medium containing 0.6 mg/ml 3-[4,5-dimethylthiazol-2-yl-]-2,5

-diphenyltetrazolium bromide (MTT), which can be reduced to the insoluble purple formazan by the viable cells. After 4h of further co-incubation, the MTT solution was replaced by 100 µl of dimethyl sulfoxide (DMSO) to solubilize the formazan. Cell proliferation was determined by monitoring the absorbance at λ =490 nm using a microplate reader (Bio-TekELx800, USA) and expressed as the percentage of untreated control cells.

Measurement the threshold for •OH to embody its cytotoxicity. 1mM of Fe^{2+} and 100, 50, 25 and 12.5 of H_2O_2 were added in 3ml of 1mM coumarin solutions. After react for 10min, the fluorescence intensity of highly fluorescent 7-hydroxycoumarin with the excitation at λ=345nm and the emission at λ=453nm. Considering the insufficient of H_2O_2 (H_2O_2: 100, 50, 25, 12.5µM; Fe^{2+}: 1 mM), we assume that the H_2O_2 will converted to ·OH with the ratio of 1:1 via the reaction $Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + (OH)^- + \cdot OH$. Thus the fluorescence intensity--·OH concentration Then, the cytotoxicity of ·OH was measure via a classical MTT assay under different incubation conditions, and the group whose cell viability lower than 90% is identified as damaged. The group incubated with FcPWNPs (40ppm) +50 µM H_2O_2 is chosen and its coumarin-trapping spectrofluorimetry is measured. At last, as measured fluorescence intensity was taken in to standard curve, the corresponding •OH concentration could be speculated.

In vivo toxicity assay. All animal experiments operations were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee (IACUC). 7-week female Kunming mice (~ 25 g) were obtained from Beijing Vital River Laboratory Animal Technology Co., Ltd., China. After the intravenous (*i.v.*) injection FcPWNPs for 70 mg/kg (in 100 µl saline) with same volume of saline as the control, their body weight was measured every two days (n = 5 for each group). There were no significant behavioral changes compared to the control group. After 3 days and 30 days injection, mice were sacrificed and their major visceral organs (heart, liver, spleen, lung and kidney) were collected in a 10% formalin solution for histopathology analysis using a typical hematoxylin and eosin (H&E) staining assay. To further evaluated potential vivo toxicity, the blood serum parameters

including aminotransferase (ALT), alanine aspartate aminotransferase (AST), alkaline phosphatase (ALP), creatinine (CREA) and nephric blood urea (UREA) together with the complete blood panel parameters including red blood cells (RBC), white blood cells (WBC), red blood cell distribution width-standard deviation (RDW-SD), hemoglobin (HGB), mean corpuscular volume (MCV), mean corpuscular hemoglobin concentration (MCHC), mean corpuscular hemoglobin (MCH), hematocrit (HCT) and lymphocyte (LYMPH%) of all groups were measured by using standard biochemistry test.

Measurment of blood circulation half-life. Three female balb/c mice at 7 weeks (Beijing Vital River Laboratory Animal Technology Co., Ltd., China) were injected intravenously (*i.v.*) with 100 μl of FcPWNPs saline solution (70 mg/kg). Subsequently, 10 μl blood was collected at the given time points (5 min, 10 min, 30 min, 1 h, 2 h, 4 h, 8 h, 12h and 24 h) and diluted to 2 ml 10 mM ethylenediaminetetraacetic acid disodium salt (EDTA-2Na) solution as anticoagulant. The W concentration was determined by ICP-OES. Origin was used for data analysis and the blood circulation half-life was calculated based on the one-component pharmacokinetic model.

Anti-cancer performace of CDT in vivo. 7 week female Balb/c mice (~20 g) were bought from Beijing Vital River Laboratory Animal Technology Co., Ltd., China. The mouse breast tumor 4T1 cells (1×10^6 cell/site) in DMEM (100 μL of dose for each mouse) and implanted subcutaneously into the right back of mice. Anti-cancer performance of CDT after intravenous (*i.v.*) and intratumoral (*i.t.*) injection were conducted when the volume of tumors reached ~100 mm^3. Three groups (n = 4 per group) which were randomly allocated for different treatment contained the following conditions: control group (injected with the dosage of 100 μL saline solution without any other treatment); *i.t.* group (intratumoral injected with the dosage of 20 μL 40 mg/kg Fe of FcPWNPs); *i.v.* group (intravenous injected with the dosage of 100 μL 50 mg/kg Fe of FcPWNPs). Then, a digital caliper is used to monitor the volume of the tumors every three days lasting 14 days after the corresponding experiments. The tumor volumes were calculated according to equation V=length × width ×

width 2^{-1} and the initial volume (V_0) of the tumor were normalized to obtain the relative tumor volume (V/V_0). The pathological tissue sections of tumors were collected for hematoxylin and eosin (H&E) staining assay after 15 h treatment. Mice were finally euthanized when the tumor volume reached 1000 mm^3.

2.Supplementary Figures

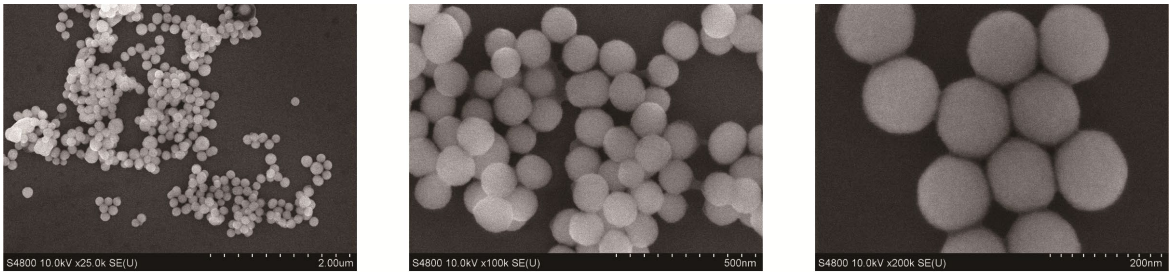
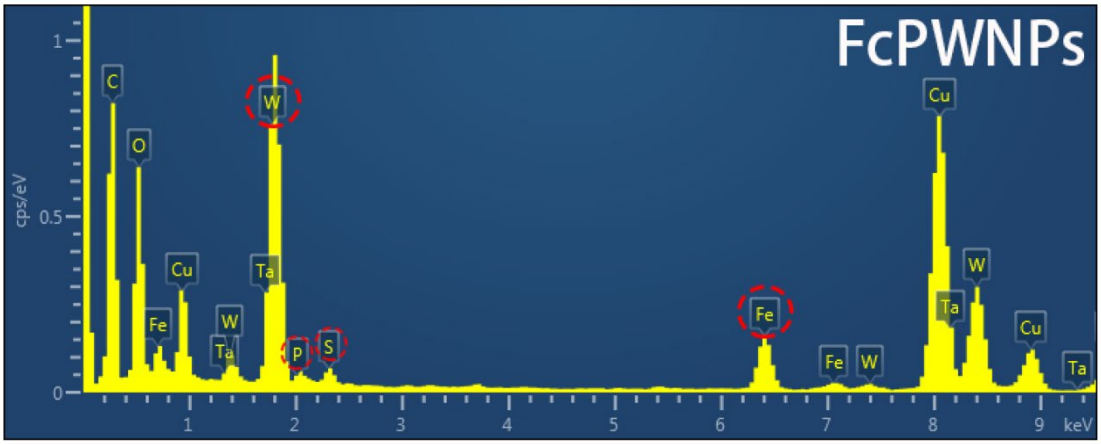


Figure S1. The SEM images of FcPWNPs



element	wt%
O	13.89
S	0.57
Fe	3.96
W	28.82

Figure S2. The EDS and element ration of Fe, W and S of FcPWNPs. Displaying the existence of every part in FcPWNPs.

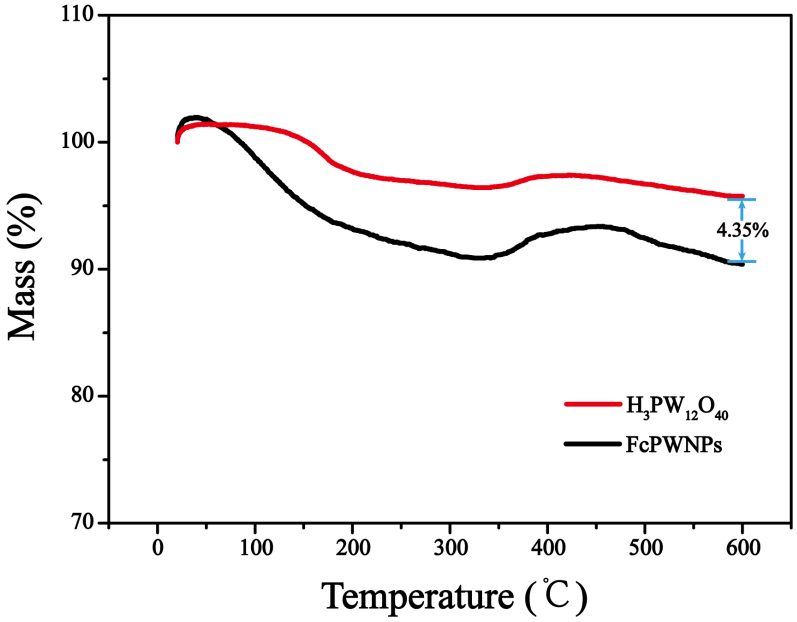


Figure S3. Thermogravimetry curve of FcPWNPs and $H_3PW_{12}O_{40}$. The concentration of cysteine is about 4.35%.

Figure S4 Digital photographs of FcPWNPs dispersed in H_2O, PBS and cell culture medium (RMPI 1640).

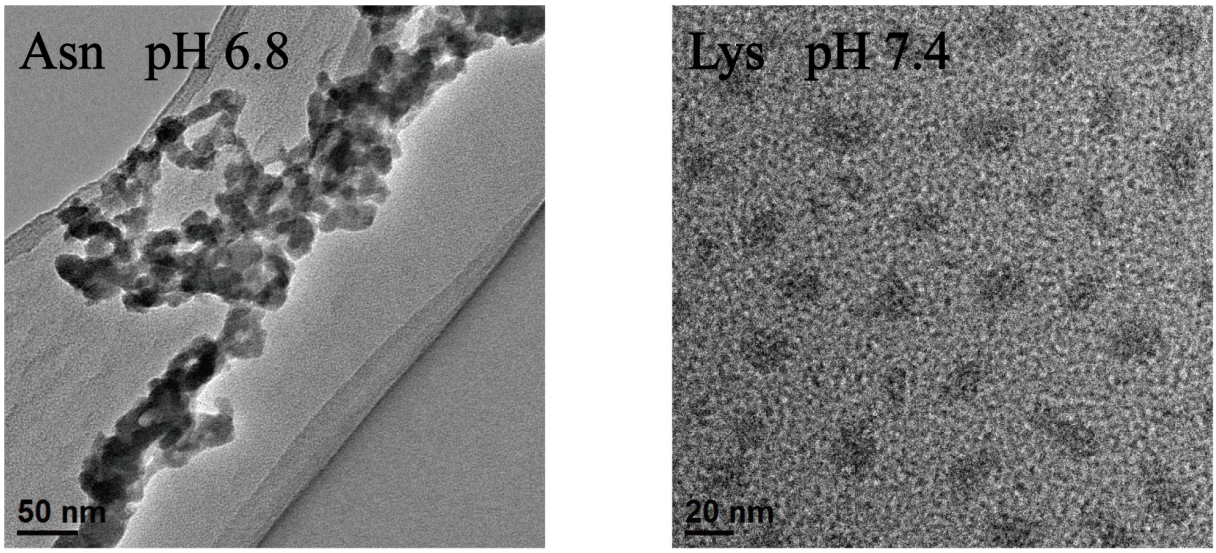


Figure S5. TEM images of Fe-asn/lys-phosphotungstate nanoparticals. The amino acid used here is replaceable after different adjusted pH condition.

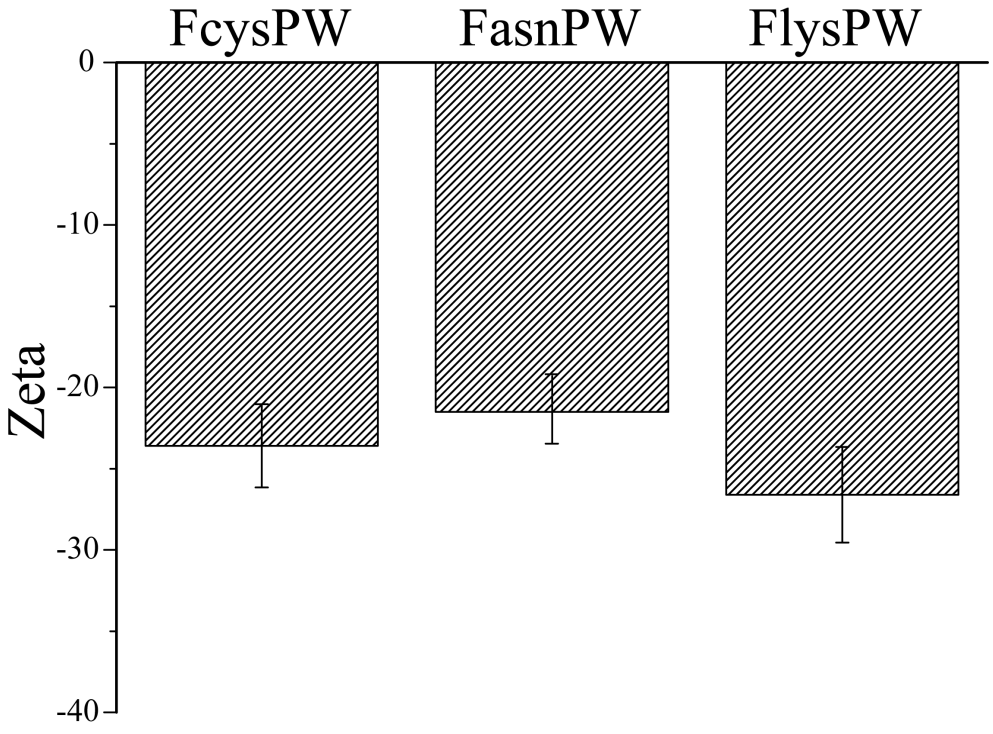


Figure S6. Zeta potentials of Fe-Cys/Asn/Lys-phosphotungstate nanoparticles.

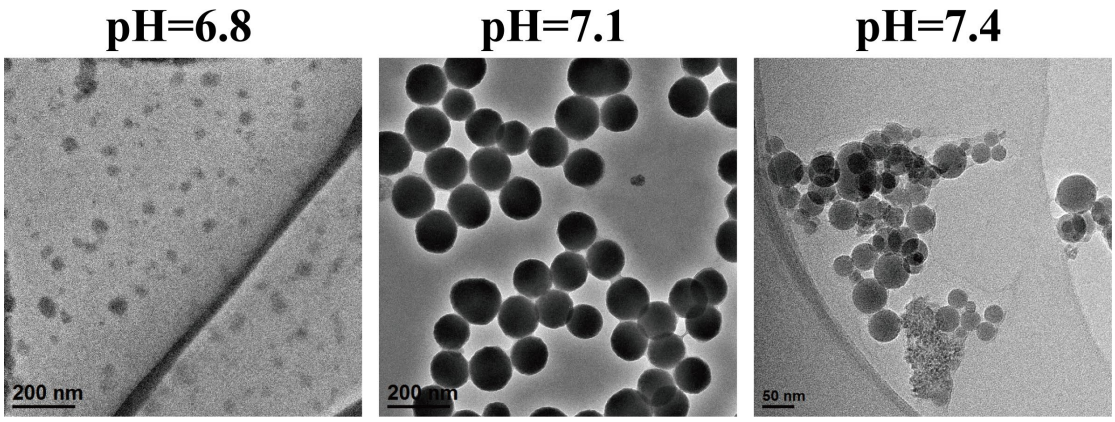


Figure S7. TEM images of FcPWNPs synthesized at different solution pH.

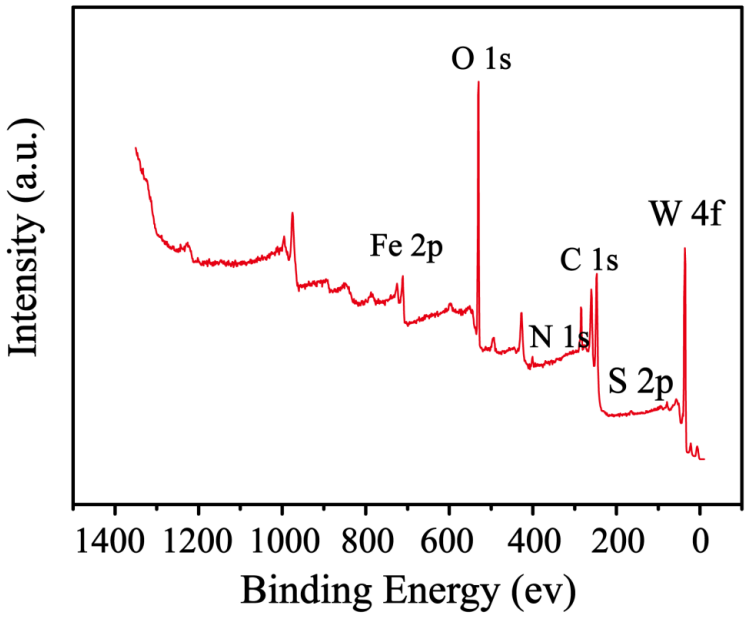


Figure S8. Over-all XPS spectrum of FcPWNPs.

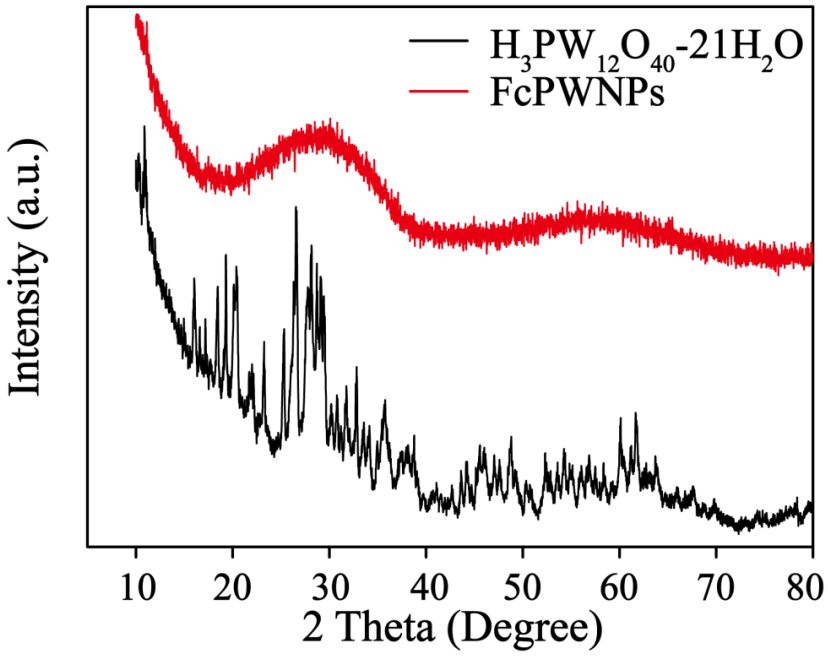


Figure S9. XRD pattern of $H_3PW_{12}O_{40}$•$21H_2O$ and FcPWNPs.

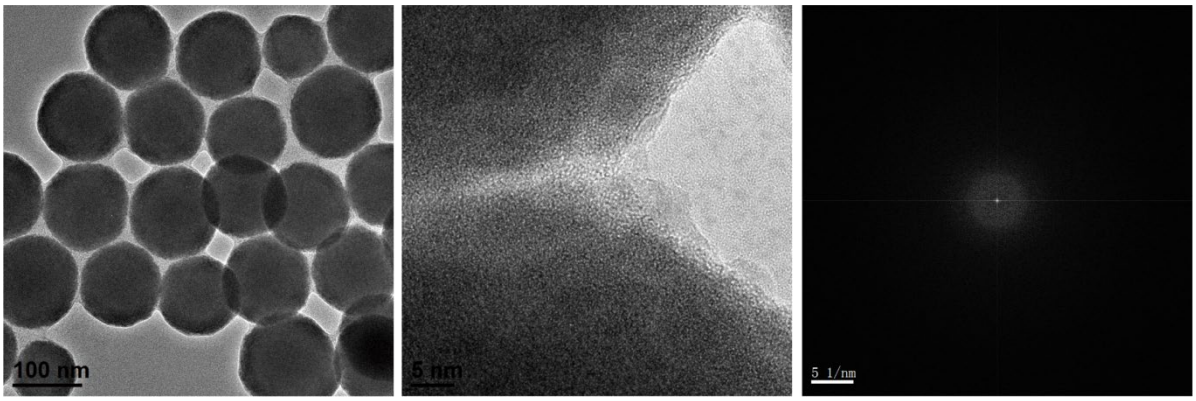


Figure S10. High resolution, HRTEM and its corresponding FFT image of FcPWNPs. Indicating the FcPWNPs were assembled by quantum dots.

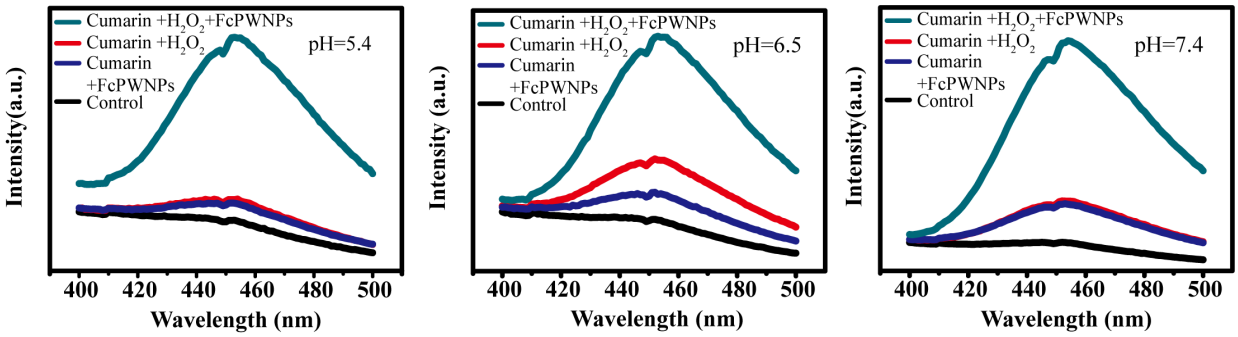


Figure S11. Spectrofluorimetry of 7-hydroxycoumarin generated by ·OH trapped coumarin. FcPWNPs (80 ppm Fe), H_2O_2 (400 μM) were added seperately or together. Showing the generation of ·OH.

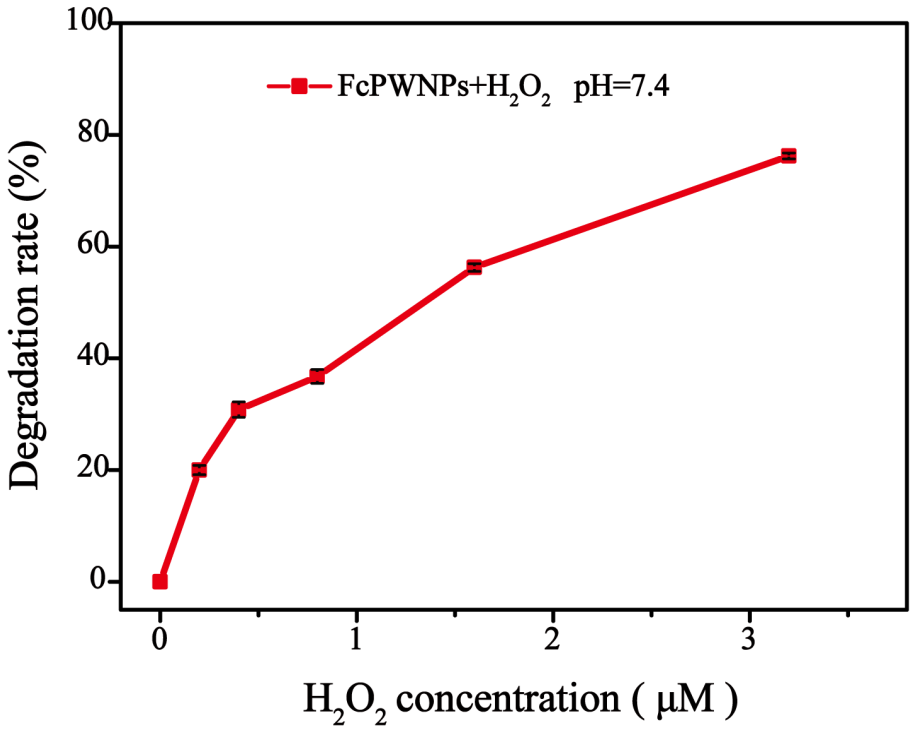


Figure S12. H_2O_2 dosage dependent degradation ratio of MO treated with FcPWNPs (Fe concentration of 80 ppm) and different concentration of H_2O_2 (0.3, 0.6, 1.2, 2.4 and 4.8 mM), respectively (n=3, mean ± SD). Exhibits H_2O_2 dose dependent degradation efficiency of FcPWNPs.

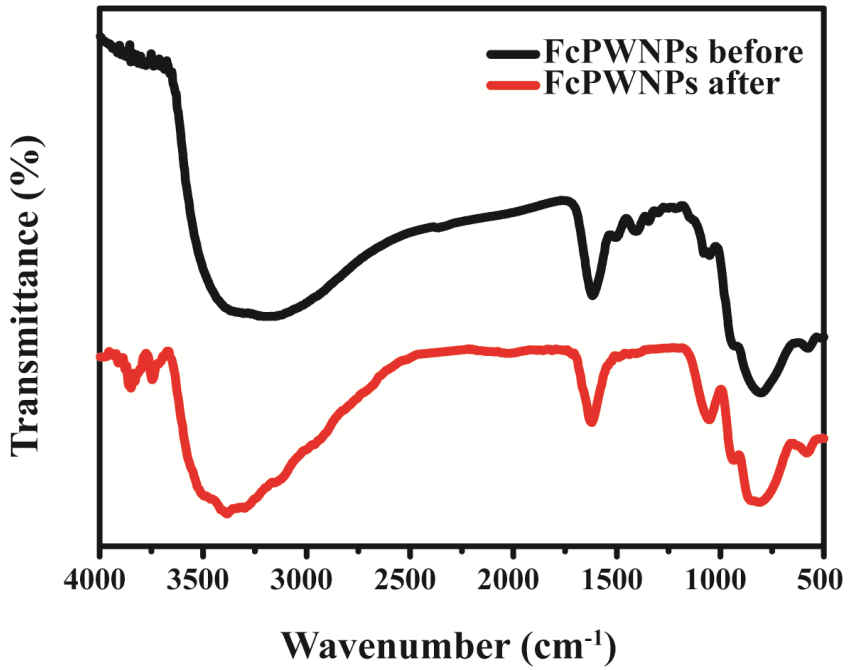


Figure S13. FT-IR spectrum of FcPWNPs, before and after treated with H_2O_2 (1mM). The overall structure of FcPWNPs did not change remarkably after reacted with H_2O_2.

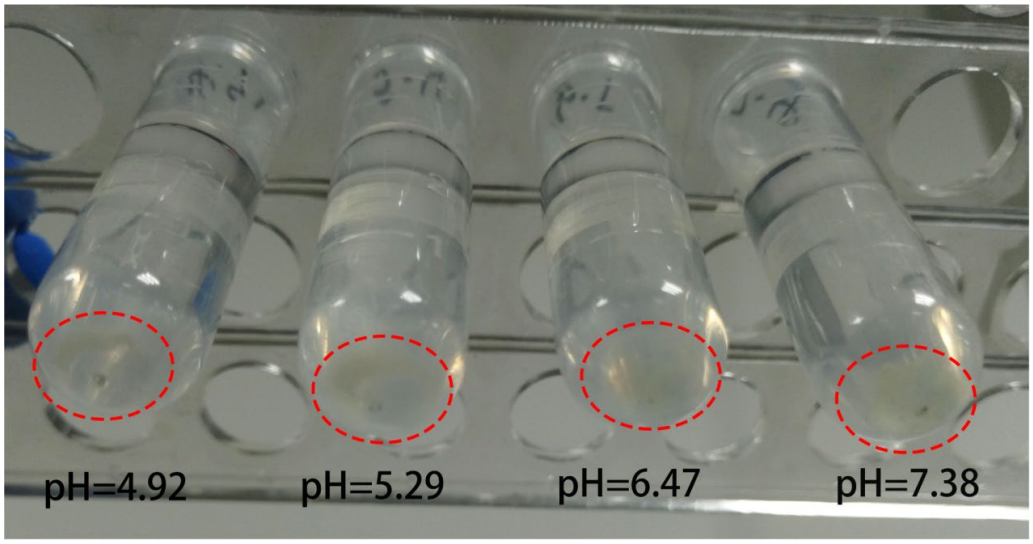


Figure S14. The digital photograph of Fe $(OH)_X$ after $Fe^{2+/3+}$ poders (1 mmol) were added into PBS solutions under different pH valve. Implying free Fe ions will soon precipitate by the –OH at neutral pH.

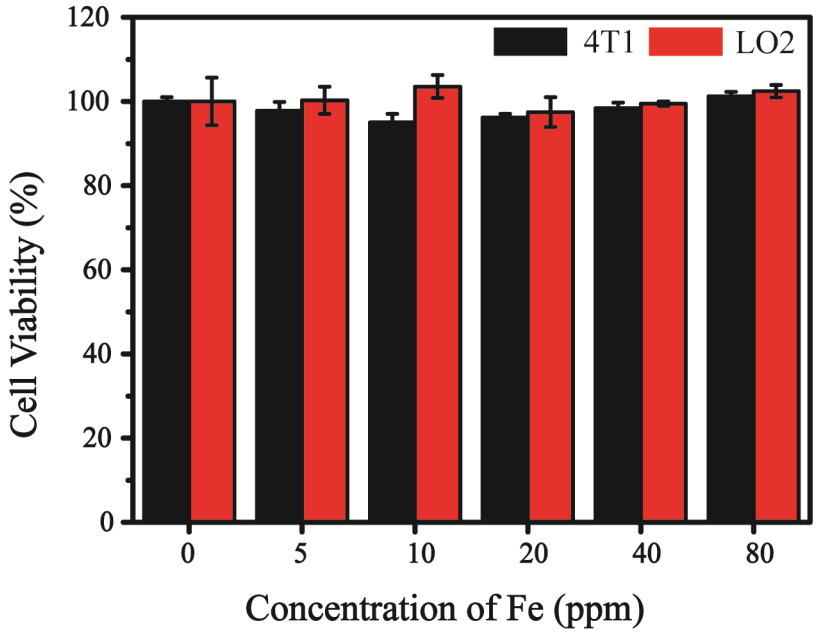


Figure S15. Cell viability of 4T1 (rat breast tumor cell) and LO2 (human liver cell) after incubated with FcPWNPs at Fe concentration of 80, 40, 20, 10, 5 ppm for 48 h, respectively. It indicates the excellente biocompatibility of FcPWNPs.

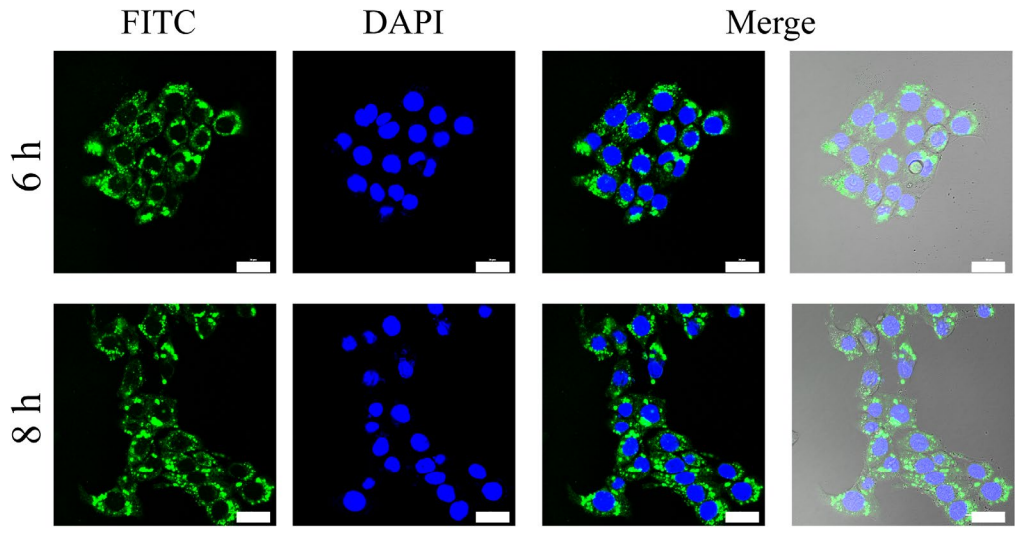


Figure S16. The co-localization picture of FITC stained FcPWNPs and DAPI stained 4T1 cells (scale bar = 20μm).

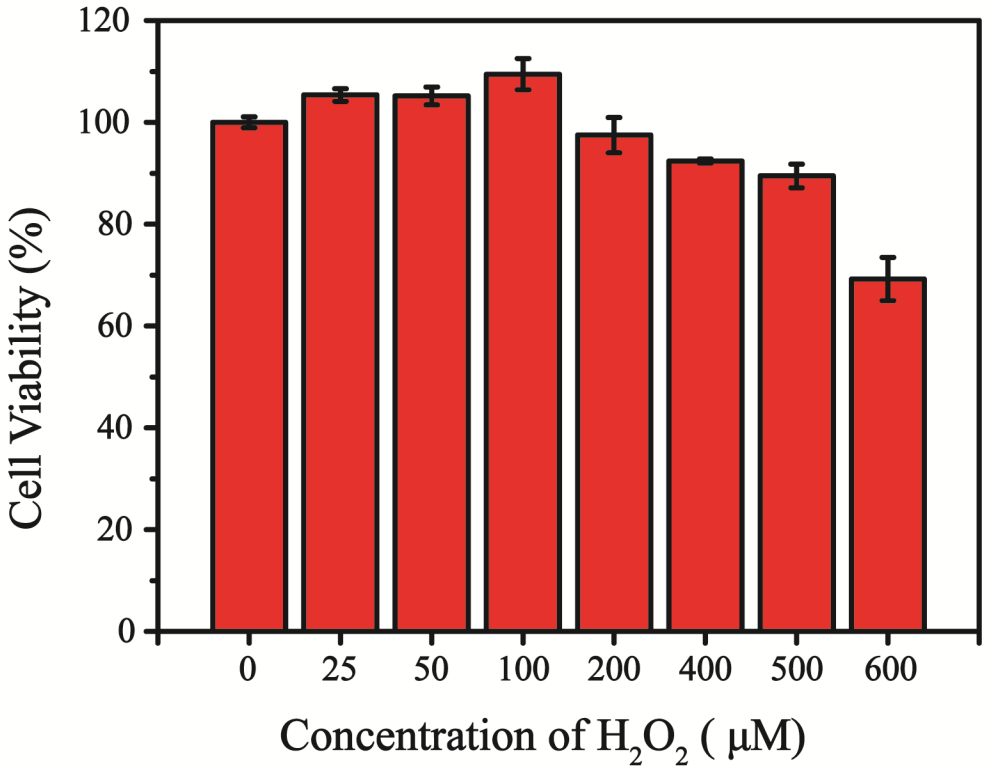


Figure S17. Cell viability of 4T1 cells after incubated with H_2O_2 of 600, 500, 400, 200, 100, 50, 25 μM for 24h, respectively. Showing no obvious decrease of cell viability was observed (more than 90%) if treated with H_2O_2 less than 200μM.

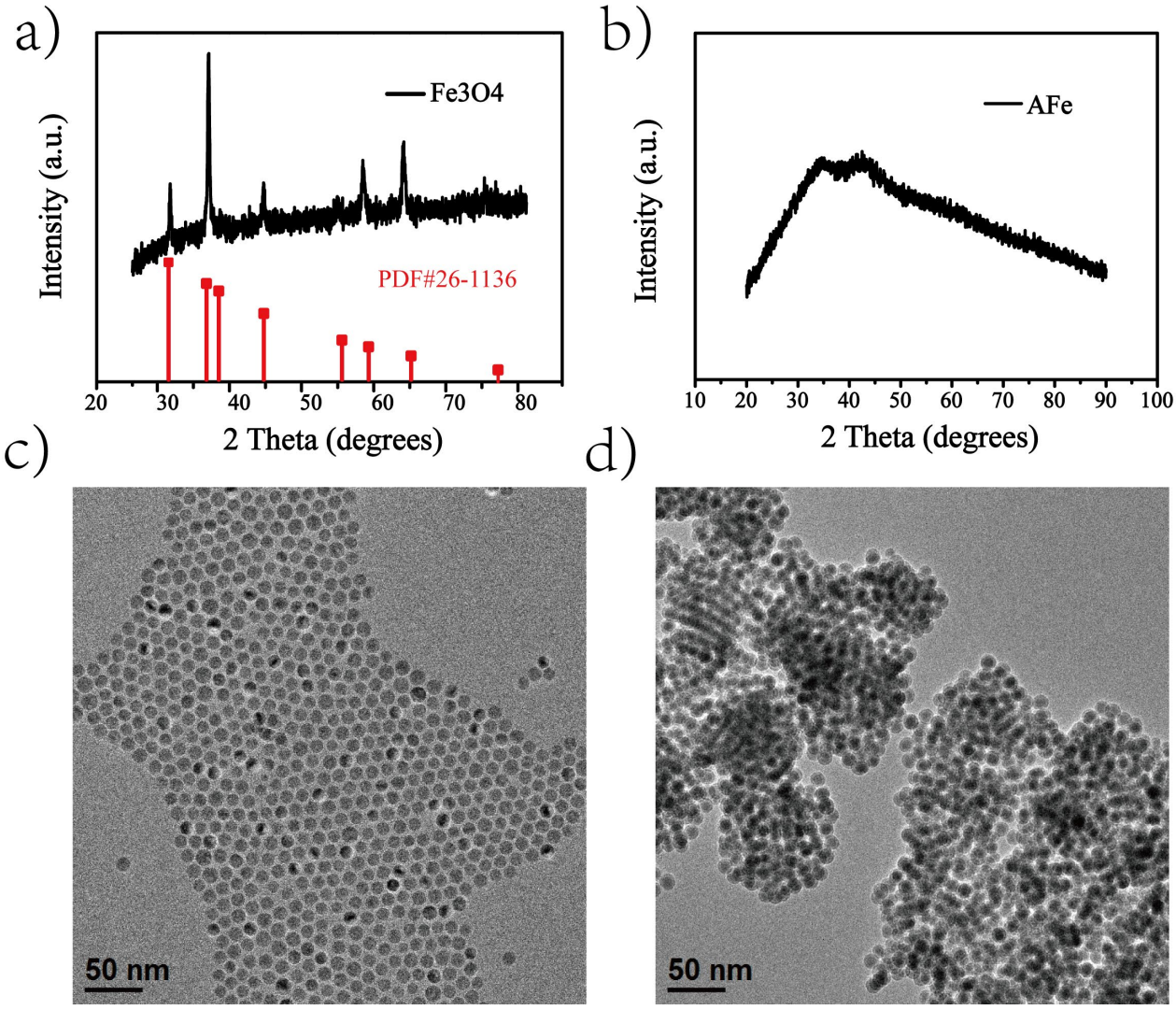


Figure S18. The characterization of Fe_3O_4 and AFe. XRD patterns of a) Fe_3O_4 and b) AFe, and their TEM images c) Fe_3O_4 and d) AFe, respectively.

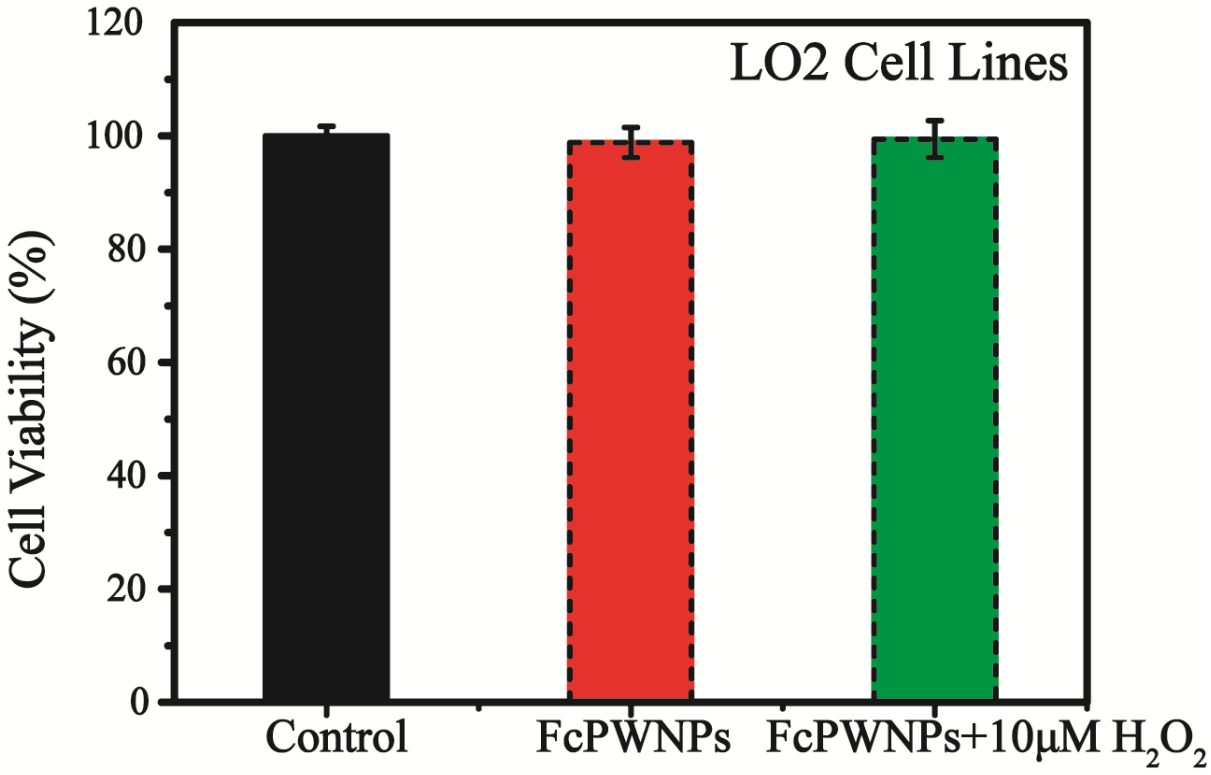


Figure S19. Cell viability of LO2 cells after incubated with FcPWNPs and 10μM of H_2O_2 respectively and simutaneously for 24h. FcPWNPs show negligible cytotoxicity to normal cells with thimbleful H_2O_2 concentration.

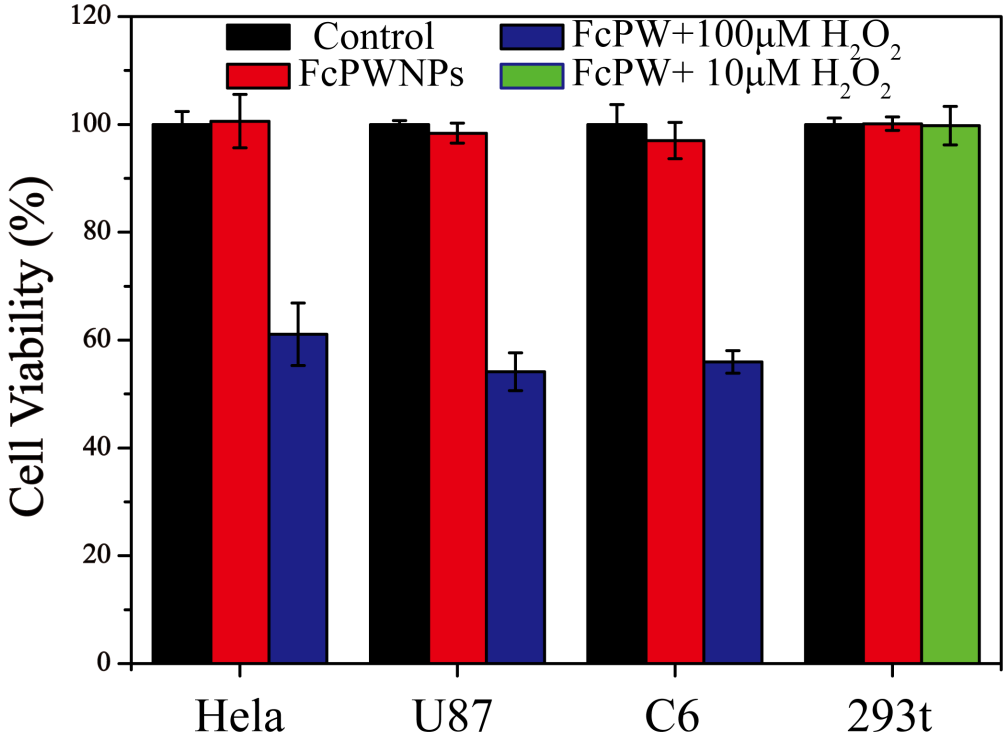


Figure S20. Cell viability of Hela, U87, C6, and 293t cells after various treatments. No cytotoxicity is observed in both cancer cell lines and normal cell lines without the participation of H_2O_2. After moderate H_2O_2 are added to mimic different tissue environment, selective cancer therapy property of FcPWNPs are observed.

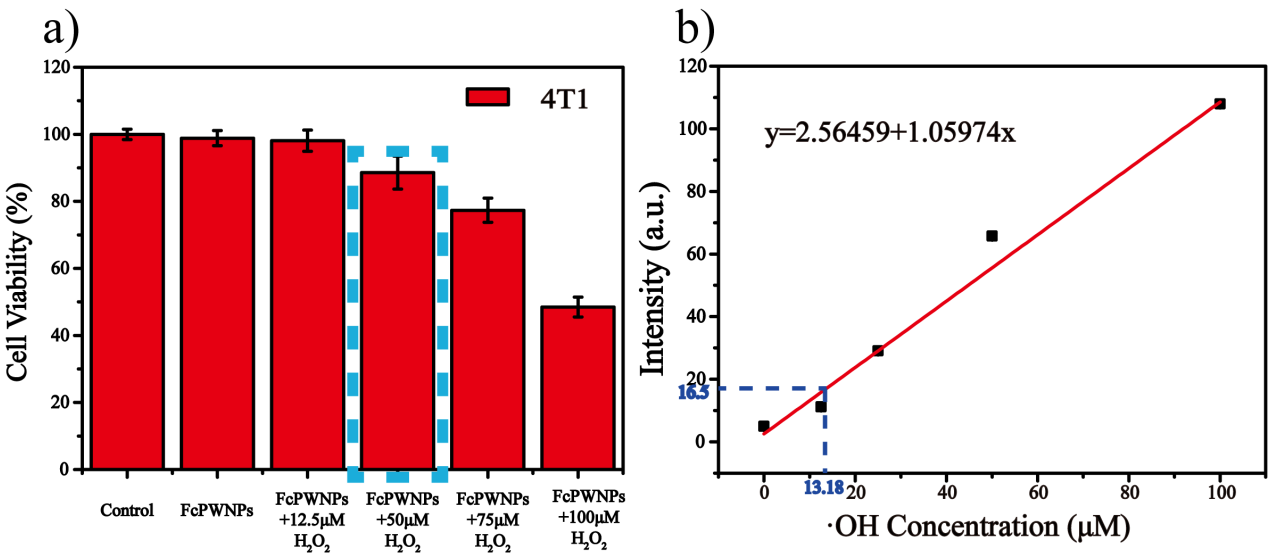


Figure S21. a) The cell viability of 4T1 cells treated with FcPWNPs and different concentration of H_2O_2 after 24h, and b) the 7-hydroxycoumarin fluorescence intensity- ·OH concentration standard curve. The threshold for ·OH to embody its cytotoxicity is speculated as 13.18μM.

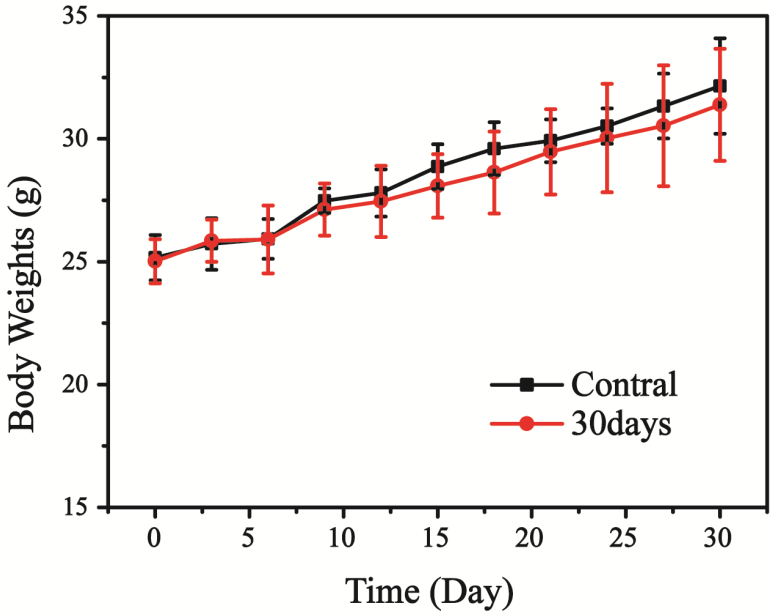


Figure S22. Time-dependent body-weight of Kunming mice treated with *i.v.* injection of FcPWNPs (70 mg Fe kg^{-1}, in 100 μl saline, n=5, mean± s.d.) and PBS (n=5, mean± s.d.). No significant body weight changes were observed.

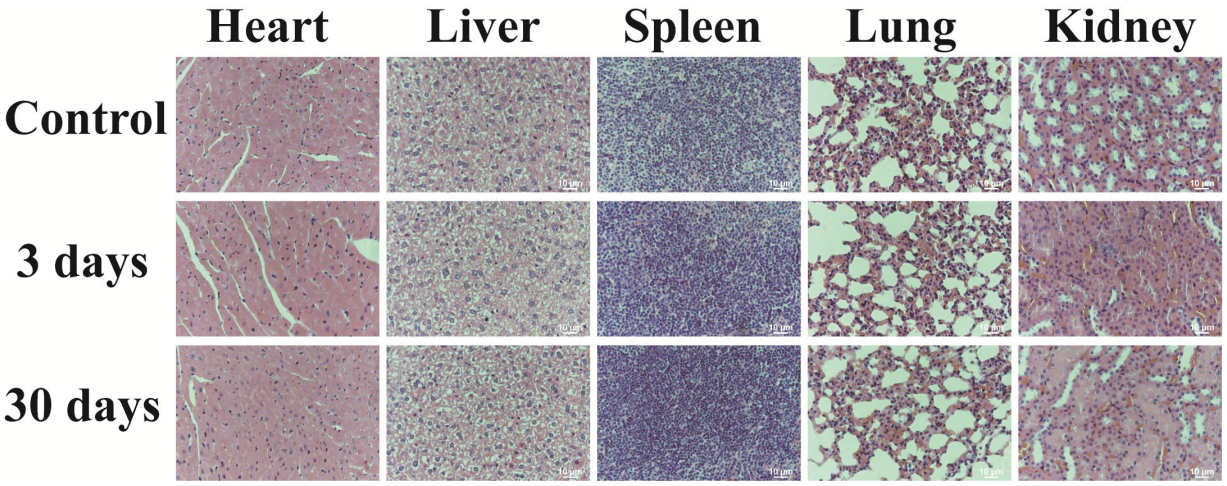


Figure S23. Pathological H&E stained images of tissue sections from heart, liver, spleen, lung and kidney of the BALB/c mice treated with FcPWNPs（70mg Fe kg^{-1}）harvested after 3 days and 30 days. The result showed that FcPWNPs possess excellent biocompatibility with negligible acute and chronic toxicity on the main organs.

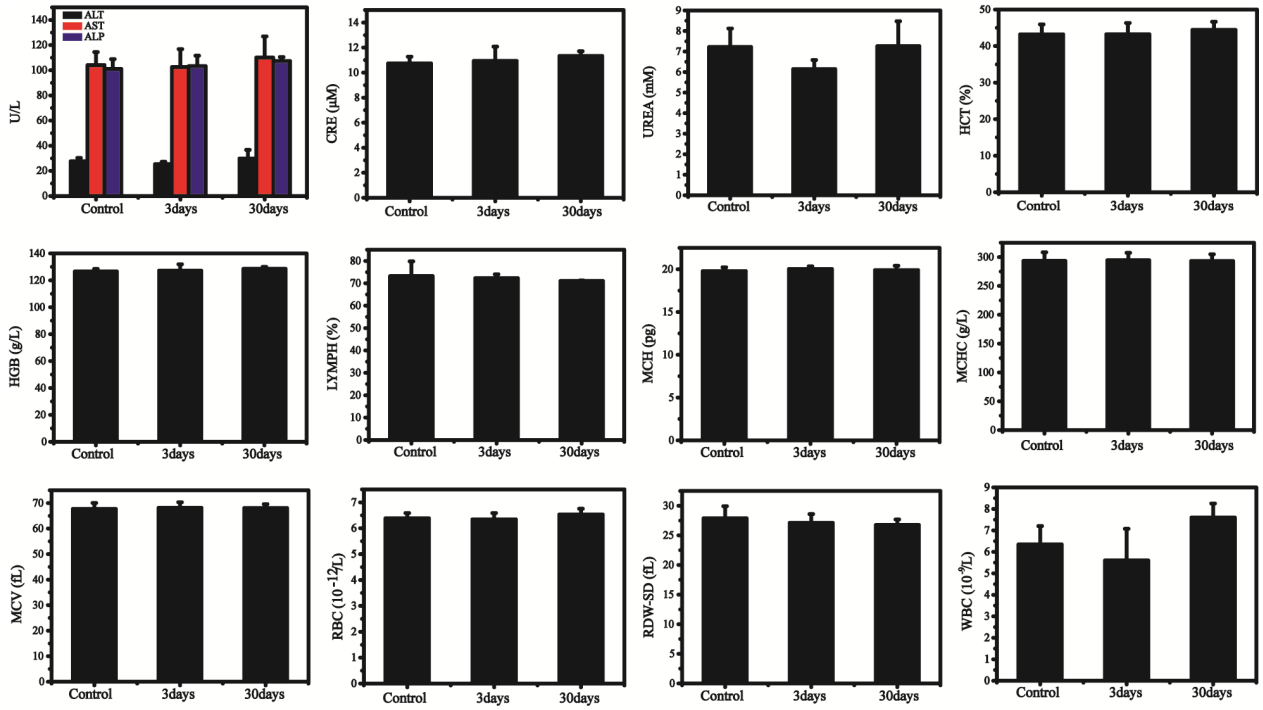


Figure S24. Blood biochemical parameters (ALT, AST, ALP, CREA and UREA) and blood routine tests of the mice after *i.v.* injection of FcPWNPs (70 mg Fe kg^{-1}, 100 μl, n=5, mean± s.d.) for 3 days and 30 days with equivalent injection of saline as control.

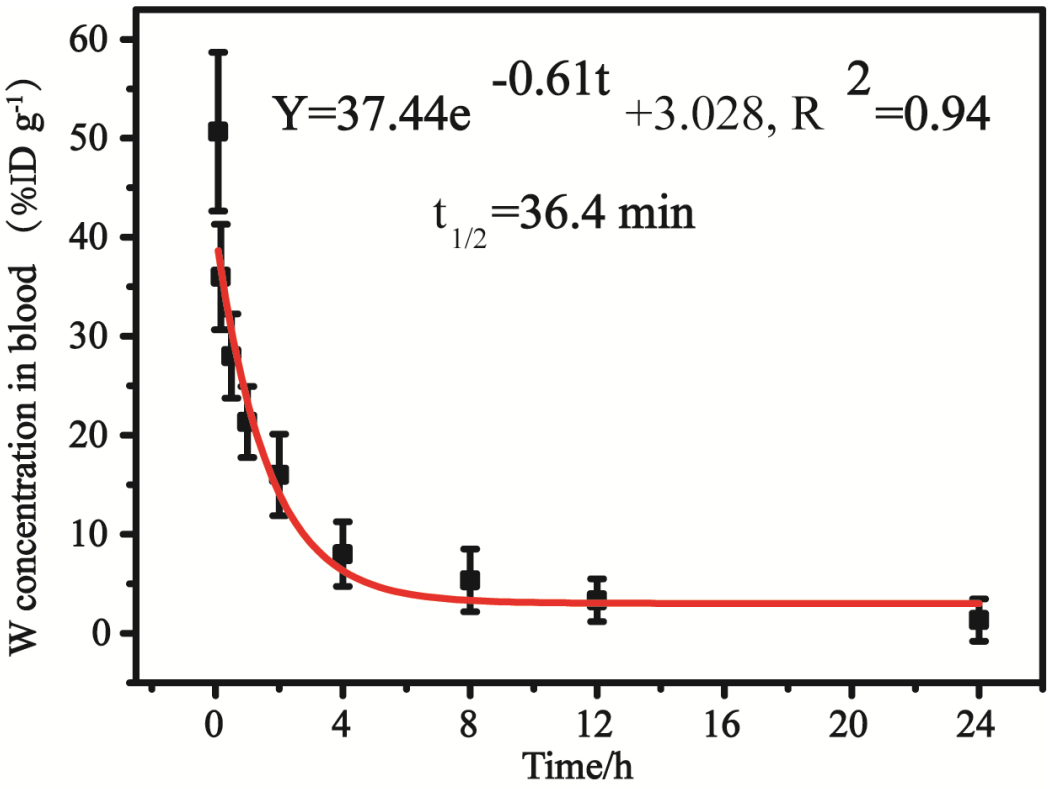


Figure S25. The blood circulation half-life of FcPWNPs was calculated to be nearly 36.4min basing on the one-component pharmacokinetic model (n=3, mean± s.d.). The usage of W element excluded the influence of intrinsic Fe element in the blood.

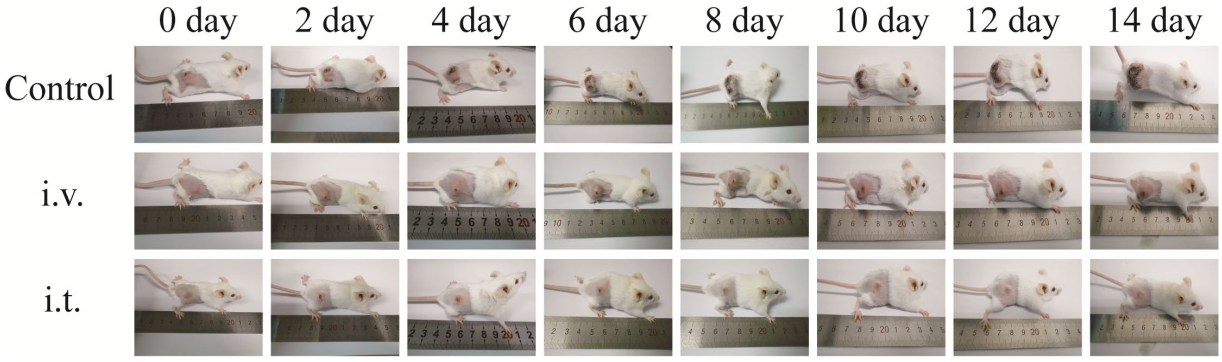


Figure S26. Representative photographs of 4T1-tumor-xenografted BALB/c mice after *i.v.* injection with saline and 100 μl, (50 mg Fe Kg^{-1}) of FcPWNPs, *i.t.* injection with 20 μl, (40 mg Fe Kg^{-1}) of FcPWNPsNPs at different times.