Electronic Supplementary Material (ESI) for Biomaterials Science.


# Supramolecular-mediated dual-functional nanocomposites for programmable cancer therapy

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## MATERIALS AND METHODS

**Chemicals:** All the chemicals were used as received without further purification. Methylene blue (MB) was purchased from TCI (Tokyo, Japan), DCFDA, Hoechst 33342, Calcein/AM double staining kit and Resveratrol were obtained from Solarbio (Beijing, China). Annexin V/PI apoptosis kit was purchased from Biolegend (CA, USA). CCK-8 was bought from Dojindo (Kumamoto, Japan). Tanic acid (TA) was purchased from Sigma (Sigma-Aldrich, USA). Hydroxypropyl-β-CD (HP-CD) was purchased from Roquette (Lestrem, France). All HPLC-purified DNA molecules were synthesized by Sangon Biotech Co., Ltd. (Shanghai, China). DMEM and FBS were purchased from Wisent Corporation (Nanjing, China). Opti-MEM was purchased from Gibco (California, USA). M-β-CD (methyl-β-cyclodextrin) and CPZ (chlorpromazine hydrochloride) were purchased from Selleck (Selleck Chemicals, USA). Matrigel was purchased from BD (BD, USA). 35 mm glass bottom dishes (Cellvis, Mountain View, CA) were purchased from Hualide Technologies Co., Ltd. (Beijing, China). The water used throughout the experiments was Millipore water (18.2 MΩ). All the experiments were conducted at room temperature unless mentioned otherwise.

**Instruments:** Transmission electron microscopic (TEM) images were captured on a Hitachi HT-7700 (Hitachi Co. Ltd., Japan) transmission electron microscope. Fluorescence spectra were collected using a Hitachi F-4600 fluorimeter (Hitachi Co. Ltd., Japan). UV/Vis spectra was recorded by a Hitachi 5300 spectrophotometer (Hitachi Co. Ltd., Japan). In vitro cytotoxicity assay was carried out on a Thermo MULTISCAN GO reader (Thermo, USA). Confocal microscopic images were obtained using an Olympus FV1000 confocal microscope (Olympus Co., Japan) at 60× magnification. The flow cytometry assays were carried out using a Guava easyCyte flow cytometer6 (Guava Technologies Inc., Hayward, CA, USA). The in vivo fluorescence images were acquired with IVIS® Spectrum *in vivo* imaging system (PerkinElmer Inc., Waltham, Massachusetts).

**Synthesis Fe-NPs and Res-Fe-NPs:** Typically, 22.8 mg of Res was added into 500 μL of absolute ethanol and 154 mg HP-CD was dissolved 5 mL of DI water. Then mixed

the two solutions together followed with an immediate vortex for 8 h (60 $^{o}$C，800 r/min) to get CD-Res (The molar ratio of HP-CD and Res is 1:1). For Fe-NPs synthesis, 37.5 μL DNA solutions (200 μM) was mixed with 15 μL $Fe^{2+}$ solutions (20 mM) in 247.5 μL DI water then the mixture was heated to 90 $^{o}$C and maintained for 3 h. After cooling to room temperature, the solution was centrifuged at 13000 rpm for 10 min and washed with DI water. The particles were re-dispersed in 500 μL DIwater and kept at 4 $^{o}$C for further use. For Res-Fe-NPs synthesis, 37.5 μL DNA solutions (200 μM) was mixed with 15 μL $Fe^{2+}$ solutions (20 mm) and 150 μL CD-Res (2.6 mg/mL) in 297.5 μL DI water then the mixture was heated to 90 $^{o}$C and maintained for 3 h, followed by the same procedure as described for Fe-NPs synthesis. The NPs obtained above were dispersed in 500 μL of tannic acid (TA) solution (3 mM) and vortexed for 1 min, then the mixture was centrifuged at 13000 rpm for 3 min to remove the supernatant, and re-dispersed in DI water. Repeat the cleaning three times to obtain TA-coated NPs, which were dispersed in DI water for later use.

**Res Release:** For drug release experiments, the obtained CD-Res (1.6 mg) were immersed in DI water (1 mL) and let it stand still for 4 h, and then filter the precipitates to get the supernatants for Res fluorescence detection. Different concentrations of Res were set as standards. At predetermined time intervals (0 h, 1 h, 2 h, 4 h, 6 h, 8 h and 12 h), DI water was removed by centrifugation and replaced with an equal volume of fresh DI water. The amount of released Res at specific time was determined by measuring the fluorescence intensity of Res in the supernatant. And for effects of pH on Res release, different pH (pH 5.5, pH 6.5 and pH 7.4) was set. As mentioned above, supernatant was collected for analysis and to determine the appropriate experimental pH conditions. For effects of temperature on Res release, 20 $^{o}$C, 37 $^{o}$C and 60 $^{o}$C were set to determine the appropriate experimental temperature conditions.

**Chemical kinetic activity of Res-Fe-NPs:** Res-Fe-NPs ([$Fe^{2+}$] = 0.5 mM) was added to a solution containing 10 μg/mL MB and 8 mM $H_2O_2$ and the mixture was incubated at 37 $^{o}$C for 30 minutes, and the absorbance change of MB at 665 nm was measured.

**Cell Culture:** Human breast cancer cells MCF-7 and murine breast cancer cells 4T1

were purchased from American Type Culture Collection (ATCC). MCF-7 cells and 4T1 cells were cultured in DMEM and RPMI 1640 medium, respectively. The media was supplemented with 10% fetal bovine serum (FBS), 100 U/mL streptomycin, and 100 μg/mL penicillin in a 5% $CO_2$, 37 °C incubator. Both cells were tested to be free of mycoplasma contamination and were authenticated by short tandem repeat fingerprinting.

**Analysis of cellular uptake:** MCF-7 cells were seeded in 35 mm glass-bottom confocal dishes (for fluorescence microscopy) or six-well plate (for flow cytometry) ($2\times10^5$ cells/well) and incubated overnight to reach 80-90% confluence. Cells were treated with PBS, CD-Res, Fe-NPs and Res-Fe-NPs (DNA was labeled with Cy5, equivalent 0.1 mg/mL) at 37 °C in Opti-MEM medium. After 4 h incubation, cells were washed twice with PBS and cellular uptake was analyzed with confocal laser scanning microscope. Nuclei were stained with Hoechst. For flow cytometry analysis, the cells were detached and washed several times with pre-cold PBS before flow cytometry assay.

**Inhibition test:** MCF-7 cells were plated on a six-well plate at a density of ~$4\times10^5$ cells per well. After 24 h incubation, cells were treated with 25 μM CPZ for 30 min, and 5 mM M-β-CD for 1 h at 37°C. Then, Res-Fe-NPs were added into wells and incubated for another 4 h. Subsequently, cells were washed with PBS twice and detached with trypsin, then cell pellets were fixed with 4% formalin solution neutral buffered (Sigma) for 20 min and collected for flow cytometry analysis.

**Cell viability test:** MCF-7 cells ($2\times10^4$ cells/well, 100 μL) were seeded in 96-well plate. After cultured for 24 h, the cells were treated with CD-Res, Fe-NPs and Res-Fe-NPs (0.1 mg/mL) for 4 h. Then, cells were cultured for another 20 h, followed by the CCK-8 assay according to the manufacturer's protocol. The cells treated with PBS were used as negative control.

**Combination index (CI) analysis:** The synergistic, additive, or antagonistic cytotoxic effects were evaluated by the combination index (CI) analysis according to the method of Chou-Talalay[1], using the following equation:

$$CI=\frac{(D)1}{(Dx)1}+\frac{(D)2}{(Dx)2}$$

In which (Dx)1 and (Dx)2 represent the IC50 value of the drug 1(Res) alone and drug 2 (Fe-DNA) alone, respectively. (D)1 and (D)2 represent the concentration of drug 1 and drug 2 in the combination system (Res-Fe-NPs) at the IC50 value. Values of CI > 1 indicate antagonistic effect, CI = 1 indicate additive effect and CI < 1 indicate synergistic effect.

**Cell apoptosis assay:** MCF-7 cells were plated on a six-well plate at a density of ~$4\times10^5$ cells per well. After 24 h incubation, the culture medium was replaced with Opti-MEM containing CD-Res, Fe-NPs and Res-Fe-NPs (0.1 mg/mL). After incubation for 4 hours, cells were washed with PBS twice to remove uninternalized materials. Then the cells were stained with Annexin V and PI using Annexin V/PI apoptosis detection kit according to the manufacturer's instructions for flow cytometry analysis.

**Live/dead cell containing:** MCF-7 cells were plated on 35-mm confocal dishes at a density of ~$1\times10^5$ cells per well and treated with materials as the same with that for cell apoptosis assay. Then, the cells were stained with calcein AM/PI double-stain kit under the instructions of the manufacturer for CLSM imaging.

**Detection of intracellular $^1O_2$ production:** MCF-7 cells were plated on 35-mm confocal dishes at a density of ~$1\times10^5$ cells per well and treated with materials as the same with that for cell apoptosis assay. After incubation for different time (1 h, 2 h, 4 h and 24 h) in the dark, cells were washed to remove noninternalized materials. Fresh culture medium containing DCF-DA (20 μM) was added for another 20 min incubation in the dark. The post treatment cells were collected for fluorescence imaging and flow cytometry analysis.

**Care and use of animals:**

Female BALB/c mice aged 6-8 weeks (16-18 g) were purchased from Vital River Animal Laboratories (Beijing, China) and maintained in a sterile environment. Xenograft tumor models were established by inoculating MCF-7 cells ($1\times10^6$ cells/100 μL in 1:1 (v/v) PBS and Matrigel, BD bioscience) into the left flank of the mice.

**In vivo imaging:** When the tumor sizes reached ~200 $mm^3$, the mice were

intravenously injected with Res-Fe-NPs (100 mg/kg, DNA was labeled with Cy5). At specified time intervals (1h, 3h, 6h, 12h, 24h), mice were anesthetized with isoflurane and imaged with an IVIS system with excitation and emission wavelengths of 640 and 680 nm, respectively. Tumors and organs were also collected and imaged at specified time intervals (1h, 3h, 6h, 12h, 24h).

**In vivo tumor therapy:** When the tumor sizes reached ~50 $mm^3$, the mice were randomly divided into four groups for the treatment with PBS, CD-Res, Fe-NPs and Res-Fe-NPs. Mice were intravenously injected with different samples every other day for three times. Then tumor volume and body weight were recorded every other day. The tumor volume was calculated using the following equation: tumor volume=length×$width^2$/2. At the end of the experiment, tumors of mice in contrast treatment groups were fixed and sectioned for H&E and TUNEL staining.

**Statistical analysis:** Data are presented as mean ± SD. Student's t-test was applied for comparison of two groups, and one-way analysis of variance (ANOVA) was used to compare the difference of multiple groups. A *"P"* value < 0.05 was considered statistically significant. All data were analyzed using GraphPad Prism version 5.

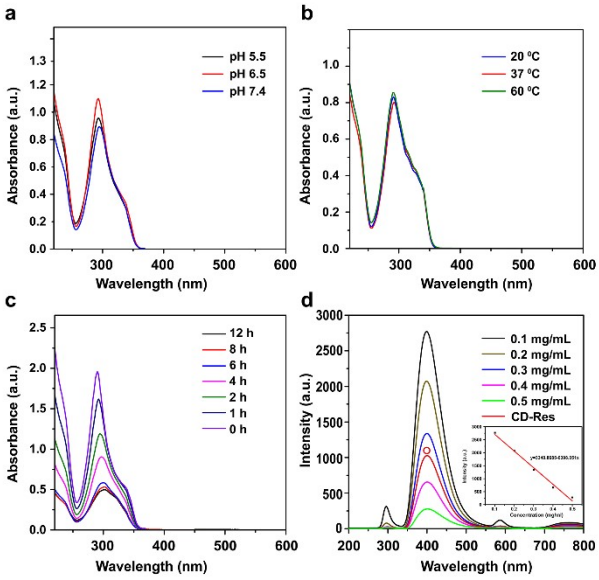


Figure S1 UV absorption of CD-Res release at different (a) pH; (b) temperature; (c) time; (d) CD-Res

fluorescence quantification at different concentration.

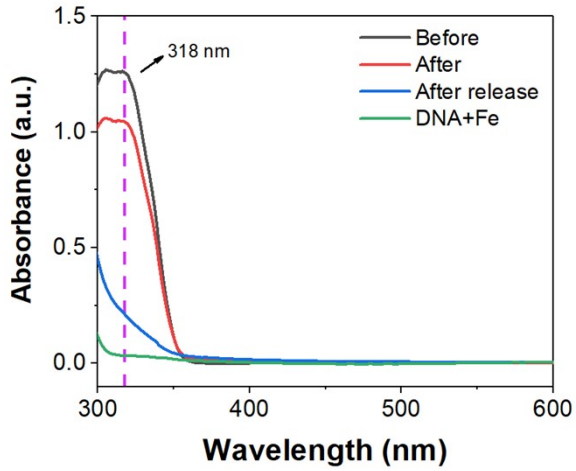


Figure S2 UV absorption of verifying the release of Res-Fe-NPs. (Before: the Res-CD solution before loading; After: the supernatant after loading; After release: the supernatant after 12 hours incubation in PBS solution with pH 6.5; DNA+Fe: the solution of DNA and Fe(II) before loading)

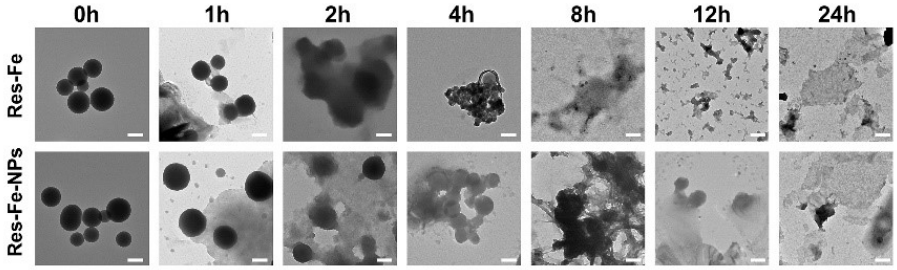


Figure S3 TEM images of Res-Fe and Res-Fe-NPs that are incubated in 1X PBS for different time.

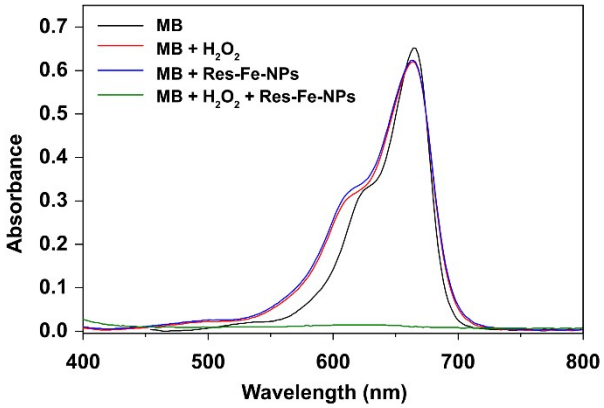


Figure S4 Fenton reaction conditions of Res-Fe-NPs in solution

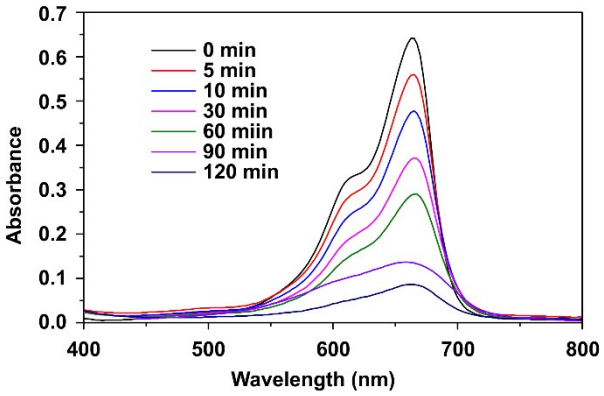


Figure S5 ·OH production and MB fading change with time.

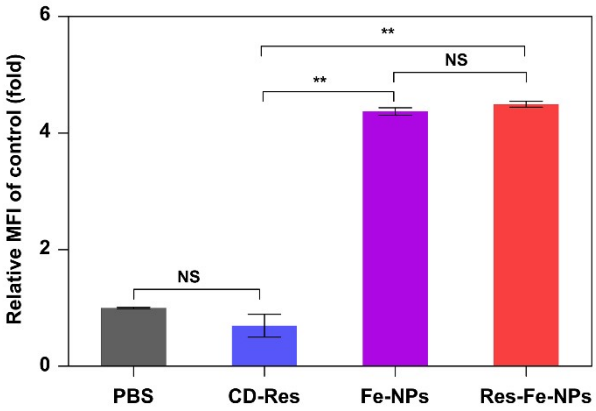


Figure S6 Flow cytometric quantification of the cellular uptake of Fe-NPs and Res-Fe-NPs. Data are represented as means ± SD (n = 3).

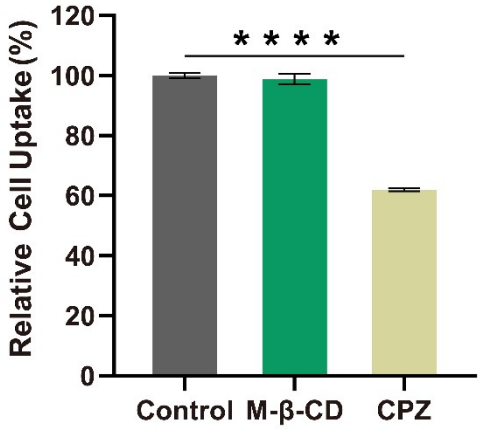


Figure S7 Endocytic mechanisms of Res-Fe-NPs. Data are represented as means ± SD (n = 3).

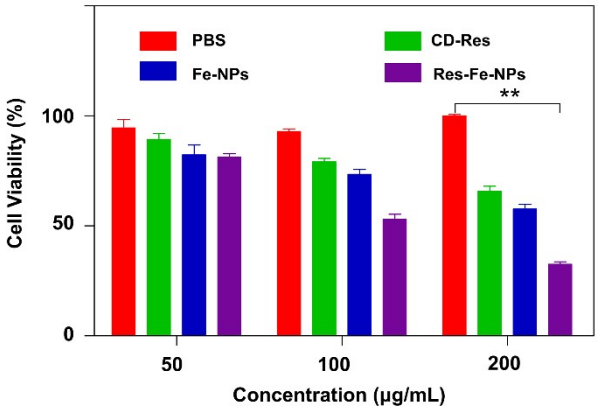


Figure S8 Cell viability of MCF-7 cells with different treatments (PBS, CD-Res, Fe-NPs, Res-Fe-NPs) at different concentrations. Data are represented as means ± SD (n =5).

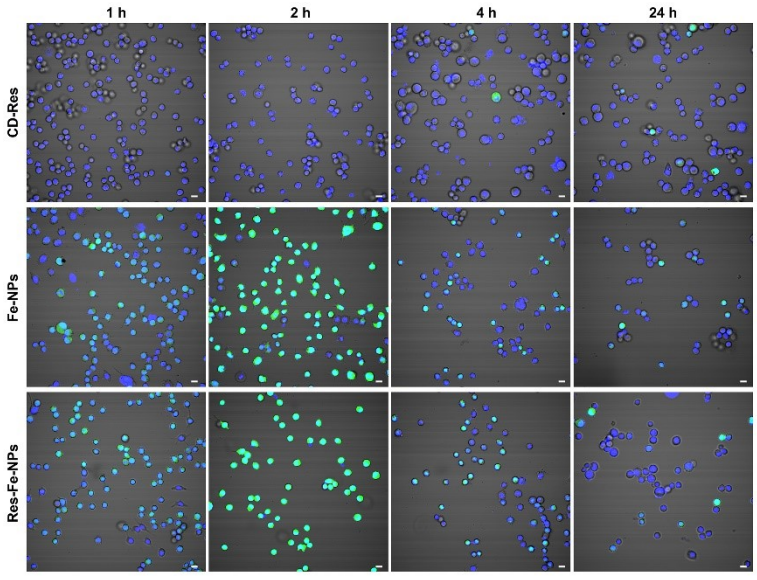


Figure S9 Confocal fluorescence images of time dependent (1 h, 2 h, 4 h and 24 h) effect of CD-Res, Fe-NPs and Res-Fe-NPs on MCF-7 cells intracellular ROS production. DCF-DA was used as an indicator of $^1O_2$ in cells. Nuclei stained with Hoechst 33258. Scale bars, 10 μm.

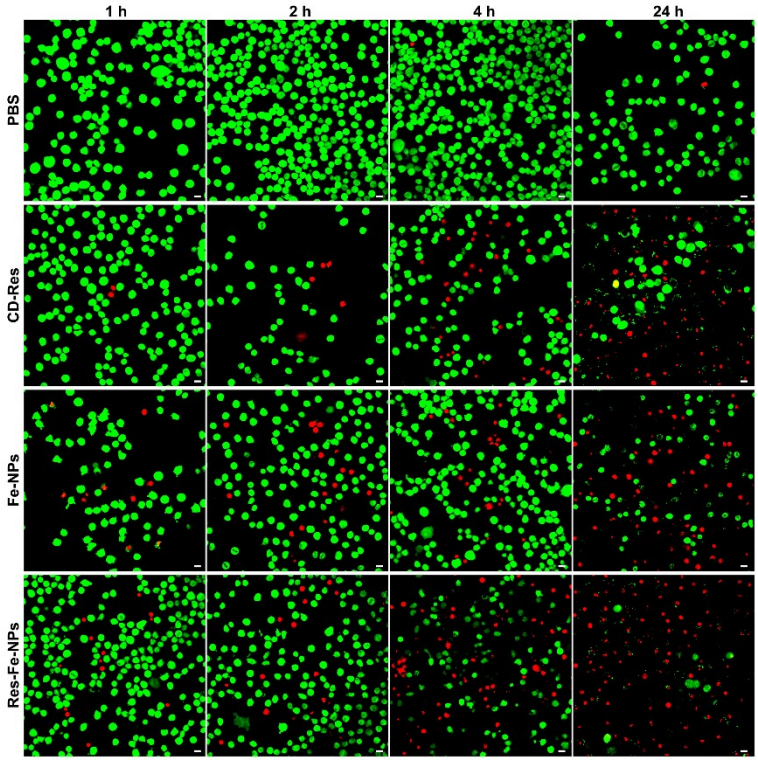


Figure S10 CLSM images of MCF-7 cells with different treatments (PBS, CD-Res, Fe-NPs and Res-Fe-NPs) for different time, and then costained with calcein AM and propidium iodide (PI). Scale bars, 10 μm.

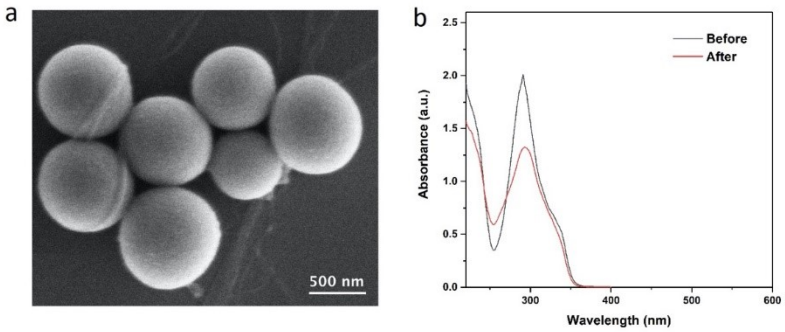


Figure S11 (a) SEM image of Res-Fe-DNA; (b) UV absorption of Res before and after the formation of NPs. **Synthesis of Res-Fe-DNA:** 37.5 μL of random DNA solution (200 μM) was added into 150 μL of Res solution (200 μM, ethanol/water (*v*/*v*): 1:49), immediately followed a quick vortex to make sure the ingredients mixed together thoroughly. 15 μL of $FeSO_4 \cdot 7H_2O$ (20 mM) was then quickly added into the above mixture and followed a one-minute vortex. The final mixture was heated to 90 °C for 3 hours. After cooling to room temperature, the as-prepared Res-Fe-DNA were centrifuged at 13000 rpm for 15 minutes and washed with DI water sufficiently. The Res-Fe-DNA were re-dispersed in 300 μL DI water and kept for future use. The SEM images proved the formation of Res-Fe-DNA nanoparticles. And the entrapment efficiency of Res in NPs was determined to be about 36% according to the UV absorption of CD-Res before and after the reactio

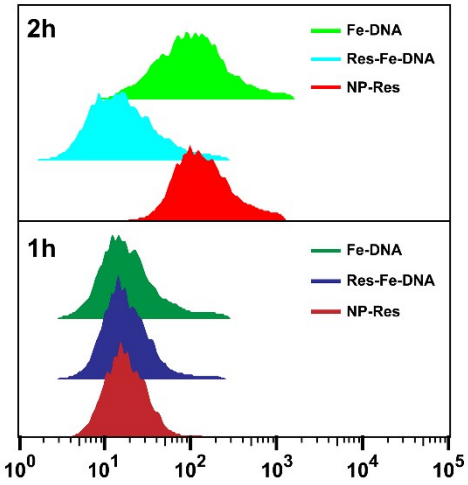


Figure S12 Flow cytometry showing of MCF-7 cells after different treatments. DCF-DA was used as an indicator of $^1O_2$ in cells.

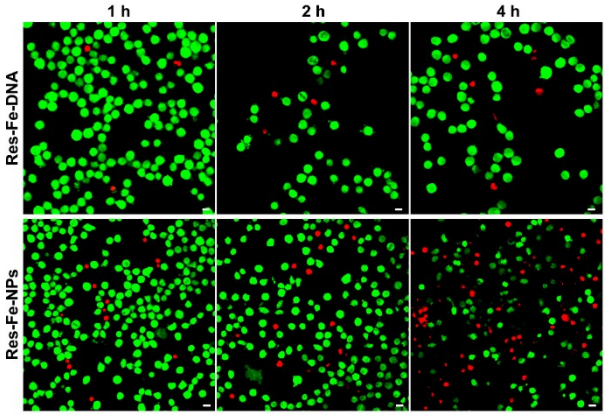


Figure S13 CLSM images of MCF-7 cells treated with Res-Fe-DNA and Res-Fe-NPs for different time, and then costained with calcein AM and propidium iodide (PI). Scale bars, 10 μm.

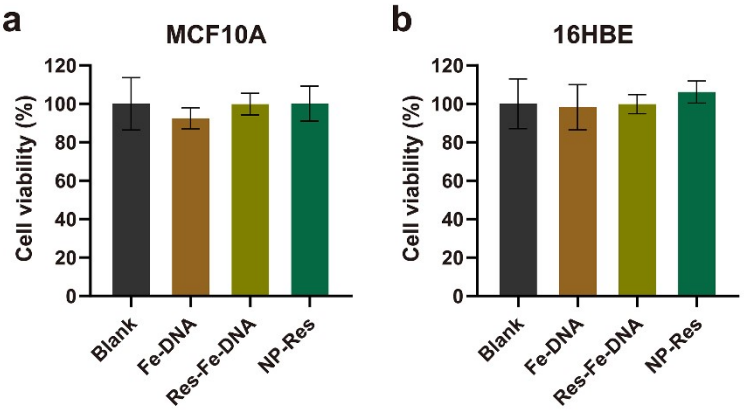


Figure S14 Cell viability of 16HBE and MCF-10A cells with different treatments (PBS, CD-Res, Fe-NPs, Res-Fe-NPs). Data are represented as means ± SD (n =5).

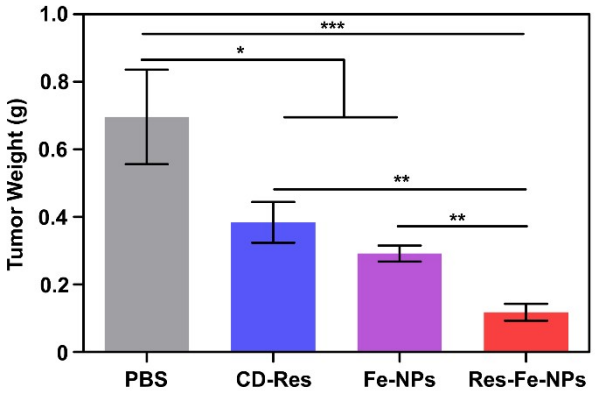


Figure S15 The average tumor weights of mice at day 16 in the different treatment groups. The data are presented as the mean ± SD (n =5). *$P$ < 0.05, **$P$ < 0.01, ***$P$ < 0.001.

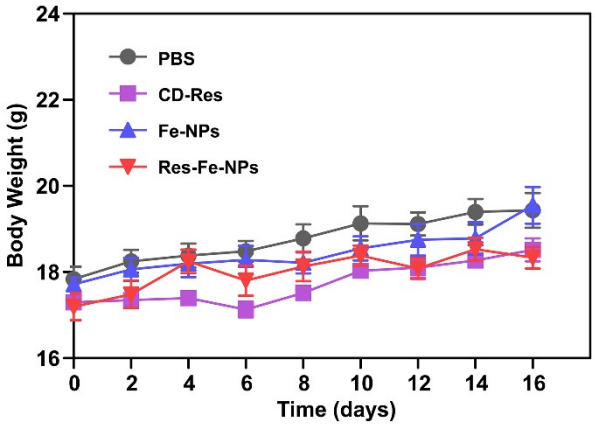


Figure S16 Body weight changes with time of BALB/c mice under different treatments. The data are presented as the mean ± SD. (n = 5).

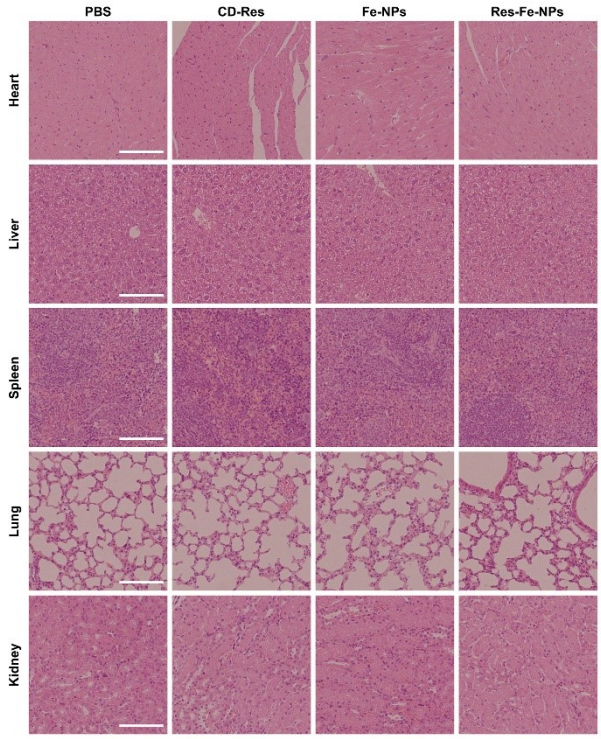


Figure S17 Histological assessments of heart, liver, spleen, lung and kidney tissues using H&E staining in different treatment groups. (Scale bars, 100 μm. The data are presented as the mean ± SD (n = 5).)

## References

1 T. C. Chou and P. Talalay, Adv. Enzyme Regul., 1984, **22**, 27-55.