Supporting information

Multi-pathway inducing ferroptosis by MnO_2-based nanodrugs for targeted cancer therapy

Shuhua Fan,[a‡] Qiangqiang Yang,[a‡] Qianna Song,[a] Min Hong,[a*] Xiaoyan Liu,[a] Haobin Chen,[a] Juan Wang,[a] Chuan Li,[a] Shuang Cheng[b*]

[a] Department of Chemistry, Liaocheng University, Liaocheng, 252059, Shandong, China. E-mail: hongmin@lcu.edu.cn

[b] Department of Agriculture, Liaocheng University, Liaocheng, 252059, Shandong, China. E-mail: chengshuang@lcu.edu.cn

‡ S. Fan and Q. Yang contributed equally.

.

1. Materials

$MnCl_2 \cdot 4H_2O$, tetramethylammonium hydroxide (TMA·OH), dihydroartemisinin (DHA), N-(3-dimethylaminopropyl)-N'-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuc-cinimide (NHS), dimethyl sulfoxide (DMSO), and 4-dimethylaminopyridine (DMAP) were purchased from Aladdin Reagent Co., Ltd. (Shanghai, China, http://www.aladdin-e.com). Distearoyl phosphatidyl ethanolamine polyethylene glycol carboxyl (DSPE-PEG-COOH, Mw = 2000) was purchased from Nanocs Inc. (USA). Tf (Mw = 80000, Purity: 95%) was purchased from Solarbio Co., Ltd. (China). 1640 RPMI cell culture medium and fetal bovine serum (FBS) was purchased from Gibco (Life Technologies, Australia). Penicillin and streptomycin, GSH and GSSG Assay Kit, Reactive Oxygen Species Assay Kit，Lipid Peroxidation MDA Assay Kit and Lyso-Tracker Red were purchased from Beyotime Co., Ltd. (China). 3-(4,5)-Dimethylthiahiazo(-2)-3,5-diphenytetrazoliumromide (MTT) was purchased from Energy Chemical Co., Ltd. (China). Other reagents were purchased from China National Pharmaceutical Group Corporation and used as received.

Antisense oligonucleotide:

Human-ASO (ASO): 5'-GATACGCTGAGTGTGGTTT-3'

Human-FAM-ASO (FAM-ASO): 5'-GATACGCTGAGTGTGGTTT-FAM-3'

Mouse-ASO (ASO'): 5'-GATA*T*GCTGAGTGTGGTTT-3'

2. Instruments

Fluorescence spectra were determined on an F-7000 spectrophotometer (Hitachi, Japan). Absorption spectra (UV-Vis) were carried out on a UV-750 ultraviolet spectrophotometer (Perkin-Elmer, USA). Fourier Transform infrared (FT-IR) spectra were carried out on a Nicolet 6700 FT-IR spectrophotometer (Thermo Scientific Ltd, USA). Transmission electron microscope (TEM) images were performed on a JEM 2100 electron microscope (JEOL Ltd., Japan). TEM worked with an acceleration voltage at 200 kV, and a micro grid was used for sample preparation. Scanning electron microscope (SEM) images were performed on a Thermo Fisher Scientific

FIB-SEM GX4. X-ray photoelectron spectroscopy (XPS) measurements were undertaken with a K-Alpha spectrometer (Thermo Scientific Ltd., USA). Atomic force microscopy (AFM) was carried out on a MultiMode 8 atomic force microscope with a Nanoscope V controller (Bruker, USA). The Raman spectrum was recorded with a Renishaw-inVia Raman microscope (UK). Confocal laser scanning microscopy (CLSM) was carried out on ZEISS LSM 880 (Germany). A Guava easyCyte 6-2L flow cytometer (Millipore, USA) was used to determine the fluorescence of the cell samples.

3. Experimental section

(1) Synthesis of manganese dioxide nanosheets

0.5937 g $MnCl_2 \cdot 4H_2O$ was dissolved in 10 mL ultrapure water to obtain 0.3 mM $MnCl_2$ (solution A). 2.1748 g TMA·OH was dissolved in 18 mL water, and then add 2 mL H_2O_2 (30 Wt%) into it to obtain the solution B. After that, pour solution B into solution A (this step is completed within 15 s), as the mixed aqueous solution is added, the solution immediately turns dark brown. Then, the suspension was stirred for 24 hours at room temperature to fully complete the oxidation reaction process. In order to purify MnO_2, the resulting solution was centrifuged for 10 min at 10000 rpm, and then washed it three times with ethanol and ultrapure water, and the sediment collected was dried with a vacuum freeze dryer at -80 °C to remove the remaining solvent. 30 mg of the as-prepared manganese dioxide was dissolved into 20 mL of ultrapure water. Finally, the solution was sonicated for at least 10 h and keep it in refrigerater (4 °C) for use in the experiment.

(2) Synthesis of DSPE-PEG-DHA

EDC (100 mM, 200 μl) and DMAP (100 mM, 100 μl) were added to DSPE-PEG-COOH (1 mM, 1 mL) solution to activate the carboxyl group, and the mixture was sonicated for 1 h to assure adequate mixing. Then add 50 μl DHA (100 mM) in DMSO into the obtained solution above, and stir the mixture for 24 h and sonicate for 10 h at room temperature. Next, the solution was treated by dialysis with dialysis bag (MWCO = 1000) for 72 h. Finally, the solution collected was dried with a vacuum

freeze dryer at -80 °C to obtain DSPE-PEG-DHA product.

(3) Synthesis of DSPE-PEG-Tf

EDC (100 mM, 100 μl) and NHS (100 mM, 200 μl) were added to the solution of DSPE-PEG-COOH (1 mM, 600 μL) to activate the carboxyl group. Then Tf (2 mg/ml, 600 μl) was added followed the pH of mixture was regulated to 7.0. The mixture was stirred at room temperature for 24 h and dialyzed for 72 h using the dialysis bag (MWCO=10000). Finally, the product of DSPE-PEG-Tf was obtained by vacuum freeze-drying.

(4) Synthesis of Tf-DHA-MnO_2, Tf-DHA-ASO-MnO_2 or FAM-Tf-DHA-ASO-MnO_2

DSPE-PEG-Tf (0.5 mg/ml) and DSPE-PEG-DHA (1 mg/ml) were sequentially added to the MnO_2 nanosheet (2 mg/ml) solution (same volume). After reacting for 24 h, the solution was centrifuged for 20 min (10000 rpm) in a centrifuge. Then the precipitate was washed three times. Then add GPX4 mRNA targeted human- or mouse-ASO at the ratio of 100 nM ASO per 40 μg/ml MnO_2-NS, and react for 2 h.

(5) UV-Vis characterization of MnO_2-NSs

The optical properties of MnO_2-NSs were measured using an UV-vis spectrometer, with a 2 nm slit width at a scan speed of 200 nm/min. The solution of MnO_2-NS was prepared to a concentration of 1 μM and scanned at 300-650 nm. The characteristic peak of MnO_2-NSs appeared at 360 nm (Fig. S1C).

(6) DLS of MnO_2-NSs and Tf-DHA-ASO-MnO_2 nanosystems

The size distribution and zeta potential of freshly prepared MnO_2-NSs (5 mg/ml) or Tf-DHA-ASO-MnO_2 nanosystems (5 mg/ml) were determined by the INan-ZS90 laser nanoparticle size analyzer.

(7) X-ray photoelectron spectroscopy of MnO_2-NSs

The XPS of a small amount of solid MnO_2-NSs was measured using a K-Alpha spectrometer. It can be seen from Fig. S1G that the binding energies of O1s, Mn ($2p_{3/2}$) and Mn ($2p_{1/2}$) in XPS pattern A are 528.7, 640.9 and 652.6 eV, respectively. Fig. S1H shows the valence state of manganese in the synthesized MnO_2-NSs is mainly +4.

(8) Ultraviolet characterization of DSPE-PEG-COOH, DSPE-PEG-DHA and DHA

The optical properties of DSPE-PEG-DHA was measured using the UV-vis spectrometer, with a scan speed of 200 nm/min. From Fig. S3A we can see that in order to confirm the combination of DHA and DSPE-PEG-COOH, a new absorbance peak at ~262 nm assigned to DSPE-PEG-DHA could be observed by dissolved it in ethanol and treated with NaOH (0.2%) for 30 min at 50 ℃. Compared with DHA (~282 nm), there is a blue shift for the characteristic peak of newly-synthesized DSPE-PEG-DHA.

Also, in order to determine the loading rate of DSPE-PEG-DHA on MnO_2-NS, the absorption spectra of samples of different concentrations were obtained using a UV-Vis spectrometer. The absorbance intensity at ~262 nm was correlated with the concentration of DSPE-PEG-DHA. DHA-MnO_2 nanosystems were prepared as mentioned above. Then, the resulting solution was centrifuged for 20 min (10000 rpm) in a centrifuge. The UV-vis of suspended solution was determined. According to the standard curve obtained from the absorbance intensity of DSPE-PEG-DHA versus its concentration, the loading rate of DSPE-PEG-DHA on MnO_2-NS could be calculated (Fig. S3).

(9) Infrared characterization of MnO_2-NSs combined with Tf and DHA

After DSPE-PEG-Tf, DSPE-PEG-DHA and Tf-DHA-MnO_2 synthesized and freeze-dried, a small number of solid samples were taken, and the infrared spectrum was determined in the range of 4000 nm-1000 nm scanning wavelength. The FT-IR spectra of DSPE-PEG-COOH gives the signal of -COOH (~1740 cm^{-1}), but it was absent in DSPE-PEG-DHA and replaced with ~1730 cm^{-1} assigned to -COO ester

group. The absorption of methyl (-CH_3) (~3000 cm^{-1}), ethyl (-CH_2) (~2900 cm^{-1}), ester bond (-COO-) (~1635 cm^{-1}) can be observed in the FT-IR spectrum of Tf-DHA-MnO_2 nanosystems, which indicate the successful absorption of DHA and Tf onto MnO_2-NSs (Fig. S2C).

(10) Fluorescence characterization of DSPE-PEG-COOH, DSPE-PEG-Tf and Tf

The fluorescence spectra of Tf (0.1 mg/ml), DSPE-PEG-COOH (0.1 mg/ml) and DSPE-PEG-Tf (0.1 mg/ml) were measured by using an F-7000 spectrophotometer with the parameter of PMT voltage at 700 v, slit set at 5, and scan speed at 240 nm/min. The 340 nm emission shows the existence of Tf. The results shown in Fig. S2D indicate that Tf had been successfully conjugated with DSPE-PEG-COOH. After absorbed onto MnO_2-NSs, the fluorescence of DSPE-PEG-Tf was quenched completely (Fig. S2E), thus indicating the complete absorption of DSPE-PEG-Tf on MnO_2-NSs.

(11) Raman spectroscopic characterization of DSPE-PEG-COOH combined with DHA

Firstly, drop the solution of DSPE-PEG-COOH, DHA, or DSPE-PEG-DHA onto a clean glass slide with a pipette. After dried naturally, the Raman spectra were measured using a Renishaw-in Via Raman microscope with the excitation wavelength at 532 nm and the wavelength range at 500 -3000 nm.

(12) Fluorescence recovery of FAM-Tf-DHA-ASO-MnO_2 nanosystems after treating with GSH

A series of GSH with different concentrations (0, 0.2, 0.75, 1, 1.25, and 1.5 mM）was mixed with the same amount of FAM-Tf-DHA-ASO-MnO_2 (40 μg/ml). After incubation at 37 °C for 6 h, the fluorescence intensity of each mixture was determined. As shown in Fig. 2A, a gradual increase of fluorescence intensity with an increasing concentration of GSH was observed, demonstrating that FAM-ASO was released from the FAM-Tf-DHA-ASO-MnO_2 nanosystems.

(13) ROS yielded by DHA-MnO_2 under the existence of GSH

2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) was used to evaluate ROS generated by the DHA-MnO_2 in solution. MnO_2 (1 mg/ml), DHA-MnO_2 (1 mg/ml) were mixed with 1mg/ml GSH, and then adjusted to pH = 5 - 5.5, and allowed to stand at room temperature for 3 h. Mixed liquor stained with DCFH-DA (50 mM) in room temperature for 2 h. The fluorescence spectrum of DHA-MnO_2 solution were detected using an F-7000 spectrophotometer. DCF was excited with 488 nm laser and the fluorescence intensity at 525 nm shows the amount of DCF, which was correlated with the level of ROS.

(14) Cell cultivation

HeLa, A549, HepG2, HBL-100, LO2 and 4T1 cells were purchased from the Chinese Academy of Sciences Cell Bank (Shanghai, China) and were cultured in DMEM/High glucose supplemented with 10% (v/v) fetal bovine serum and 1×antibiotics at 37 ^oC in 5% CO_2 atmosphere. Dulbecco's Phosphate-Buffered Saline (DPBS) without Ca^{2+} and Mg^{2+} (Invitrogen) was used to wash cells.

(15) Intracellular GSH levels

Intracellular GSH levels were analyzed using the commercial GSH and GSSG assay kits. The level of GSH was not altered significantly in HeLa cells and A549 or after incubation with MnO_2 (40 μg/ml) and Tf-DHA-ASO-MnO_2 (40 μg/ml) for 48 h. However, GSH levels were substantially reduced in HeLa and A549 cells after 72 h incubation. This finding might be explained by the ubiquitous expression of GSH in cells and its various other functions, such as its roles as an antioxidant and in detoxification. Additionally, the expenditure of GSH induced by the Tf-DHA-ASO-MnO_2 treatment consumes much more GSH than the amount saved by GPX4 knockdown.

(16) Tf mediated tumor cell targeting

To investigate the functions of Tf on the nanoparticle delivery system, combination of Tf-MnO_2 and FAM modified GPX4 antisense oligonucleotide. A549 Cells were cultured on confocal dishes at a density of 1×10^5 per dish in RPMI DMEM with 10% FBS, penicillin (100 units/ml), and streptomycin (100 mg/ml) in 5% CO_2 and 95% air at 37 ℃ in a humidified incubator overnight and divided into three groups. FAM-Tf-DHA-ASO-MnO_2, FAM-DHA-ASO-MnO_2 and MnO_2 were added to A549 cells individually and incubated for 2 h. In addition, A549 Cells were treated with FAM-Tf-DHA-ASO-MnO_2 suspension (40 μg/ml) for 2 h pre-blocked by Tf for 30 min in Tf block group and the blank group was as Control. Cells were rinsed with PBS for three times, and further observed by CLSM (ZEISS LSM880, Germany). FAM was excited with an argon-ion laser at 488 nm (reflected by a beam splitter HFT 488 nm), and emission wavelength is at 525 nm.

The results show that the fluorescence was significantly weakened when TfR was blocked in the Tf block group that was also treated with FAM-Tf-DHA-ASO-MnO_2 nanosystems for 2 h (Fig. S4B). While, there is no obvious fluorescence signal for the sample treated with MnO_2-NSs (Fig. S4A). As a control, FAM-DHA-ASO-MnO_2 nanosystems without the loading of DSPE-PEG-Tf were also determined for its delivery of DHA and FAM-ASO. The weaker fluorescence signals can be observed when cells were treated the same time as FAM-DHA-ASO-MnO_2 nanosystems (Fig. S4C). These results suggest that the FAM-Tf-DHA-ASO-MnO_2 nanosystems could actively target and enter into cancer cells high-efficiently with TfR-mediated endocytosis.

(17) Fluorescence response difference of FAM-Tf-DHA-ASO-MnO_2 in normal cells and cancer cells

FAM-Tf-DHA-ASO-MnO_2 (20 μg/mL) was added to HepG2 and LO2 cells, respectively. After incubating in the incubator for 6 h, the culture medium was removed and the cells were washed three times with PBS buffer for confocal

microscopic measurement. The excitation wavelength was selected to be 488 nm, and the appropriate parameters were set for observing. The results show that FAM-Tf-DHA-ASO-MnO_2 can enter HepG2 cells more easily, while the FAM fluorescence in LO2 cells is very weak (Fig. S5). This indicates that the high expression of TfR on the cell membrane surface of HepG2 promote the uptake of FAM-Tf-DHA-ASO-MnO_2 in cancer cells.

The cell samples used for CLSM have also been collected for the flow cytometric fluorescence quantitative analysis.

(18) Subcellular distribution of FAM-ASO and lysosome in tumor cells

Firstly, A549 cells were inoculated in confocal dishes. After the cells were incubated overnight, FAM-Tf-DHA-ASO-MnO_2 (40 μg/ml) was added and reacted for 4 h. The control group and probe group were incubated with Lyso-TrackerRed staining solution at 37 ℃ for 40 min. Lyso-TrackerRed staining solution was removed, fresh cell culture medium was added, and then observed by laser confocal microscope. The fluorescence of Lysotracker Red was detected with the CLSM under the magnification of 63 using oil immersion lens (excitation: 543 nm; signal: 570-590 nm band pass-filter). Meanwhile, the fluorescence FAM was also recorded (excitation: 488 nm; signal: 500-530 nm band pass-filter).

(19) ROS burst induced by MnO_2-NS nanosystem in A549 cells

2',7'-dichlorodihydrofluorescein diacetate (H_2DCFDA) was used to evaluate ROSgenerated by the endocytosed nanoparticles MnO_2-NS nanosystem. A549 cells (5×10^3 per well) were cultured overnight, incubate in 40 μg/ml MnO_2-NS nanosystem for 5 h, and stained with H_2DCFDA (50 mM) in DMEM medium for 30 min and then washed for three times with PBS solution, observation under Confocal laser scanning microscopy (CLSM).

Mn^{2+} could catalyze DHA to generate ·OH. It has been testified by the above fluorescence assay of DCFH-DA. Here an enhancement for the fluorescence intensity can be observed in the DHA-MnO_2 nanosystem treated cell samples. The enhancements are even more obvious for the Tf-DHA-MnO_2 and Tf-DHA-ASO-

MnO_2 nanosystems treated group. It indicates that DHA can enhance the ROS generation caused by the reaction between DHA and Mn^{2+}. Obviously, here Mn^{2+} comes from the degradation of MnO_2-NSs resulting from the reduction effect of intracellular over-expressed GSH. Comparatively, Tf and ASO alone show little effect on the generation of intracellular ROS caused by MnO_2-NSs (Fig. S6).

The cell samples used for CLSM have also been collected for the flow cytometric fluorescence quantitative analysis.

(20) PCR determination intracellular GPX4 mRNA level

A549 cells were firstly inoculated in 6-well plates and cultured overnight, then Tf-DHA-MnO_2 and Tf-DHA-ASO-MnO_2 were added respectively and incubated for different times. The blank group cells were not treated with any nanosystems. Then, total RNA from the tested cells was extracted using Trizol total RNA isolation reagent (TIANGEN) according to the manufacturer's instructions. The cDNA was reverse-transcribed using a QuantiNova Reverse Transcription Kit (Qiagen, Duesseldorf, Germany). The reactions were incubated in a thermal cycler for 2 min at 45 °C, 3 min at 25 °C, 10 min at 45 °C, and 5 min at 85 °C and then held at 4 °C. Real-time PCR was performed using the QuantStudio™ 5 Real-Time PCR system (Applied Biosystems). The primers were as follows: GPX4 mRNA forward primer 5'-AGAGATCAAAGAGTTCGCCGC-3', GPX4 mRNA reverse primer 5'-TCTTCATCCACTTCCACAGCG-3'; GAPDH forward primer 5'-CTCAGACACCATGGGGAAGGTGA-3', GAPDH reverse primer 5'-ATGATCTTGAGGCTGTTGTCATA-3'. The reaction proceeded as follows: 1 cycle of 50 °C for 2 min and 1 cycle of 95 °C for 2 min were followed by 40 cycles of 95 °C for 15 s, 55 °C for 15 s, and 72 °C for 1 min.

As shown in Fig. S7A, we can see that the level of GPX4 mRNA quantitatively measured using RT-PCR was significant decreased in A549 cells with the prolong of Tf-DHA-ASO-MnO_2 nanosystem treatment time. After treatment with Tf-DHA-ASO-MnO_2 nanosystem for 72 h, the relative level of GPX4 mRNA was decreased into 50% of untreated sample. But Tf-DHA-MnO_2 nanosystem without loading ASO has no

such effect.

(21) Immunofluorescence labeling

A549 cells were inoculated in laser confocal dishes. 24 h later, MnO_2 (40 μg/ml) and TF-ASO-MnO_2 (40 μg/ml) were added for 48 h. The control group and the probe group were fixed, sealed, incubated with the first antibody, incubated with the second antibody, and finally washed and observed under the laser confocal microscope. The expression of the GPX4 protein (pink fluorescence) was detected in A549 cells using immunofluorescence staining. As shown in Fig. S7B, after treatment with 40 μg/ml Tf-DHA-ASO-MnO_2 nanosystems for 48 h, the GPX4 expression was significantly decreased in A549 cells compared to control, PBS-treated cells.

(22) LPOs detection

A549 cells were inoculated in a 6-well plate and incubated overnight. Then, MnO_2 (40 μg/ml), DHA-MnO_2 (40 μg/ml), Tf-DHA-MnO_2 (40 μg/ml) or Tf-DHA-ASO-MnO_2 (40 μg/ml) were added respectively and treat for 48 h and 72 h. The control group and probe group were digested and lysed at the same time. After the sample was prepared, the protein concentration was determined by BCA protein concentration detection kit. According to the results, lipid oxidation (MDA) detection kit was used to determine lipid oxides in each probe group and control group. The MDA content of the sample solution was calculated, and it was found that the MDA content of the Tf-DHA-ASO-MnO_2 group was the highest, and the longer the probe incubated the cells, the more MDA content.

(23) Cytotoxicity of MnO_2-NSs

MnO_2-NSs after being sonicated adequately were firstly prepared to a concentration of 5 mg/ml. Then, they were diluted with cell culture medium to various concentrations, and then added to a 96-well plate at 37 °C and 5% CO_2 concentration. After 48 h of reaction with HeLa, A549, HepG2, or HBL-100 cells in the incubator, the cells were treated with MTT and left for 4 h more. Then remove the

MTT solution in the 96-well plate and add DMSO, and the absorbance value was measured on the microplate reader. The cell viability of each well was calculated according to the absorbance value.

(24) Cytotoxicity Tf-DHA-ASO-MnO_2 and Erastin

Firstly, A549, HeLa, HepG2 or 4T1 cells were inoculated into 96-well plates. After cultured for 24 h, the cell samples were fed with different concentration of Tf-DHA-ASO-MnO_2 / Erastin (0, 1, 2, 5, 10, 15, 20, 30 and 40 μg/ml) and the plates were incubated at 37 °C, 5% CO_2 for a period of 48 h. Then the cell viability of each sample was measured with the same method as the above mentioned.

(25) Mn uptake

HeLa and A549 cells were seeded in 6-well plates, and Tf-DHA-ASO-MnO_2 was incubated in both cells for different times (0.5, 1, 2, 4, and 6 h), and then the cells were digested and collected. Count the number of cells by cytometry to ensure that the cell density is basically the same. Then, all cell samples were ultrasonically disrupted in a cell disrupter for 20 min, and centrifuged at 12000 rpm for 30 min. In order to accurately determine the concentration of Mn^{2+} in the supernatant, a standard solution of $MnCl_2$ was prepared, and the corresponding Mn^{2+} concentrations of the standard samples and the collected supernatants were determined by Atomic Absorption Spectrometer (TAS-990, China). The final results are shown in Table S1.

(26) Xenograft tumor mice model

Balb/c female mice (4 weeks old) were purchased from SPF (Beijing) Biotechnology Co., Ltd. All procedures were conducted in accordance with the " Guiding Principles in the care and Use of Animals (China) and were approved by the Laboratory Animal Ethics Committee of Liaocheng Universitv (L20210111) 4T1 cells (5×10^6) in 200 μL solution were subcutaneously injected into the left flank region of the mice. Animals were used in the experiments when the tumors reached

approximately about 100 mm^3.

(27) Antitumor effect *in vivo*

30 Balb/c female mice bearing 4T1 tumor were divided into six groups, minimizing the differences of weights and tumor sizes in each group. The mice were administered with PBS, MnO_2-NS (20 mg/kg), Tf-MnO_2 (20 mg/kg), DHA-MnO_2 (20 mg/kg), Tf-DHA-MnO_2 (20 mg/kg) and Tf-DHA-ASO-MnO_2 (20 mg/kg, in terms of MnO_2), respectively. All drugs were intravenously injected repeatedly through tail veins with a 48 h interval for 12 days. The tumor sizes were measured with a caliper every one days and calculated as Volume = (tumor length)$\times$(tumor width)^2/2. The body weight of the mice were also recorded for evaluating the therapeutic effect of all drugs.

(28) Statistical analysis

Experiments were repeated three times. Data were analyzed using IBM SPSS Statistics 25, and presented as mean values ± standard deviations from 3 independent measurements. Statistical comparisons between different treatments were assessed by using the one-way ANOVA method assuming significance at $*P < 0.05$ and highly significant at $**P < 0.01$.

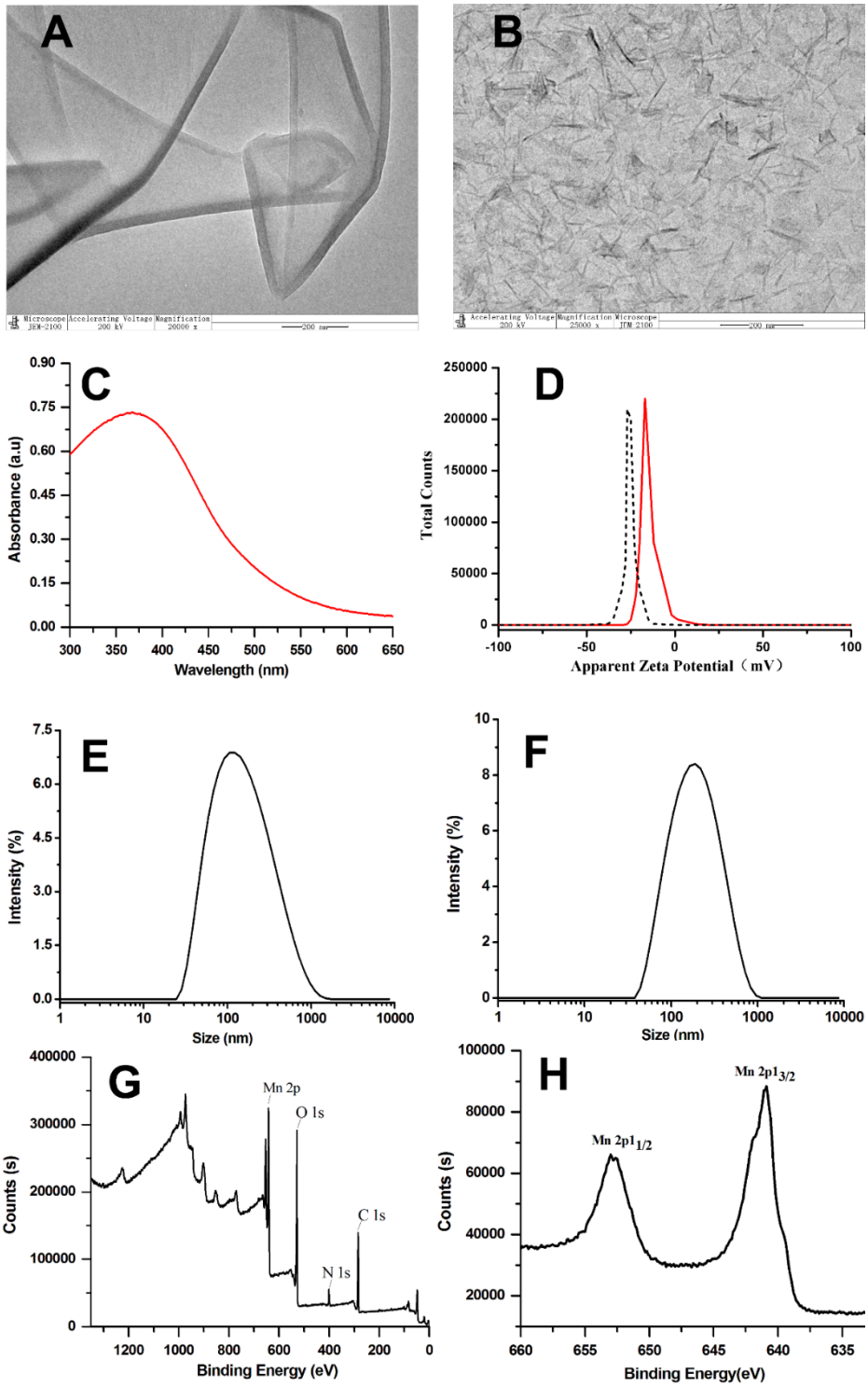

Fig. S1 (A) TEM of newly-synthesized MnO_2-NSs. (B) TEM of MnO_2-NSs after fully crushed by sonification. (C) UV-Vis spectra of MnO_2-NSs. (D) Zeta potential of MnO_2-NSs and Tf-DHA-ASO-MnO_2 nanosystems. Size distribution of MnO_2-NSs (E) and Tf-DHA-ASO-MnO_2 nanosystems (F) determined by DLS. (G, H) XPS of MnO_2-NSs.

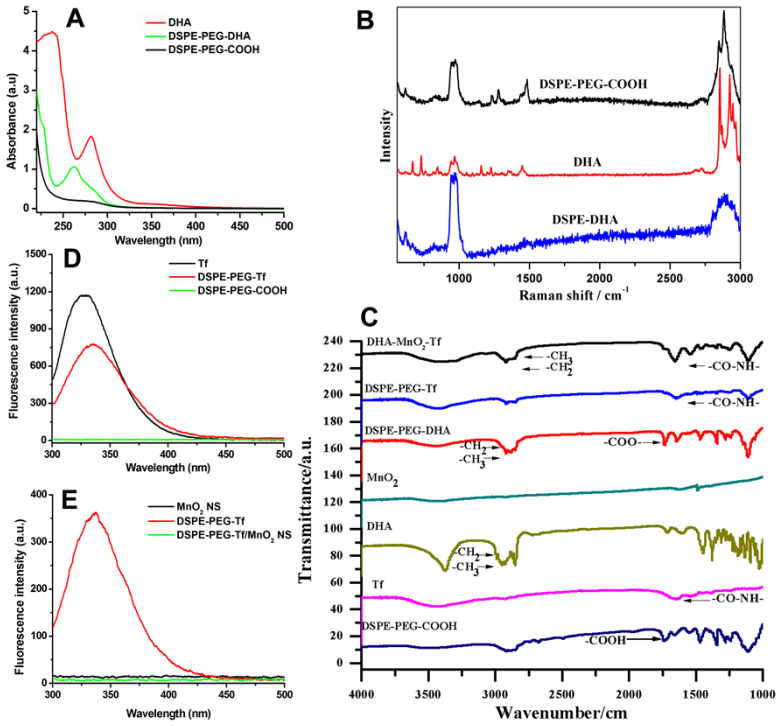

Fig. S2 (A) UV-Vis spectra of DSPE-PEG-COOH, DHA, and the synthesized DSPE-PEG-DHA. (B) Raman spectra of DSPE-PEG-COOH, DHA, and the synthesized DSPE-PEG-DHA. (C) IR spectra of different samples. (D) Fluorescence spectra of DSPE-PEG-COOH, Tf, and the synthesized DSPE-PEG-Tf. (E) Fluorescence quenching of DSPE-PEG-Tf after absorbed on MnO_2-NSs.

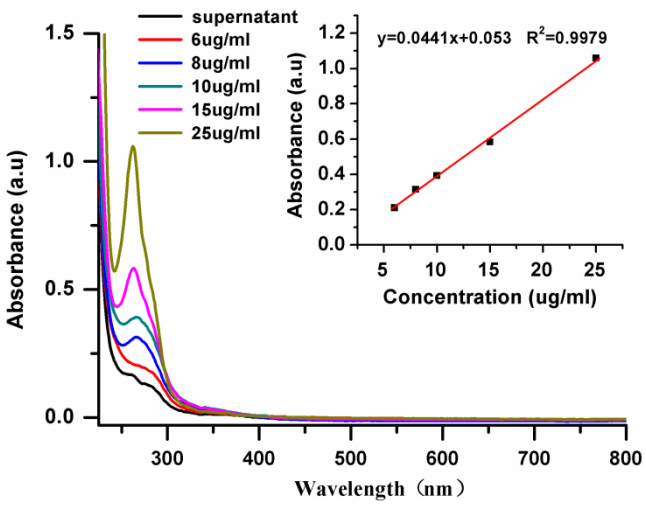

Fig. S3 UV-Vis spectra of different concentration of DSPE-PEG-DHA and the supernatant after centrifuging the reaction mixture of DSPE-PEG-DHA and MnO_2-NS. Insert: the standard curve of UV-Vis spectra of different concentration of DSPE-PEG-DHA.

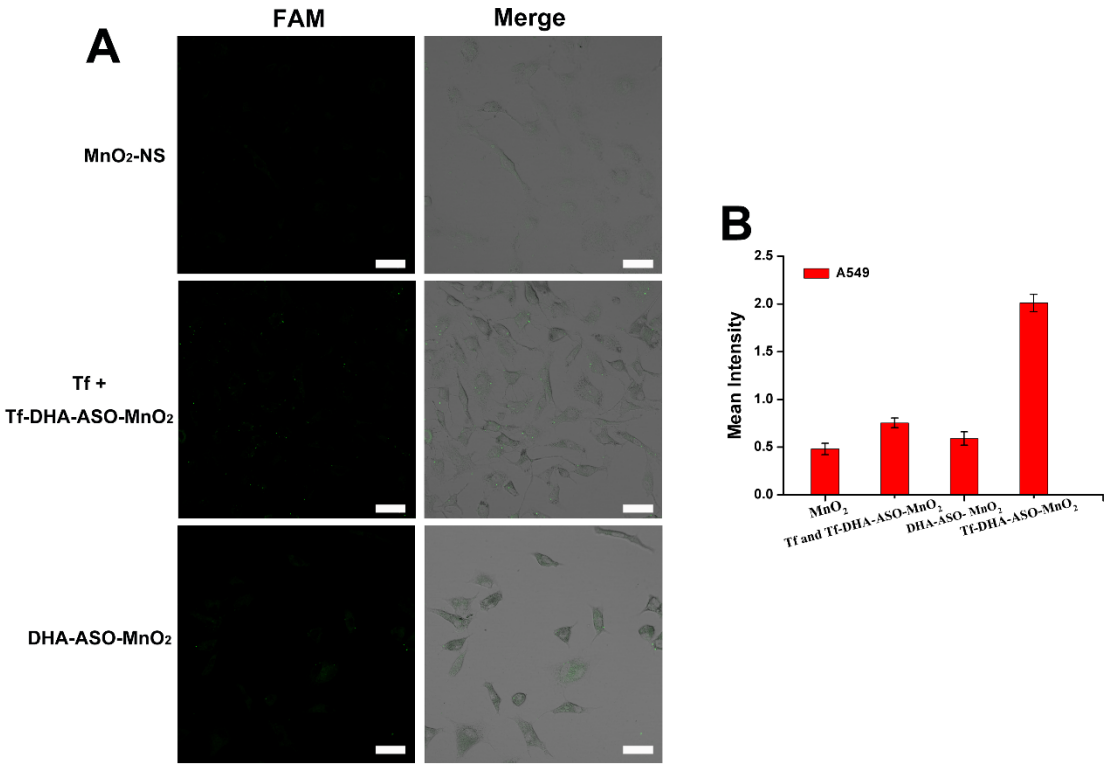

Fig. S4 (A) Confocal images of A549 cells after incubation with MnO_2-NSs (40 μg/ml), Tf and FAM-Tf-DHA-ASO-MnO_2 nanosystems (40 μg/ml), and FAM-DHA-ASO-MnO_2 nanosystems (40 μg/ml) for 2 h. Merge means the merged image of FAM fluorescence and bright field of cells. Scale bars represent 50 μm. (B) Mean fluorescence intensity of CLSM images of A549 cells treated with different MnO_2-NS based nanosystems shown in (A).

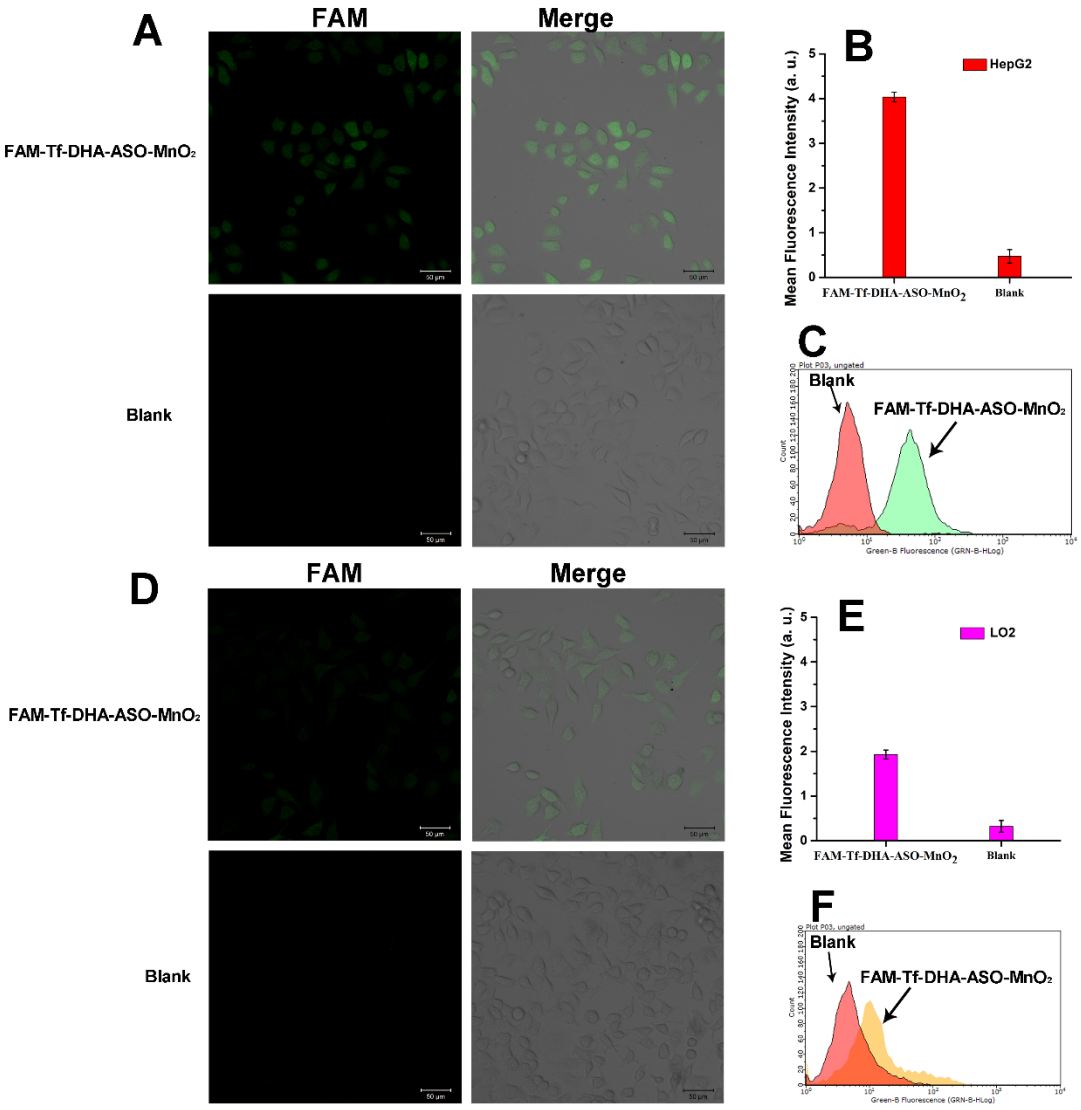

Fig. S5 Confocal images (A or D), mean fluorescence intensity of confocal images (B or E), and flow cytometric analyses (C or F) of HepG2 or LO2 cells after incubation with PBS and FAM-Tf-DHA-ASO-MnO_2 nanosystems (20 μg/ml) for 6 h. Merge means the merged image of FAM fluorescence and bright field of cells. Scale bars represent 50 μm. Error bars show the standard deviations of three images.

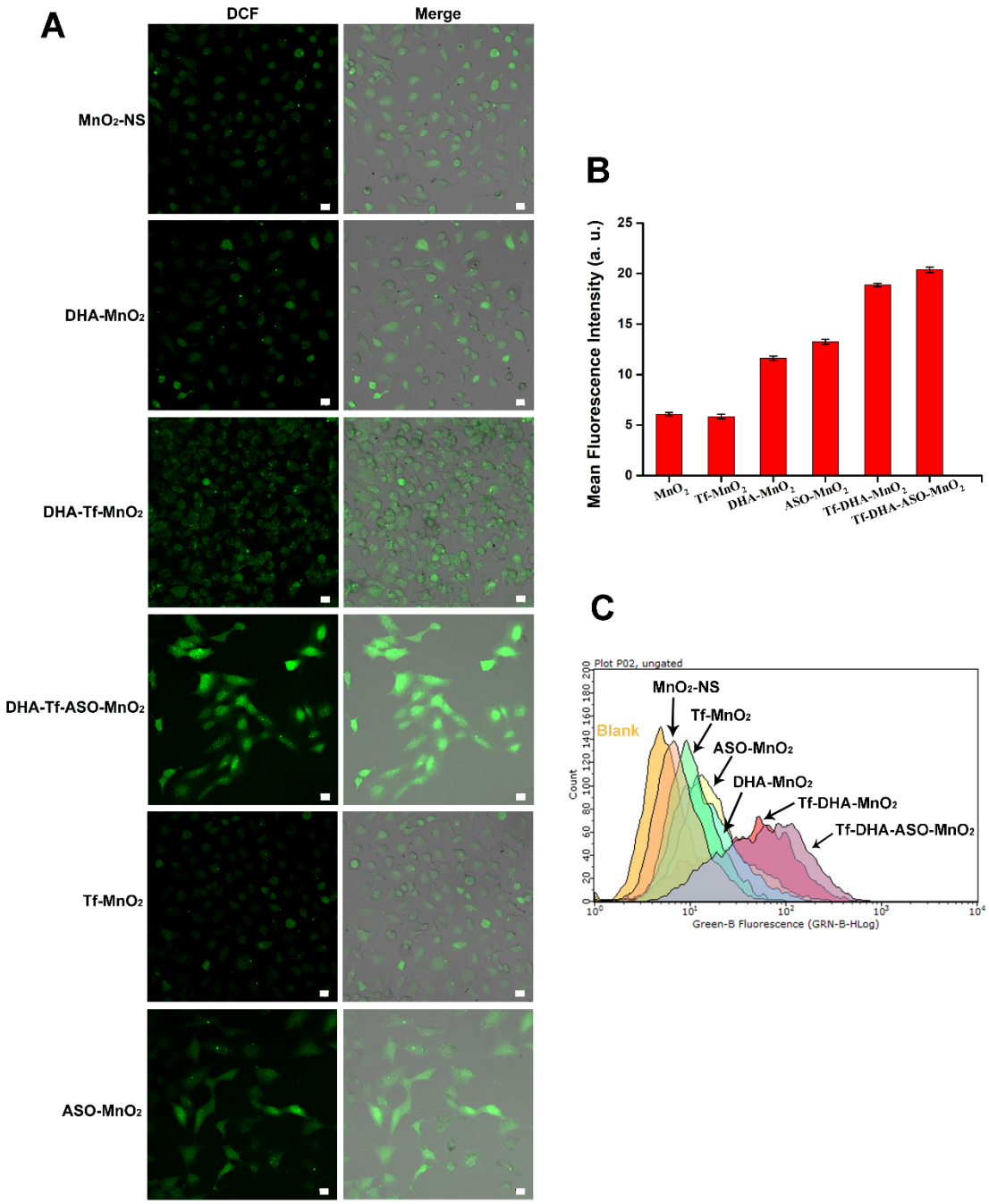

Fig. S6 (A) Confocal images of A549 cells showing the ROS response when cells were treated with different MnO_2-NS based nanosystems (40 μg/ml) for 5 h. Scale bars represent 20 μm. (B) Mean fluorescence intensity of CLSM images of A549 cells treated with different MnO_2-NS based nanosystems shown in (A). Error bars show the standard deviations of three images. (C) Flow cytometric analyses of cell samples after incubation with different MnO_2-NS based nanosystems (40 μg/ml) for 5 h.

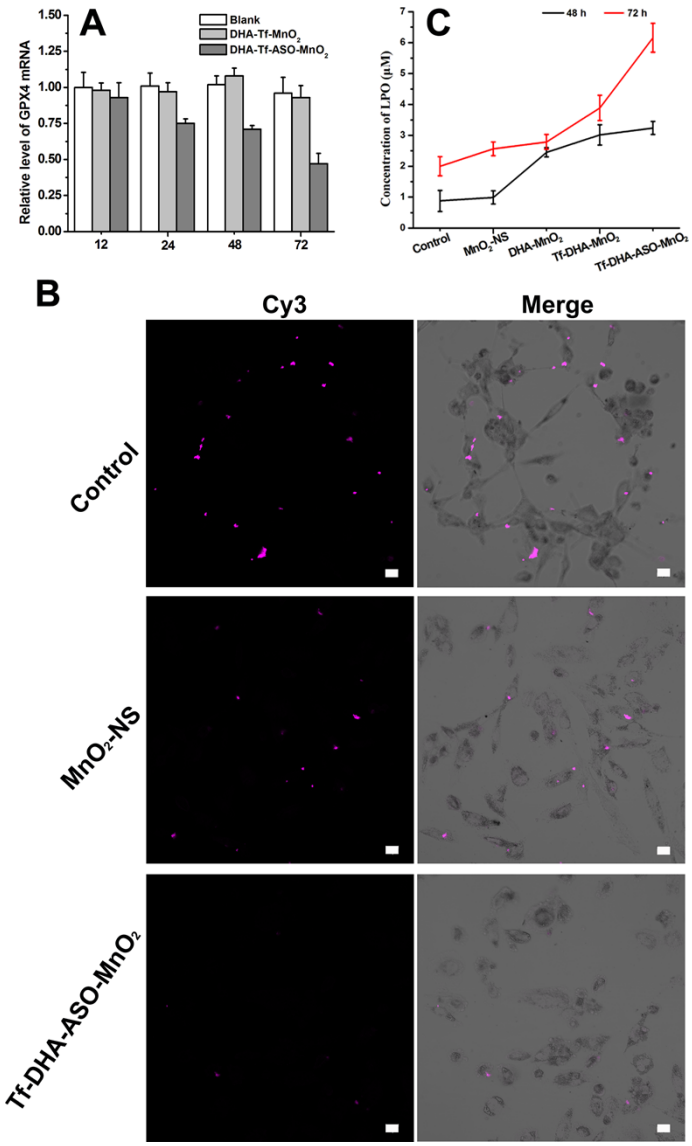

Fig. S7 (A) Level of GPX4 mRNA in A549 cells quantitatively measured using RT-PCR when treated with Tf-DHA-ASO-MnO_2 nanosystem for different times. (B) Expression of the GPX4 protein (pink fluorescence) detected in A549 cells using immunofluorescence staining after treatment with 40 μg/ml Tf-DHA-ASO-MnO_2 and MnO_2-NS nanosystem for 48 h. (C) Content of LPO in A549 cells after treated with different MnO_2-NS based nanosystems. Scale bars represent 20 μm. Error bars show the standard deviations of three experiments.

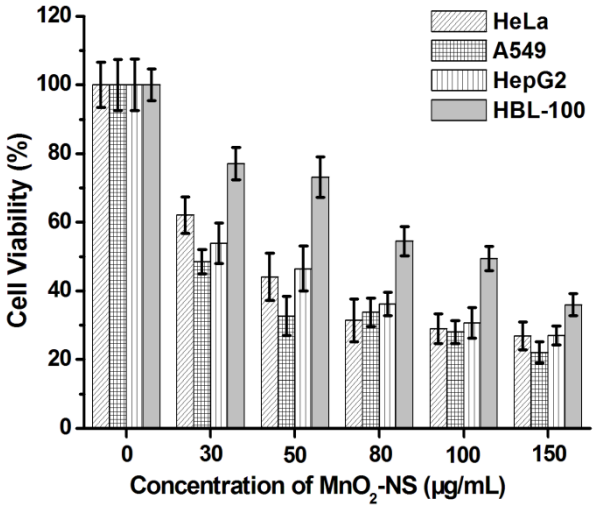

Fig. S8 Cytotoxicity of different concentrations (0, 30, 50, 80, 100, and 150 μg/ml) of MnO_2-NSs to HeLa, A549, HepG2, and HBL-100 cells. Error bars show the standard deviations of three experiments.

Table S1 Intracellular uptake of manganese ions in A549, HeLa, HepG2 and 4T1 cells at different times

Concentration / Cell / Time	A549	HeLa	HepG2	4T1
0 h	0	0	0	0
0.5 h	0.75 μg/ml	0.61 μg/ml	0.26 μg/ml	0.58 μg/ml
1 h	1.5 μg/ml	2.66 μg/ml	0.87 μg/ml	1.33 μg/ml
2 h	3.94 μg/ml	3.73 μg/ml	1.93 μg/ml	2.84 μg/ml
4 h	20.18 μg/ml	23.79 μg/ml	14.62 μg/ml	20.13 μg/ml
6 h	31.54 μg/ml	30.58 μg/ml	21.74 μg/ml	28.23 μg/ml

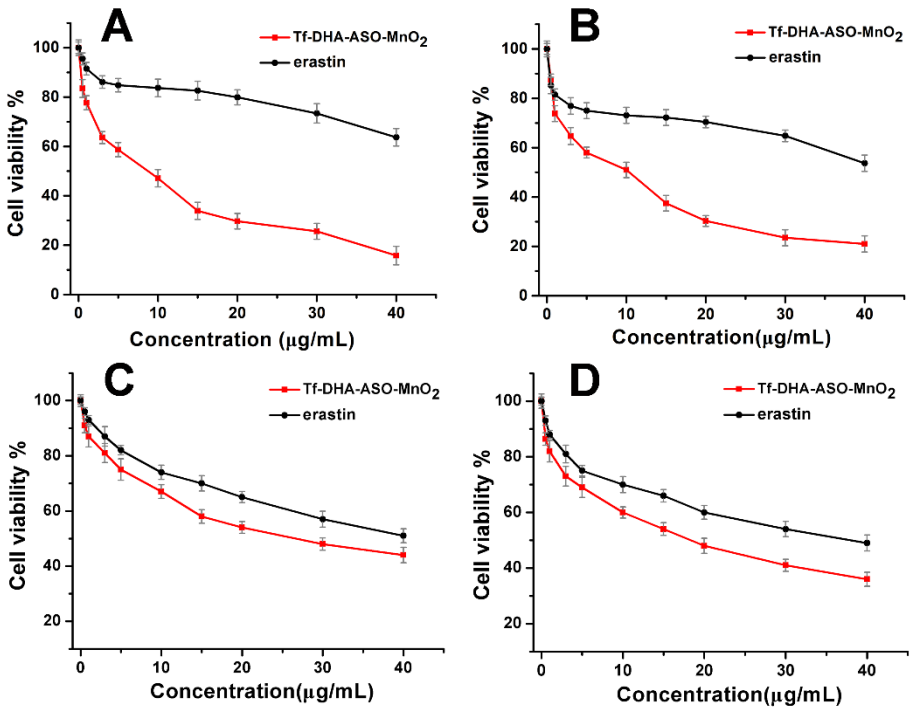

Fig. S9 Cytotoxicity of different concentrations (0, 1, 2, 5, 10, 15, 20, 30 and 40 μg/ml) of Tf-DHA-ASO-MnO_2 and erastin (0, 1, 2, 5, 10, 15, 20, 30 and 40 μg/ml) to A549 (A), HeLa cells (B), HepG2 (C), and 4T1 (D). Error bars show the standard deviations of three experiments.

Table S2 IC_{50} values of Tf-DHA-ASO-MnO_2 and erastin against A549, HeLa, HepG2 and 4T1 cells.

IC_{50} Cell / Drug	A549	HeLa	HepG2	4T1
Tf-DHA-ASO-MnO_2	12.277 μg/ml	13.675 μg/ml	32.633 μg/ml	25.475 μg/ml
Erastin	83.805 μg/ml	52.552 μg/ml	49.112 μg/ml	44.725 μg/ml

REFERENCES

1 L. J. Liua, Y. C. Wei, S. D. Zhai, Q. Chen and D. Xing, Biomaterials, 2015, **62,** 35.