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Supplementary Information

Vehicle-saving theranostic probes based on hydrophobic iron oxide nanoclusters using doxorubicin as a phase transfer agent for MRI and chemotherapy

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Experimental Section

Materials

Doxorubicin (DOX), 4′,6-diamidino-2-phenylindole (DAPI), hyaluronidase (HAase) and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (USA). Cell Counting Kit-8 (CCK8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Calcein-AM was purchased from J&K Scientific Ltd (Beijing, China). Hyaluronic acid (HA, 90 kDa) was purchased from Freda Biochem Co., Ltd. (Shandong, China). Iron(III) chloride hexahydrate ($FeCl_3 \cdot 6H_2O$), oleic acid (OA), methanol, ethanol, dichloromethane, dimethyl sulfoxide (DMSO), 1-octadecene, hexane and sodium hydroxide were purchased from Sinopharm Chemical Reagent Co. Ltd. (China). Ultrapure water is prepared by a Milli-Q Gradient System (18.2 M·Ω resistivity, Millipore Corporation, Bedford, MA, USA).

Cell Culture and animals

HeLa cells (ATCC, Manassas, VA) were cultured in DMEM medium that were supplemented with 10% fetal bovine serum and 100 U/ml 1% antibiotics penicillin/streptomycin at 37℃ in a humidified atmosphere containing 5% CO_2 incubator. Babl/C nude mice were obtained from Wushi, Inc. Corp, and the animal experiments were strictly carried out accordance with "National animal management regulations of China" and approved by the Animal Ethics Committee of Fujian Medical University.

Synthesis of hydrophobic IONPs

IONPs was synthesized according to our previously reported methods.[1] Firstly $FeCl_3 \cdot 6H_2O$ (26 mmol) and oleic acid (80 mmol) were dissolved in 200 mL methanol and stirred vigorously at room temperature. Subsequently, 200 mL methanol solution containing sodium hydroxide (80 mmol) was added dropwise into the above solution. After 1 hour, the reaction mixture was filtered, then the filter residue was washed 3 times by deionized water, acetone and acetic acid aqueous solution (10%), respectively. The iron-oleate complex was obtained by drying the filter residue in a vacuum overnight. Then, iron-oleate complex (3.6 g), oleic acid (2.0 mmol), and 1-octadecene (79 mmol) were uniformly mixed in a 250 mL round bottom flask. Under argon atmosphere, the mixture was heated to 100 ℃ and refluxed for 60 min, before heated to 320 ℃ and refluxed for another 30 minutes. Afterwards, the reaction mixture was cooled to room temperature, and added with a large amount of ethanol. The

resulting precipitate was collected by centrifugation and washed three times with ethanol and hexane in sequence. Finally, the as-prepared IONPs were stored in CH_2Cl_2.

Synthesis of the DINCs@HA

DINCs@HA was synthesized by a microemulsion method. Firstly, 150 μL IONPs in dichloromethane solution (50 mg/mL) were mixed with 50 μL DOX (20 mg/mL) in DMSO. This mixture was then added into 4 mL water. The above solution was sonicated until the mixture became homogenous dispersion. Dichloromethane was then evaporated in a ventilated place overnight. Then 2 mL HA solution (2 mg/mL) was added and the solution underwent vigorous stirring at room temperature for 6 hour. The formed HA-coated DINCs clusters were centrifuged at 8500 rpm for 25 min. The precipitate was washed three times with deionized water.

DINCs@HA characterization

The synthesized clusters were characterized by UV-vis spectrometer on a microplate reader (Spectra Max M5, Molecular Devices, USA), Transmission electron microscopy (JEOL, Japan), Dynamic light scattering (Malvern Instruments Ltd, Malvern, UK), NMR analyzer (MQ60, Bruker, GEM), Inductively coupled plasma spectrometry (iCAP 7000, Thermo, USA), Fourier transform infrared spectrometer (Perkin-Elmer, Spectrum-2000, USA), Vibrating-sample magnetometer (VSM, Westerville, OH, USA).

Determination of the DOX loading contents: The loaded DOX was quantified by fluorescence analysis using a microplate reader. First, DINCs@HA nanoparticle clusters solutions were lyophilized to yield the solid. Then, the dried samples were weighed and re-dissolved in DMSO, and precipitation was removed from the solution by centrifugation. The emission of DOX in supernatant was measured at 590 nm to calculate the DOX loading content. The Fe content in DINCs@HA was quantified using ICP measurement. Drug loading efficiency (DLE) and drug loading content (DLC) can be calculated according to:

$$\text{DLC (\%)} = W_{\text{loaded DOX}}/W_{\text{DINCs@HA}} \times 100\%$$

$$\text{DLE (\%)} = W_{\text{loaded DOX}}/W_{\text{feeding DOX}} \times 100\%$$

Where $W_{loaded\ DOX}$ is referred to as the weight of loaded DOX, $W_{DINCs@HA}$ is referred to as the weight of DINCs@HA, $W_{feeding\ DOX}$ is the feeding amount of DOX. Thus, DLC and DLE was calculated to be 30% and 20%, respectively.

Release of DOX *in vitro*

The *in vitro* DOX release from DINCs@HA was evaluated in phosphate bu□ered saline (PBS, pH 7.4) and acetate bu□ered saline (ABS, pH 5.5), and the HAase (final concentration of 5 mg/mL) was added into the above release media to further explore HAase-sensitive drug release, while the media without HAase was set as a control. Typically, 1mL of the DINCs@HA nanocluster solutions (containing 0.23 mg of DOX) was placed into a dialysis tubing (MWCO: 3500 Da) and then completely immersed in the release media. The whole assembly was incubated in the air bath at 37 °C under shaking. In order to determine the release amount of DOX from DINCs@HA, 5mL of solution was taken from the centrifuge tube at a preset time point for fluorescence detection. Subsequently, an equal amount of fresh medium was added. Fluorescence values of DOX were measured from spectrophotometer. The amount of drug released was plotted against the time point.

Cytotoxicity of DINCS@HA *in vitro*

The in vitro cytotoxicity of DINCS@HA was evaluated on the HeLa cells by CCK-8 assay. In detail, HeLa cells were seeded in 96-well plate at a density of 1×10^4 cells per well. After 24 h of incubation, the original medium was replaced by fresh one containing the DINCS@HA at the concentration of 0.5, 1, 2, 3, 4, 5 and 6 μg/mL, with or without the magnet beneath the plate for 2 h. Afterwards, all cells were washed three times with PBS. Then 100 μL of fresh medium was added in each well for further 22 h of incubation. At last, the original medium was replaced by mixed solution of containing 90 μL fresh medium and 10 μL CCK-8 working solution. The cells were incubated for 1 hour at 37 ℃ and the absorption value was immediately measured by a Spectra Max M5 microplate reader (Molecular Devices, USA) at 450 nm. The cell viability can be calculated according to:

$$\text{Cell viability} = (A_1 - A_{blank})/(A_0 - A_{blank}) \times 100\%$$

where the absorbance values of cells with or without treatment of DINCS@HA is referred to as A_1 and A_0, the absorbance values of CCK-8 itself at 450 nm is indicated as A_{blank}. The final absorbance value of every group is the average of four replicate wells.

Cellular uptake

Cellular uptake behavior was observed by confocal laser scanning microscope (CLSM) and prussian blue staining. For CLSM observation, 1.5×10^5 HeLa cells were seeded in CLSM-specific dish and cultivated for 24 hours, DINCS@HA at an equivalent DOX dose of 3 μg/mL was added to the dish. The HeLa cells were continuously cultivated for 2 hours in the presence or absence of a magnetic field before washed three times with PBS. Then 4% of paraformaldehyde solution was added to each well for cell fixing, 20 minutes later, the well were washed three times with PBS. Next the cells nuclei were stained by DAPI and then observed with CLSM (Zeiss LSM780).

Prussian blue staining, a qualitative method for the determination of iron content, was used to visually observe the content of iron in HeLa cells. 8×10^4 HeLa cells per well were seeded in 24-well plate and continue to cultivate for 24 hours. Then the original medium was replaced by fresh one including the DINCS@HA at the concentration of 3 μg/mL in the presence or absence of a magnetic field. 1-4 h later, all wells were washed three times with PBS. Then 4% paraformaldehyde solution was added to each well. After 20 minutes, all wells were washed three times with PBS. Afterwards, all wells were stained with Perls reagent (4% potassium ferrocyanide/12% HCl, v/v = 1:1). After 30 minutes, the cells were washed three times with PBS and observed using an inverted microscope (Zeiss Axio Vert. A1, Germany).

MR imaging *in vitro*

In order to verify the T_2-weighted MRI contrast enhancement of DINCS@HA in aqueous solution. 1 mL of DINCS@HA at an equivalent iron concentrations ranged from 0 to 0.8 mM aqueous solution were added into glass bottle. The nuclear magnetic resonance imaging ability of samples was performed by a 7.0T MRI scanner (Bruker Biospoin GmbH, Germany).

To validate T_2-weighted MRI contrast ability at the cellular level, the following experiments were performed. First of all, 2.5×10^5 cells per well were seeded in 6-well plate and cultured for 24 h at 37

°C. DINCS@HA at an equivalent DOX dose of 3 μg/mL was added to 6-well plate and underwent the same treatment as indicated above. After treatments, the cells were uniformly dispersed in an aqueous solution of 1% agarose. Then the nuclear magnetic resonance imaging ability of cells were imaged by a 7.0T MRI scanner (Bruker Biospoin GmbH, Germany).

MR Imaging and monitoring biodistribution of drug *in vivo*

Babl/C nude mice were obtained from Wushi, Inc. Corp, and the animal experiments were strictly carried out accordance with "National animal management regulations of China" and approved by the Animal Ethics Committee of Fujian Medical University. To establish the HeLa tumor model, 1×10^7 of HeLa cells were subcutaneously injected into right flank area of nude mice. When volume of tumor grew about 70-100 mm^3, DINCS@HA was intravenously injected into the experimental mice (100 μL, 1 mg/mL of DOX concentration). Then the tumor site was attached with a magnet though a bandage for 24 hours, and then T_2-weighted MR signals ability were imaged by a 7.0T MRI scanner (Bruker Biospoin GmbH, Germany).

For monitoring biodistribution of DINCS@HA, the tumor-bearing nude mice were underwent the same treatment as indicated above. And then, the mice were sacrificed for collecting tumors and vital organs (heart, liver, spleen, lungs and kidneys). After that, the major organs and tumors were imaged to study the biodistribution of DINCS@HA through observing the fluorescence signal from DOX detected by the IVIS imaging system. Meantime, the fluorescence intensities of major organs and tumors were also recorded.

Antitumor Effect *in vivo*

For the evaluation of antitumor effect, 4 groups with 4 tumor-bearing nude mice in every group experienced the following treatments : (1) PBS control (100μL); (2) DOX (100 μL, 1 mg/mL); (3) DINCS@HA (100 μL, 1 mg/mL of DOX concentration) with magnetic field; (4) DINCS@HA (100 μL, 1 mg/mL of DOX concentration) without magnetic field. The administration approach was intravenous injection every three days. The antitumor effect was observed through Body weight and tumor volume that were monitored once every two days. And the tumor volume was calculated according to:Tumor volume=length×width^2/2.

In addition, the tumors of experimental mice were removed and weighed after 14 days of treatments. The efficacy of chemotherapy was evaluated though investigating the histological changes. Tumors and vital organs (heart, liver, spleen, lungs and kidneys) that was removed from the mice were fixed in formalin solution, embedded in paraffin sectioned (4 μm thickness) and stained with hematoxylin-eosin (H&E) and Ki-67.

Statistical analysis

The experimental results are presented with the mean±standard deviation. The one-way of variance (ANOVA) method or Student's t-test was used to analyze statistical significance. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. The value of $p < 0.05$ indicates statistical significance.

Results

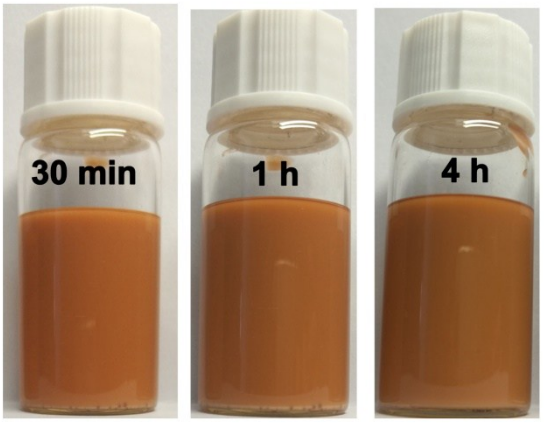

Fig. S1 A photograph of microemulsion formed by mixing DMSO/CH_2Cl_2 (1/3, V/V, containing of DOX and IONPs) and water, after storage for 30 min to 4 h.

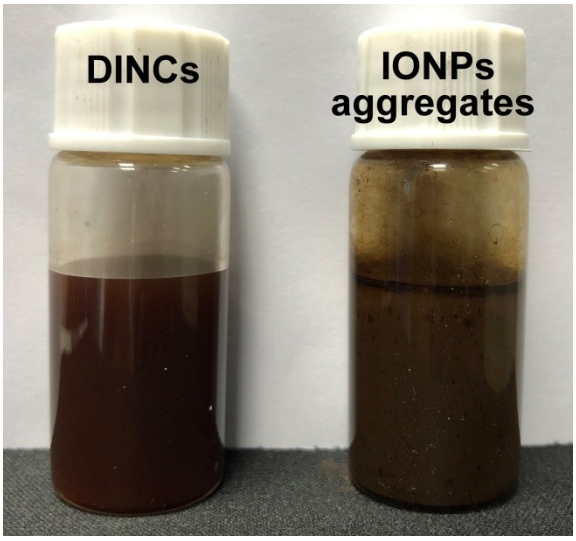

Fig. S2 Water dispersity of DINCs (left) or IONPs aggregates (right) transferred with or without DOX.

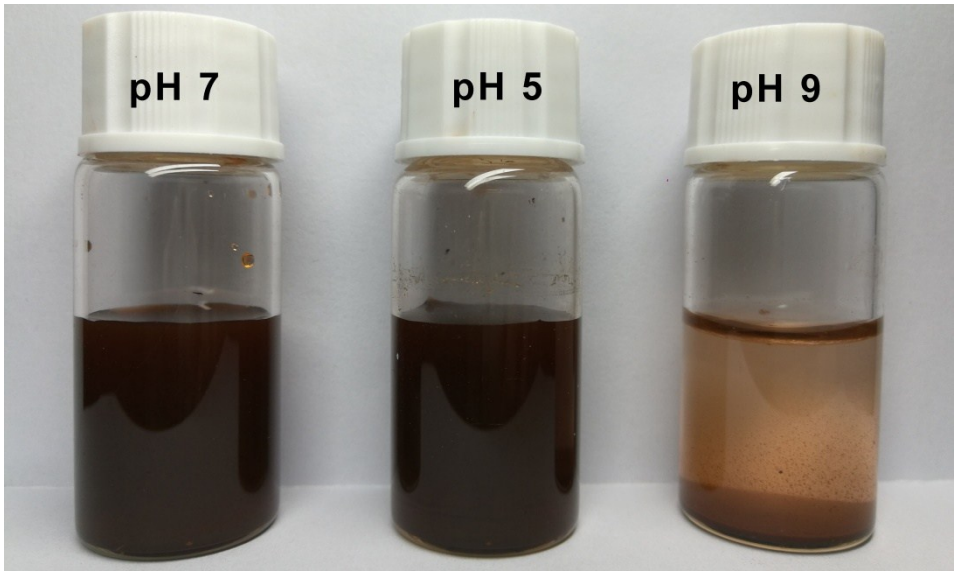

Fig. S3 Water dispersity of DINCs in different pH.

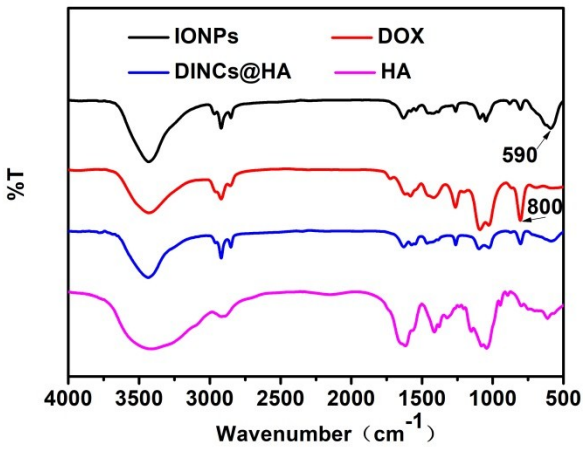

Fig. S4 FT-IR spectra of IONPs, DOX, HA and DINCs@HA. The typical bands at 590 cm^{-1} (Fe-O deformation in IONPs) and 800 cm^{-1} (deformation vibration of benzene in DOX) are both presented in the spectrum of DINCs@HA, indicating the successful incorporation of IONPs and DOX in DINCs@HA. However, the modification of HA DINCs could not be detected by FT-IR due to the less characteristic peaks compared with IONPs and DOX.

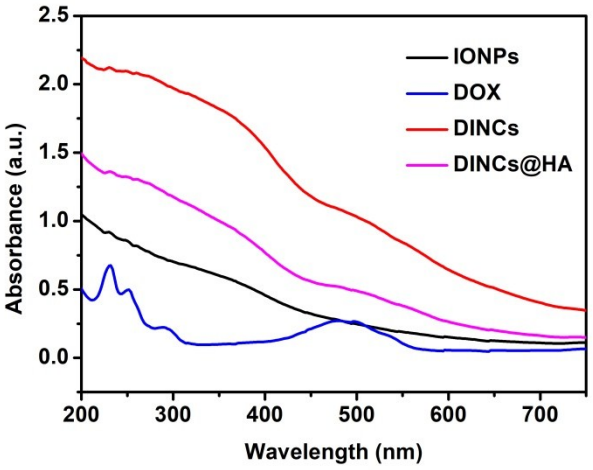

Fig. S5 UV-Vis spectra of IONPs, DOX, DINCs and DINCs@HA.

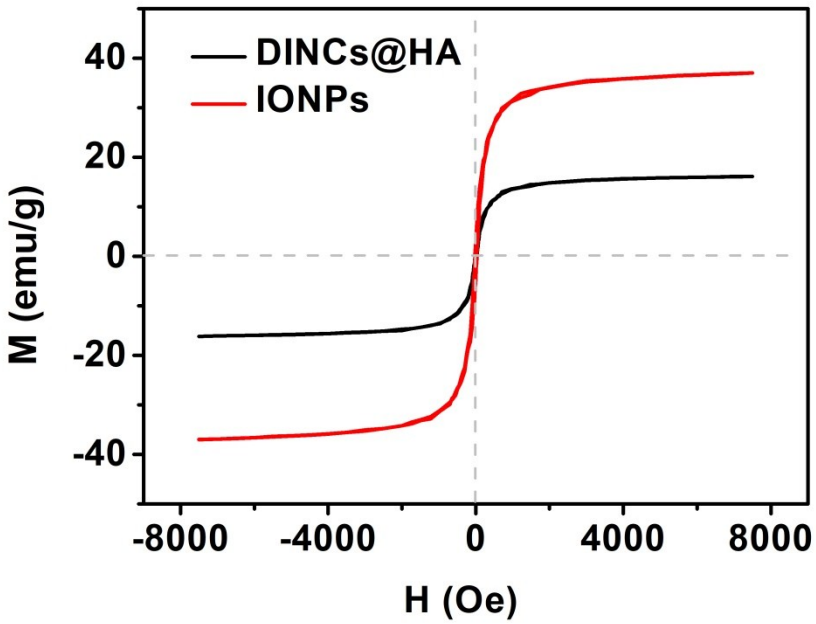

Fig. S6 Mass magnetization curves at 300 K of the IONPs (red curve) and the the DINCs@HA (black curve).

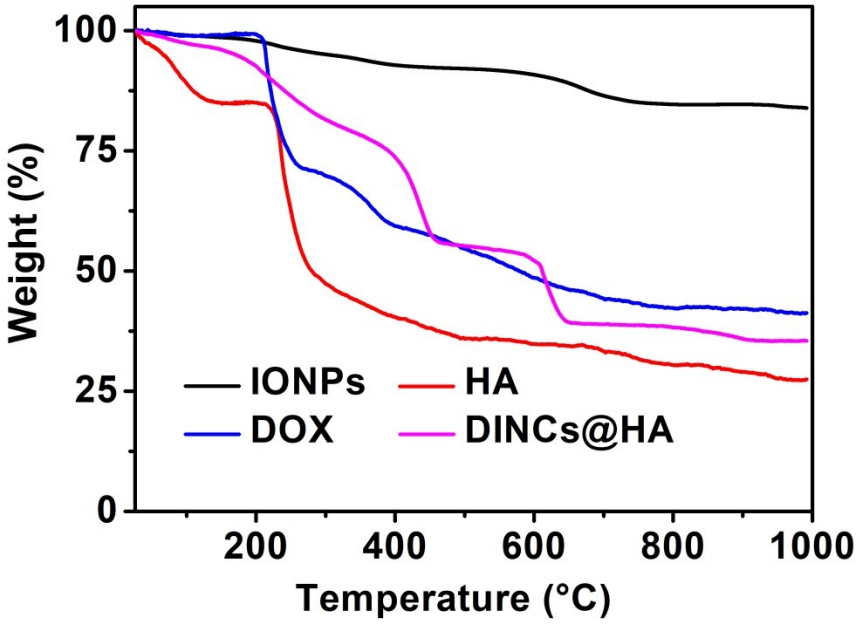

Fig. S7 TGA curves of IONPs, free DOX, free HA, and DINCs@HA under nitrogen atmosphere.

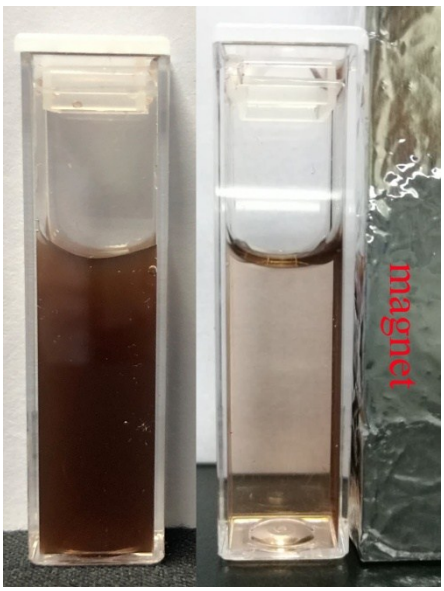

Fig. S8 A photograph of DINCs@HA aqueous solution with or without the magnetic field.

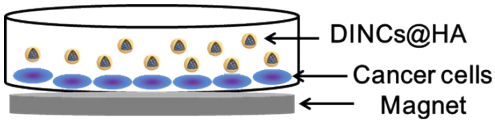

Fig. S9 Illustration of the magnet applied for enhancing cellular uptake. We fixed a cylinder-shaped neodymium magnet beneath the cell culture well, so the DINCs@HA would quickly deposit onto the cell membrane, benefiting subsequently cellular uptake.

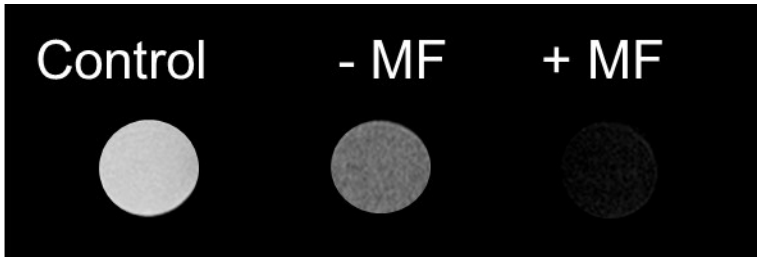

Fig. S10 T_2-weighted MRI images of HeLa cells after incubation with DINCs@HA for 2 h with or without the magnetic field, control: non-treated cells.

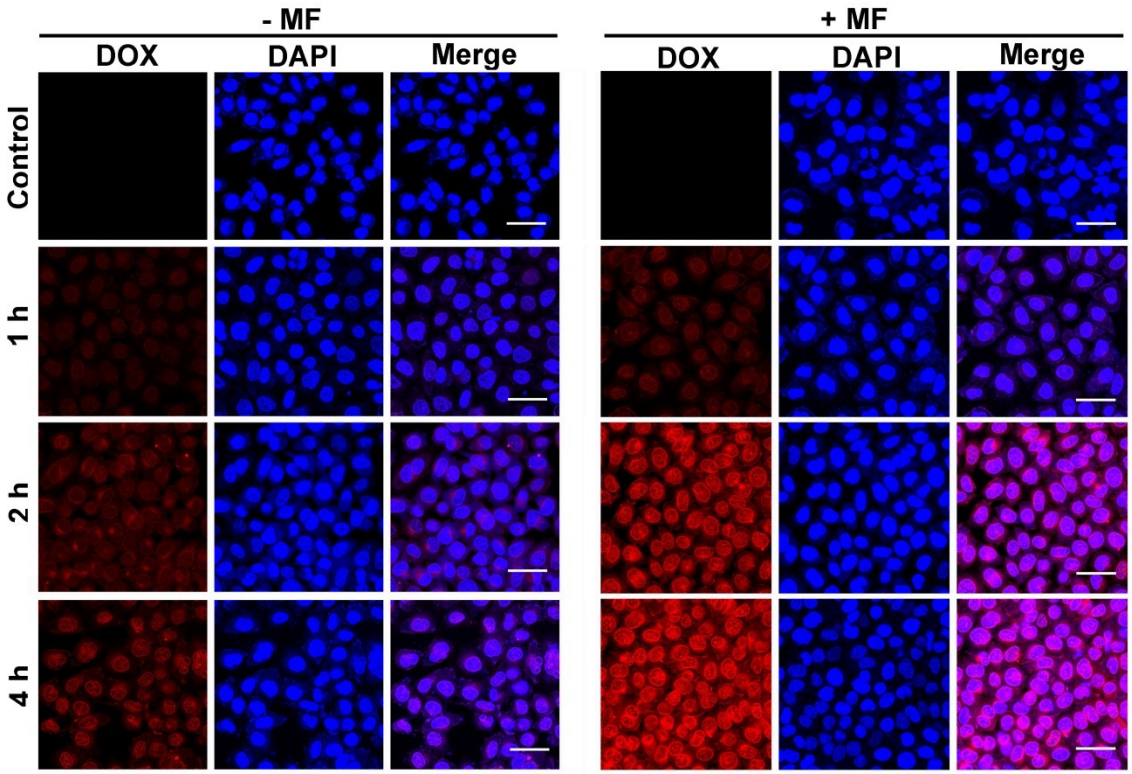

Fig. S11 CLSM images of HeLa cells incubated with DINCs@HA in the presence (+ MF) or absence (- MF) of the magnetic field, blank control: non-treated cells. (A) Prussian staining images, Scale bar: 50 μm.

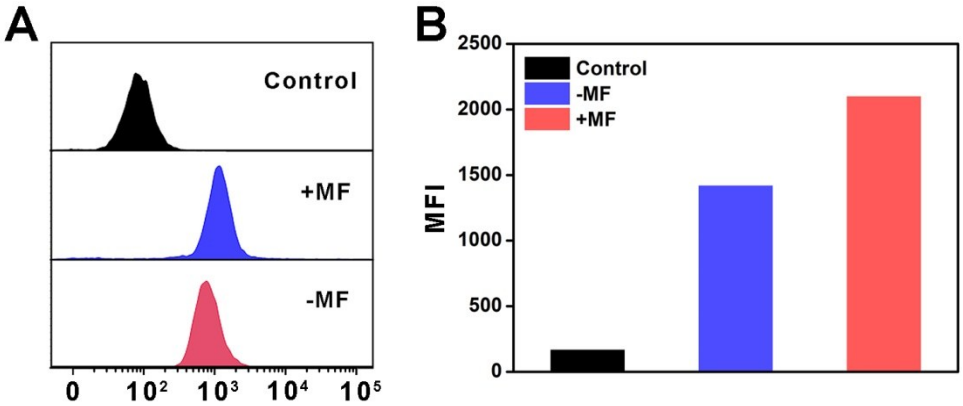

Fig. S12 (A) Cell uptake analyzed by flowcytometry, after incubation with DINCs@HA for 4h with/without magnet navigation. (B) Quantification of cellular uptake shown as mean fluorescence intensity (MFI).

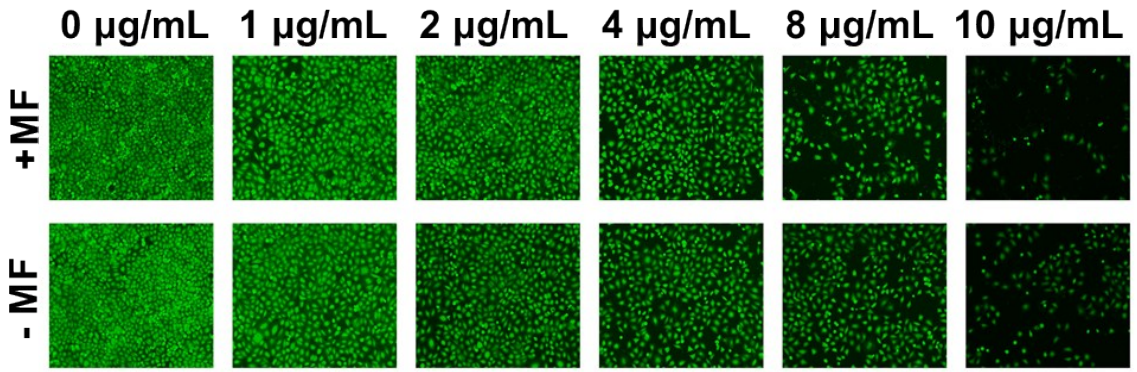

Fig. S13 Fluorescence microscopic images of HeLa cells stained by Calcein AM with different treatments as mentioned above.

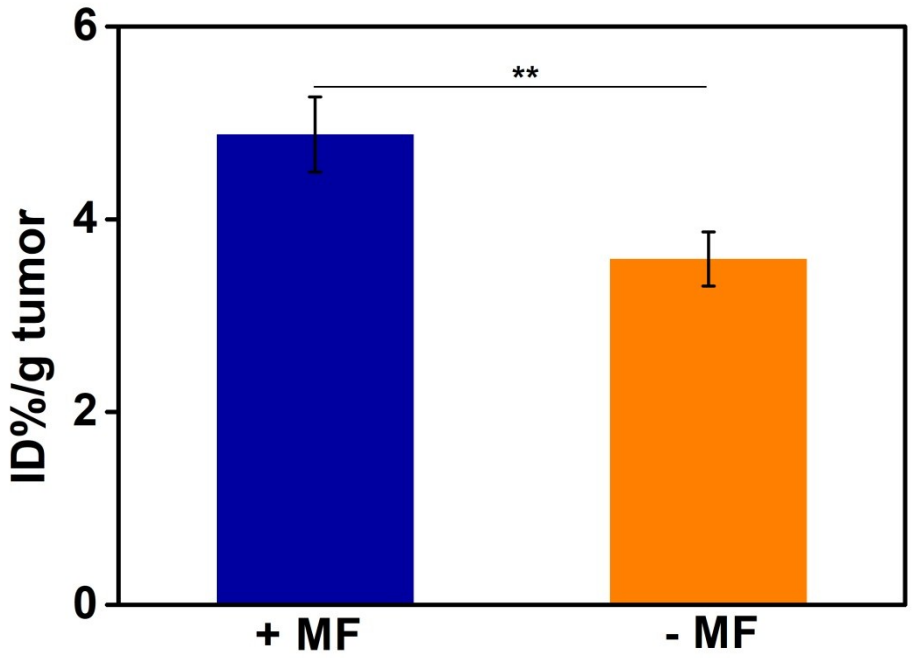

Fig. S14 The accumulation amount of DINCs@HA in tumor site at 24 h post i.v. injection of DINCs@HA, with tumor site attached by a magnet or not (*P < 0.05, **P < 0.01, ***P < 0.001; n = 3 per group). The accumulation data of DINCs@HA in the MF group based on the DOX levels at 24 h-post injection was measured to be 4.88 ± 0.39 %ID/g, which was nearly 1.4-folds higher than that in non-MF group (3.59 ± 0.28 %ID/g).

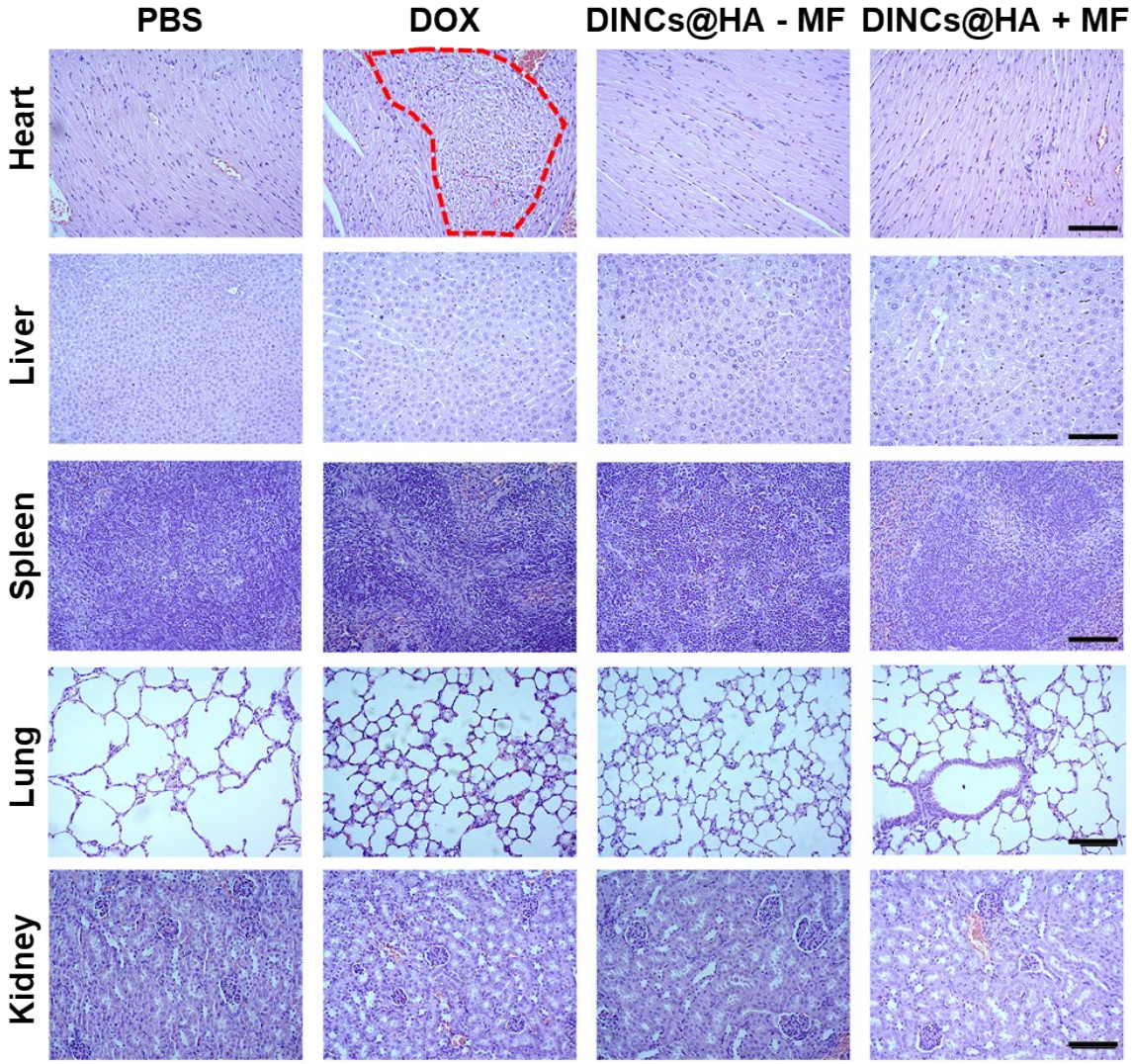

Fig. S15 The H&E staining of mice major organs with different treatments as mentioned above. Scale bar: 100 μm.

Reference

1. J. Li, X. Wang, D. Zheng, X. Lin, Z. Wei, D. Zhang, Z. Li, Y. Zhang, M. Wu and X. Liu, *Biomater. Sci.*, 2018, **6**, 1457-1468.