Supramolecular Magnetonanohybrids for Multimodal Targeted Therapy of Triple-Negative Breast Cancer Cells

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Electronic Supplementary Information

Results and Discussion

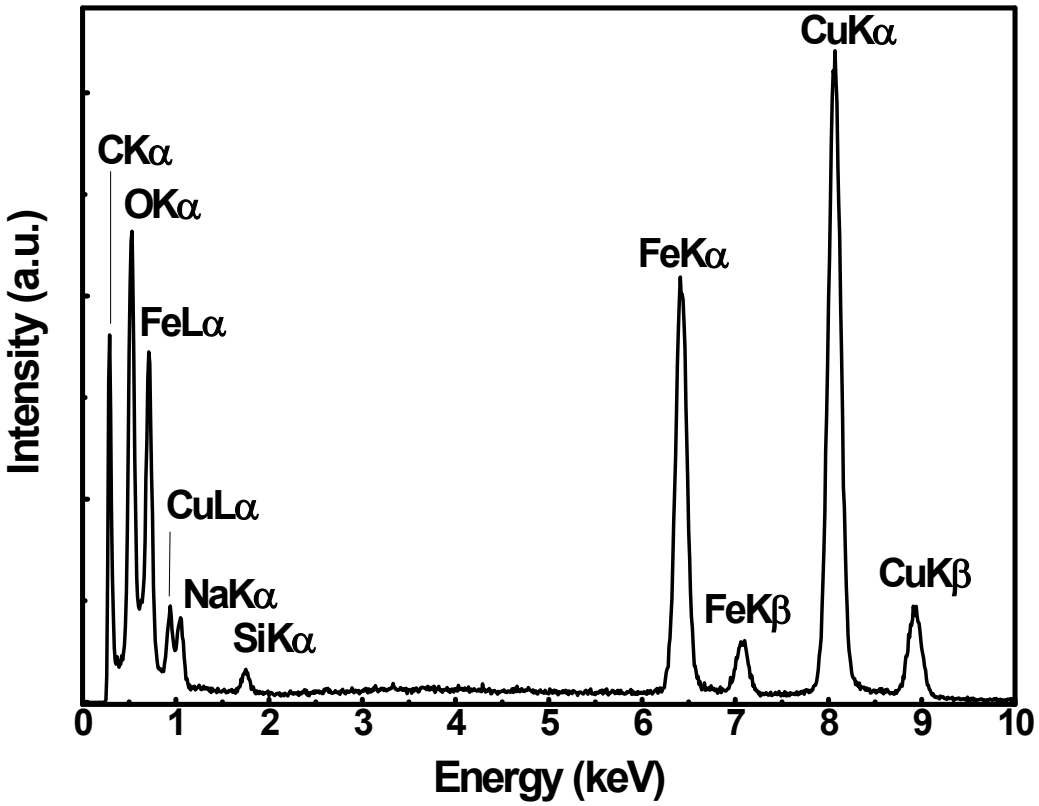

Fig. S1 EDX analysis of MNP-CMC nanoparticles.

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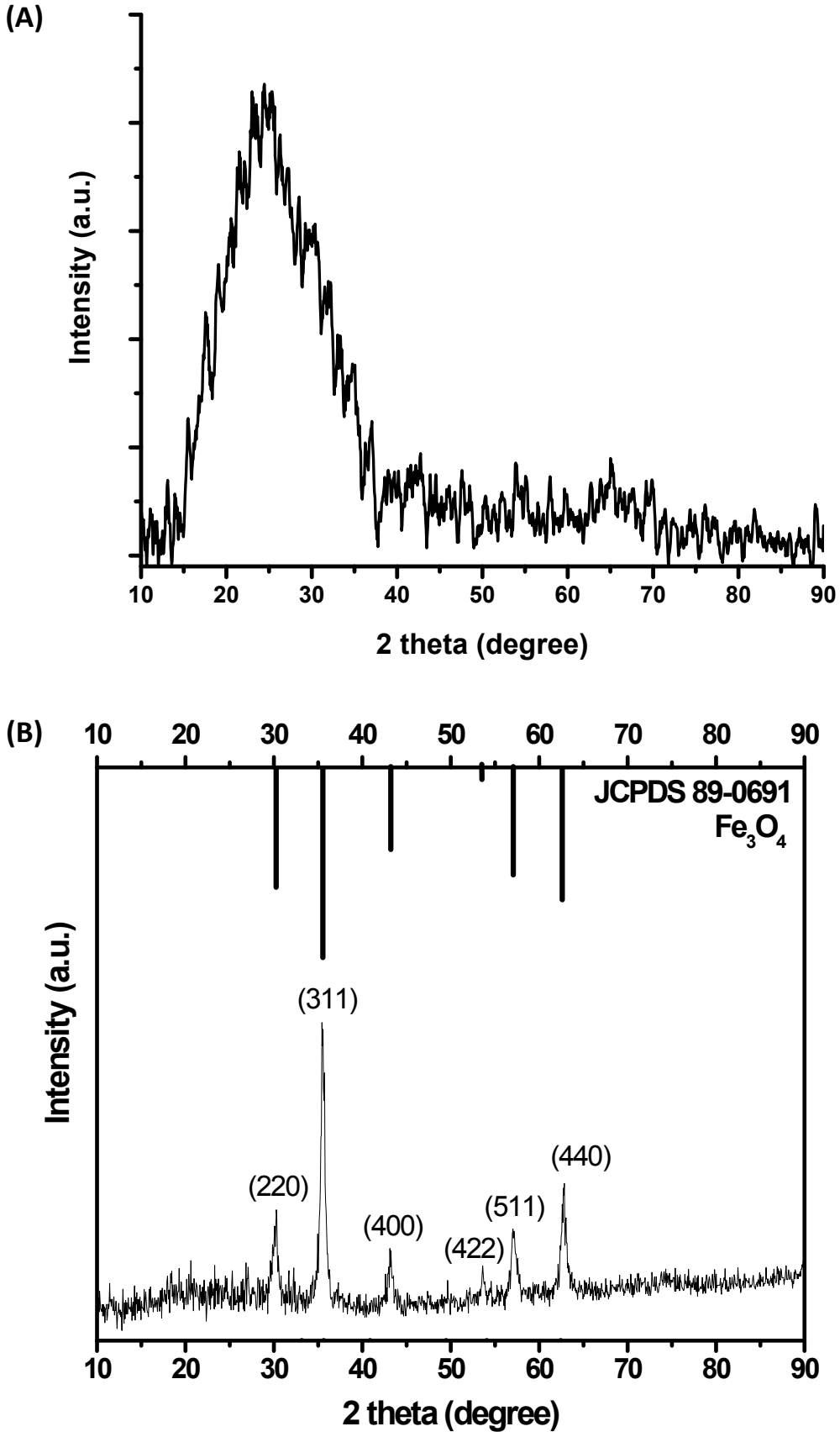

Fig. S2 XRD pattern of CMC and "bare" MNP (without CMC layer) in comparison to Fe_3O_4 magnetite JCPDS file (86-0691).

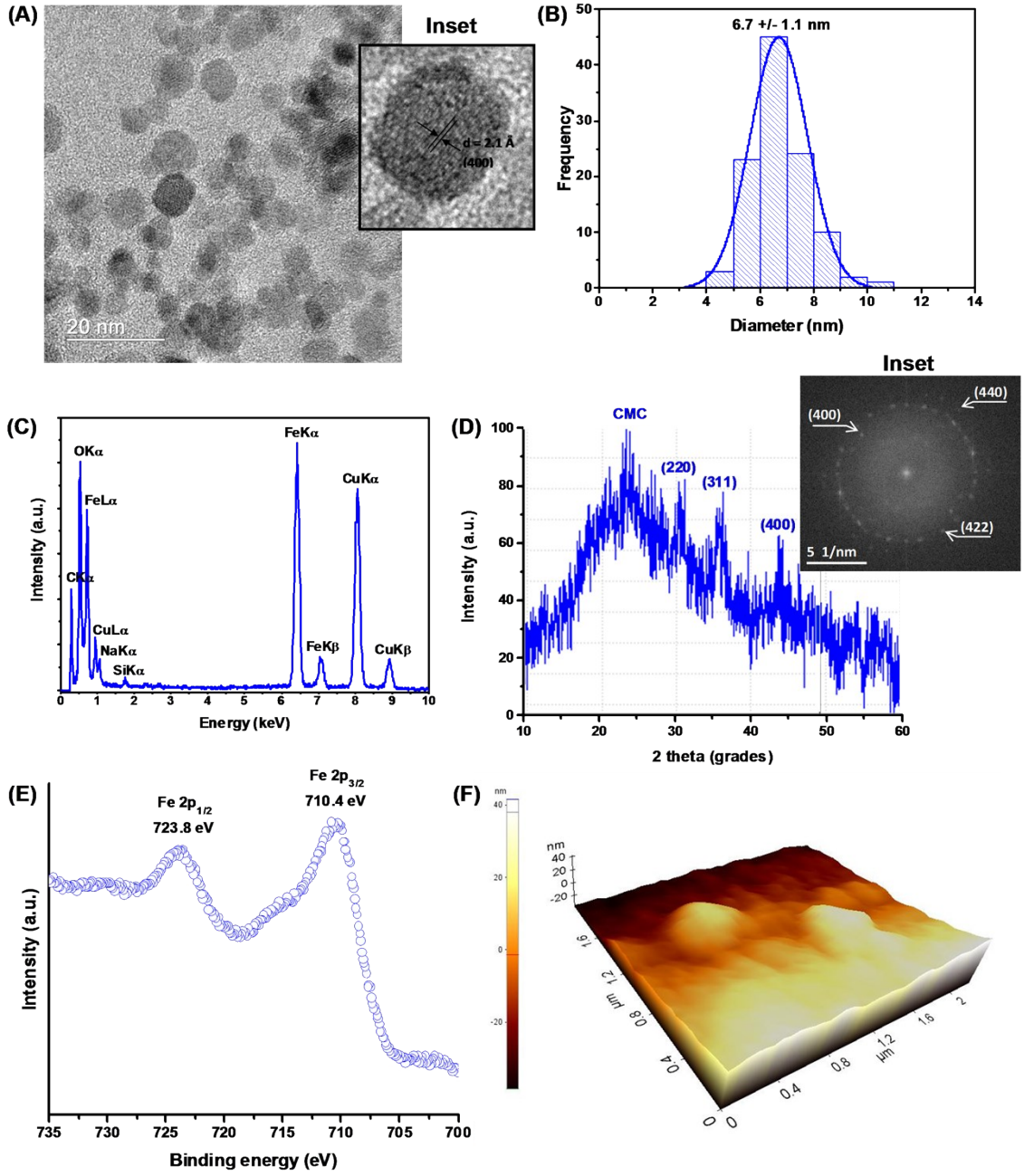

Fig. S3 MNP-CMC-ARG-FA_DOX nanocarrier: (A) TEM image (inset: HRTEM image with interplanar distance from plane (400) of magnetite) and (B) Histogram of MNP inorganic core size distribution; (C) EDX analysis; (D) XRD pattern (inset: SAED pattern with corresponding magnetite planes); (E) XPS spectra of Fe 2p region; and (F) Typical AFM 3D topographic image (inorganic core is not well-defined due to an increase of the thickness of the organic shell related to the macromolecular structure of CMC with L-Arginine and folic acid, and complexation with DOX).

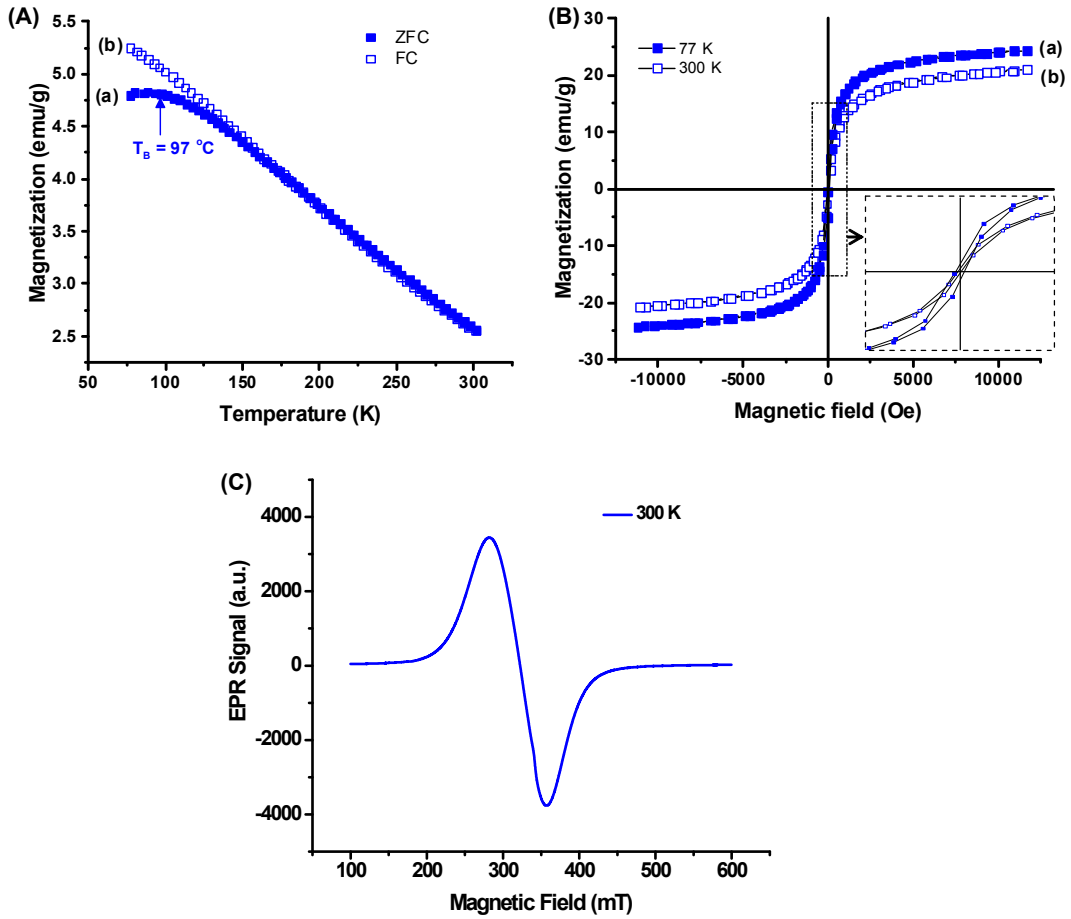

Fig. S4 (A) Magnetization curves of MNP-CMC-ARG-FA_DOX nanocarrier as a function of the temperature range (from 300 K to 77 K) for zero-field cooling ((a) ZFC) and after applying a field of 70 Oe for field cooling ((b) FC). (B) Magnetization *versus* magnetic field at (a) 77 K and (b) 300 K. (C) EPR signal at 300 K.

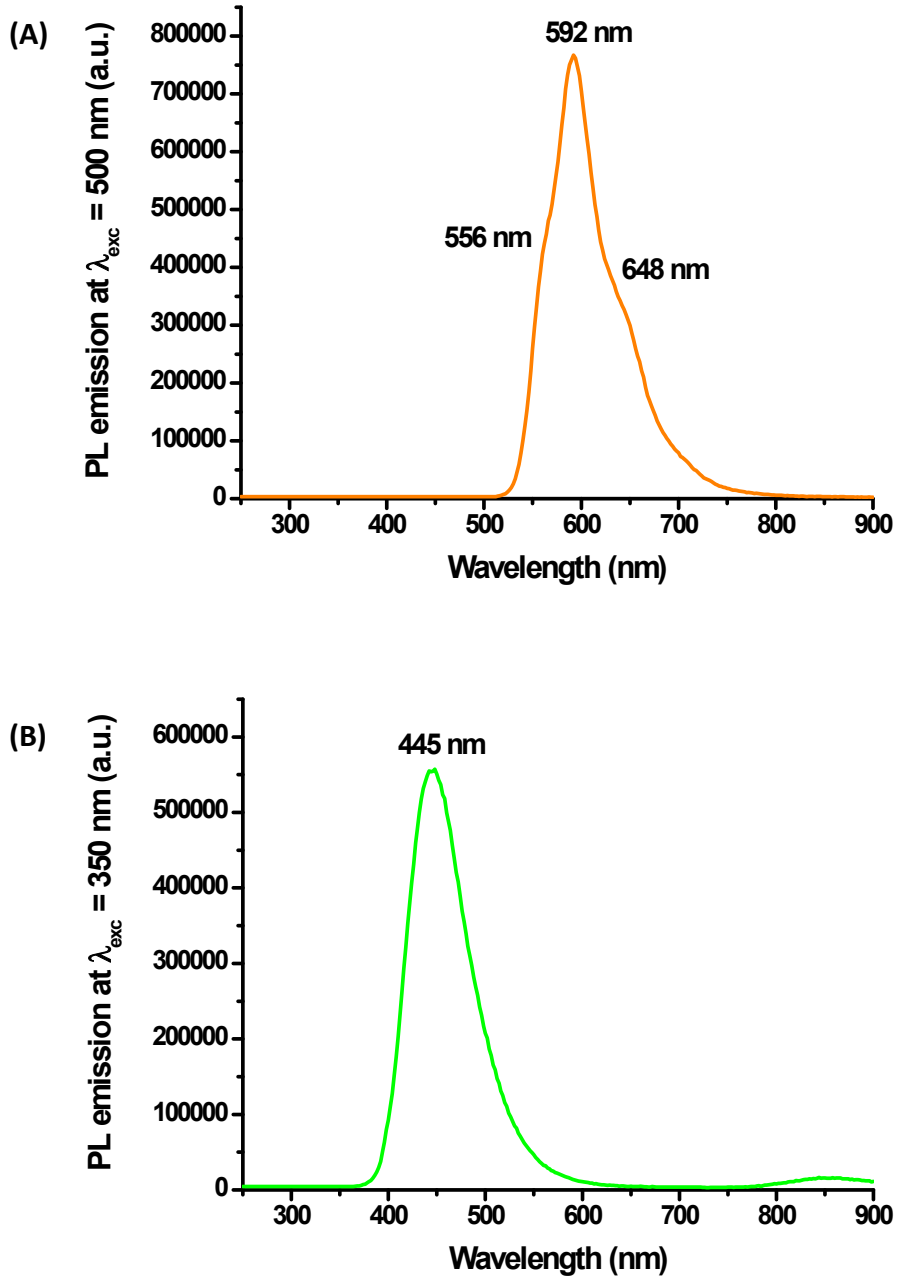

Fig. S5 PL emission spectra of (A) DOX (λ_{exc} = 500 nm) and (B) folic acid (λ_{exc} = 350 nm).

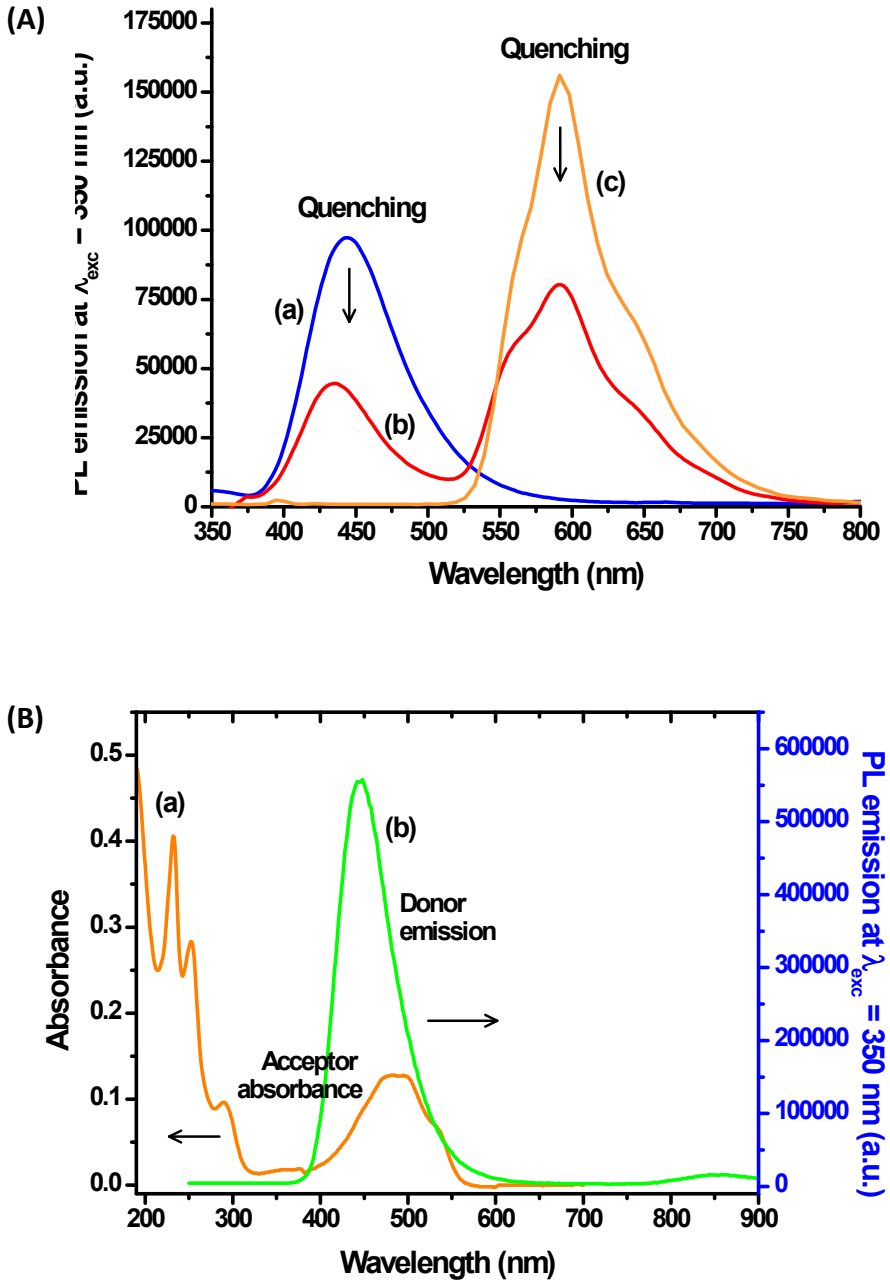

Fig. S6 (A) PL spectra of (a) MNP-CMC-ARG-FA, (b) MNP-CMC-ARG-FA_DOX, and (c) DOX. (B) Spectral overlapping of (a) DOX absorption and (b) folic acid emission.

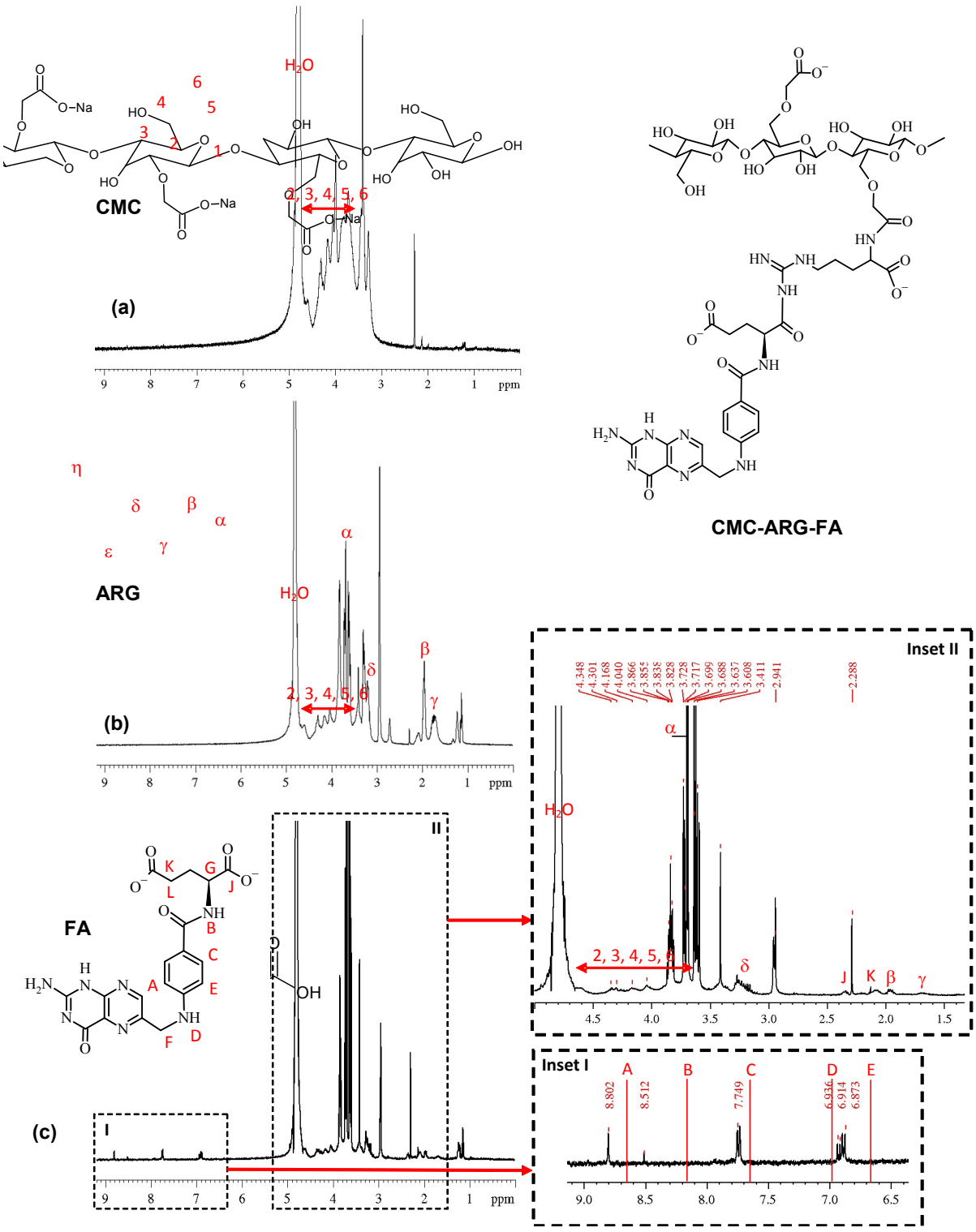

Fig. S7 ^{1}H-NMR spectra of (a) CMC, (b) CMC-ARG, and (c) CMC-ARG-FA.

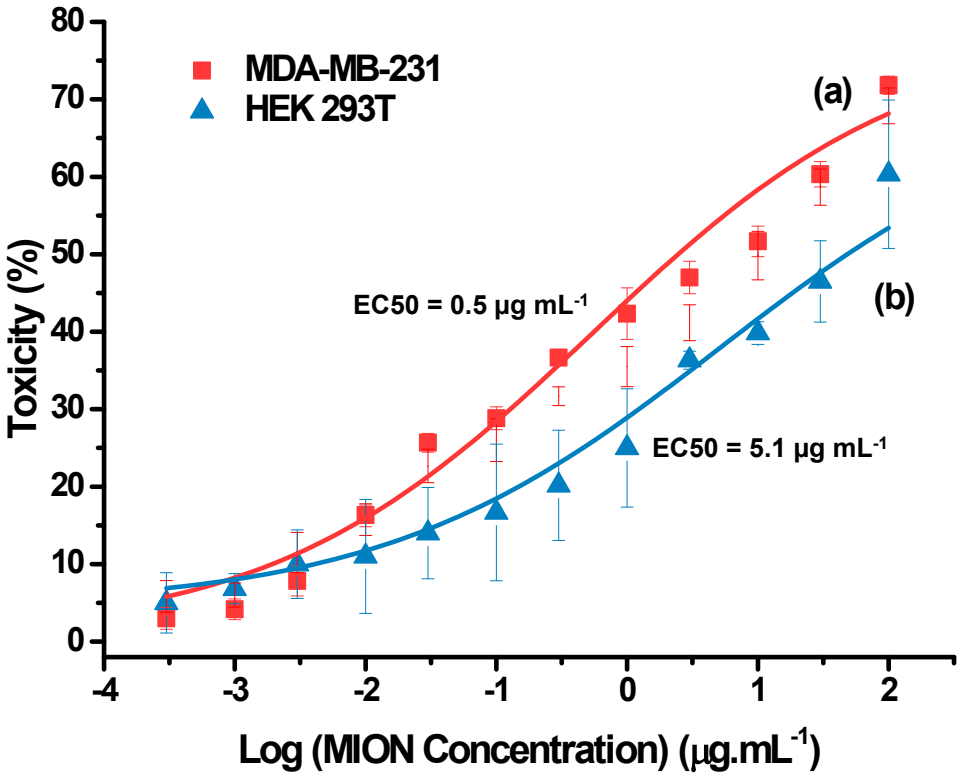

Fig. S8 Dose-response curves for (a) MDA-MB-231 and (b) HEK 293T cells (n = 6; Mean ± SD).

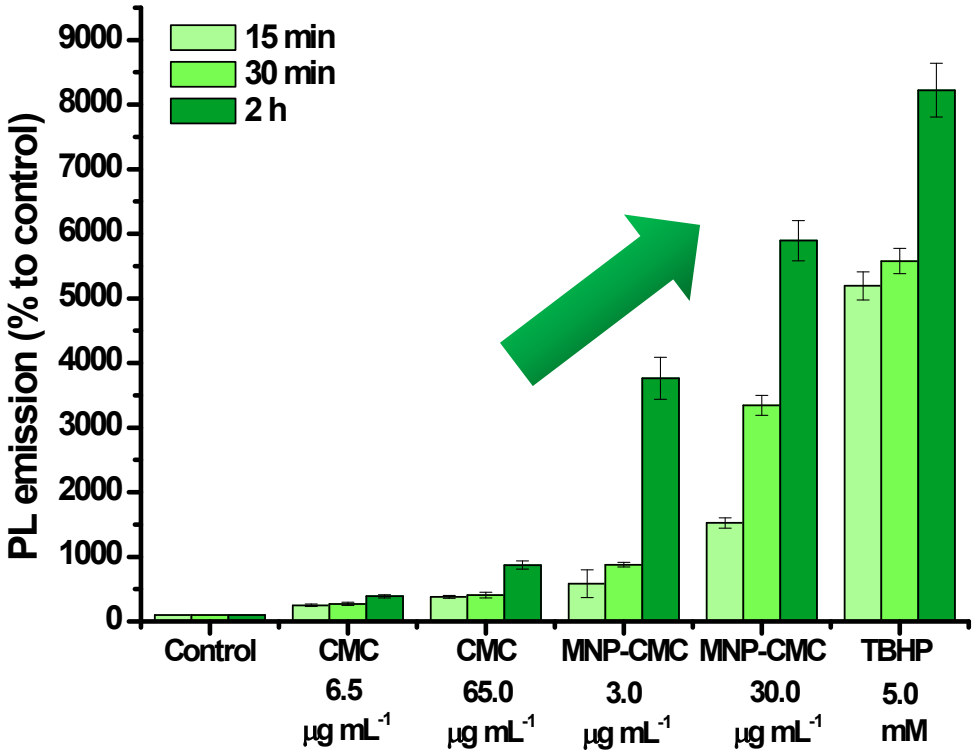

Fig. S9 Production of intracellular ROS in normal HEK 293T cell line induced by CMC and MNP-CMC after 15 min, 30 min, and 2 h of exposure (mean ± SD; n = 8).

Experimental

. Materials

Sodium carboxymethyl cellulose (CMC, degree of substitution DS = 0.7, average molar mass M_M = 90 kDa, viscosity of 180 cps, at 4% in H_2O at temperature=25 ˚C), ferric chloride hexahydrate ($FeCl_3.6H_2O$, 97%), ferrous sulfate heptahydrate ($Fe_2SO_4.7H_2O$, 99%), ammonium hydroxide (NH_4OH, 28-30 % NH_3 in H_2O), L-arginine (ARG, ≥ 98 %), folic acid (FA, ≥ 97 %), 2-(N-Morpholino)ethanesulfonic acid (MES, > 99 %, low moisture content), 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC, ≥ 98 %), N-hydroxysulfosuccinimide sodium salt (Sulfo-NHS, ≥ 98 %) doxorubicin hydrochloride (DOX, ≥ 98 %), ethalonamine hydrochloride (≥ 99 %), 3-(4,5-dimethylthiazol-2yl) 2,5-diphenyltetrazolium bromide (MTT, > 98 %), Triton™ X-100, sodium dodecyl sulfate (SDS, ≥ 99 %), hydrochloric acid (HCl, 37 %), 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA, ≥ 99 %), tert-butyl hydrogen peroxide (TBHP, 5.5 mM in decane), and paraformaldehyde (95 %) were procured from Sigma-Aldrich (USA). Fetal bovine serum (FBS), dulbecco's modified eagle medium (DMEM), phosphate-buffered saline (PBS), Leibowitz medium (L-15) penicillin G sodium, streptomycin sulfate, amphotericin-b, and trypsin-EDTA were supplied by Gibco (USA). Hydromount was purchased from Fisher Scientific Ltd. (USA). Isopropanol (≥ 99.8 %) was supplied by Merck (Germany). The aforementioned chemicals were used without further purifications, deionized water (DI water, Millipore Simplicity™) with the resistivity ≥ 18 MΩ.cm was used to prepare the solutions. The protocols and procedures were performed at room temperature (RT, 25 ± 2 ˚C), unless specified otherwise.

Human embryonic kidney (HEK 293T, American Type Culture Collection - ATCC® CRL 1573), human cervical carcinoma (HeLa, ATCC® CCL-2), human hepatocellular carcinoma (HepG2, ATCC® HB-8065™), human breast adenocarcinoma (MCF7, ATCC® HTB-22), and triple-negative human breast adenocarcinoma (MDA-MB-231, ATCC® HTB-26) cells were provided by Federal University of Minas Gerais (UFMG).

. Synthesis of magnetic nanoparticles

Magnetic iron oxide nanoparticles stabilized with CMC (MNP-CMC) were prepared by dissolving 0.02 M of Fe_2SO_4 and 0.04 M of $FeCl_3$ in CMC solution (1.0 % w/v). The mixture was homogenized by vigorous stirring and heated to 40 ± 2 ˚C. Subsequently, the mixture was heated to 80 ± 2 ˚C under nitrogen atmosphere, and 12.0 mL of a 25.0 % ammonia solution was added into the flask. After 20 min, the solution was let to cool down to room temperature and, in the sequence, it was dialyzed for 24 h using a cellulose membrane with cut-off (MWCO) of 12 kDa under moderate stirring at room temperature. After purification, the colloidal solution was stored at 6 ± 2 ˚C until further use.

. Characterization of magnetic properties of nanoparticles

Electron paramagnetic resonance spectroscopy analysis (EPR) was carried out on commercial X-band EPR spectrometer (Miniscope MS400, Magnettech) coupled to helium flux cryosystem (ESR 900, Oxford). All of the measurements were performed using the following parameters: 350 mT center field; 500 mT sweep range; 30 dB microwave power (~100 μW); 9.44 GHz microwave frequency; and 100 kHz field modulation. All samples were measured in powder form after drying inside borosilicate tubes at 120 ˚C (Wilmad Labglass).

Magnetization curves as a function of temperature were measured with a vibrational sample magnetometer (VSM; 7404, Lake Shore). For the measurements, the following protocol was used: (i) cooling the samples in zero-field from 300 K to 77 K; (ii) after reaching 77 K, a field of 70 Oe was applied and the magnetic moment measured as a function of temperature from 77 K to 300 K (zero-field cooling, ZFC); and (iii) after reaching 300 K, the magnetic moment of samples was measured again from 300 K to 77 K with the applied field (field cooling, FC). Magnetic hysteresis curves M(H) were measured in the field range -10.000 Oe < H < 10.000 Oe at 77 K and 300 K from which coercivity field and saturation magnetization were determined.

Magnetic hyperthermia analyses were performed using a Magnetherm™ instrument (solenoid diameter: 50 mm; number of turns: 17). The samples were submitted to an alternating magnetic field (AMF) with the amplitude of 19.9 kA/m and frequency of 112.6 kHz for 30 min, and the temperature increase in the sample was recorded over this period of time.

. Biological Characterization

All of the biological tests were performed according to ISO 10993-5:2009/(R)2014 (Biological evaluation of medical devices: Tests for *in vitro* cytotoxicity). HEK 293T (passage 25, 28, and 32), MDA-MB-231 (passage 8, 11, and 15), MCF7 (passage 12, 13 and 17), HepG2 (passage 7), and HeLa (passage 28) cells were cultured in DMEM (Dulbecco's Modified Eagle Medium) with 10% FBS (fetal bovine serum), penicillin G sodium (10 units mL^{-1}), streptomycin sulfate (10 mg mL^{-1}), and amphotericin-b (0.025 mg mL^{-1}) in a humidified atmosphere of 5 % CO_2 at 37 ˚C.

.. Toxicity *in vitro* - Mitochondrial activity (MTT) assay

All of the cells were plated (1×10^5 cells/well) in 96-well plates. Cell populations were synchronized (arrested in the G0 cell phase, quiescence state) by nutrient deprivation for 24 h (culture medium without FBS). After that, the total volume of media was suctioned and replaced with culture medium containing 10 % FBS and incubated for 24 h. Then, nanocolloidal solutions and DOX were added to individual wells. After 6 h and/or 24 h, the total volume of solution of each well was aspirated and replaced with 60 μL of culture media containing serum to each well. The MTT reagent (5 mg mL^{-1}) was added to each well and incubated for 4 h in an

oven at 37 ˚C and the atmosphere of 5 % CO_2. Then, 40 µL of SDS solution/4 % HCl was placed in each well and incubated for 16 h in an oven at 37 ˚C and an atmosphere of 5% CO_2. Then, the volume of 100 µL from each well was aspirated and transferred to a similar blank 96-well plate, and the absorbance was measured using iMark™ Microplate Absorbance Reader (Bio-Rad®) with a filter of wavelength at λ=595 nm. The toxicity responses were calculated according to Equation 1.

Toxicity (%) = 100 – [(Absorbance of sample)/(Absorbance of control) × 100 %] (1)

Control samples were designed for the experimental procedure as follows: control group (cell cultured with medium and 10 % FBS); positive control (cell cultured with medium, 10 % FBS and 1.0 % v/v Triton™ X-100; and negative control (cell cultured with medium, 10 % FBS and chips of sterile polypropylene Eppendorf®, 1 mg mL^{-1}).

Statistical significance was tested through One-way ANOVA followed by Bonferroni's method using the Graphpad Prism software with a significance level (α) 0.05. At p-value (or probability value) < 0.05, the result was considered statistically significant. The experiments were performed using at least in sextuplicate (n ≥ 6).

.. Evaluation of Reactive Oxygen Species (ROS) formation

Healthy (HEK 293T) and cancer (MDA-MB-231) cells (1 × 10^4 cells/well on 96-well plates) were incubated with 100 µL of 2',7'-dichlorodihydrofluorescein diacetate (DCF-DA) solution at 100 µM (diluted in culture medium) for 40 min in an oven at 37 ˚C and at the atmosphere of 5 % CO_2. After this period, the probe was removed, and the cells were exposed to 100 µL of the following samples: CMC solution at 6.5 µg mL^{-1}; CMC solution at 65.0 µg mL^{-1}; MNP-CMC suspension at 3.0 µg mL^{-1} of MNP nanoparticles and 6.5 µg mL^{-1} of CMC polymer; MNP-CMC suspension at 30.0 µg mL^{-1} of MNP nanoparticles and 65.0 µg mL^{-1} of CMC polymer. For the negative control sample, cells were incubated with DCF-DA, and positive control cells were treated with tert-butyl hydrogen peroxide (TBHP, 5.0 mM in water). After the incubation times of 15 min, 30 min, and 2 h, at 37 ˚C/5 % CO_2, fluorescence intensity at each well was measured using λ=485 nm excitation and λ=528 nm emission filters on Varioskan™ LUX multimode microplate reader (Thermo Scientific). The PL intensity values were expressed as a percentage of fluorescence intensity relative to the negative control wells (100 %). Data were presented as means and standard deviations (SD) of eight replicates.

For detection of DCF using fluorescence microscopy, MDA-MB-231 cells were plated (1×10^5 cells/well, 6-well plate) and synchronized for 24 h. Then, cells were incubated with 400 µL of DMEM medium 10% FBS and 400 µL of samples or controls (CMC solution at a final concentration of 65.0 µg mL^{-1}; MNP-CMC suspension at a final concentration of 30.0 µg mL^{-1} of MNP nanoparticles and 65.0 µg mL^{-1} of CMC polymer; DCF-DA at a final concentration of

100 μM in DMEM, negative control; and TBHP at a final concentration of 5.0 mM in water, positive control) for 30 min at 37 ˚C and 5 % CO_2. Then, the samples/controls were removed, and cells were treated with 500 μL of DCF-DA solution at 100 μM (diluted in culture medium DMEM) for 40 min. In the sequence, the probe was removed, and cells were washed with PBS before being fixed with paraformaldehyde solution (4.0 % in PBS) for 30 min, washed three times with PBS, and coverslips were mounted with Hydromount®. Digital images were captured with a Ti-U fluorescence microscope (Nikon Instruments) using FITC filter (λ_{exc} = 488 nm, and emissions were collected at 506-550 nm).

.. Cell uptake/Internalization

For internalization experiments, MCF7 and MDA-MB-231 cells were plated (5×10^5 cells per well) in a 6-well plate, incubated for 24 h in 5 % CO_2 at 37 ˚C and synchronized for 24 h. Then, MNP-CMC-ARG-FA_DOX magnetonanohybrid suspensions with the medium solution with 10 % FBS (1:1, v/v) were added to the wells with cells and incubated for 30 min and 2 h, using 5 % CO_2 at 37 ˚C, followed by washing with PBS solution. For the reference control, cells were incubated with the original medium with 10 % FBS (*i.e.,* no samples).

In the sequence, for steady-state fluorescence spectroscopy analysis, cells were trypsinized (250 μL of 0.2 % trypsin for 7 min), collected, centrifuged (5 min at 1400 rpm Hettich Mikro 200R centrifuge), and resuspended in 200 μL of PBS. Cell concentration was determined by counting the number of cells in Neubauer Chamber using CH30 microscope (Olympus Corporation). Quantitative analysis of DOX uptake into cells was performed based on the PL emission spectrum of suspended cells acquired at RT using FluoroMax- Plus - CP (Horiba Scientific) at λ_{exc} = 500 nm (slit = 2 nm). PL intensity per cell was calculated by the ratio of the intensity of the DOX emission band at 555 ± 5 nm by the number of cells. The autofluorescence of suspended cells was measured in control samples.

For confocal scanning laser microscopy (CLSM), the cells were fixed with paraformaldehyde solution (4.0 % in PBS) for 30 min, washed three times with PBS, and coverslips were mounted with Hydromount®. In the sequence, digital images were taken with an Eclipse Ti confocal microscope (Nikon Instruments, USA) using the oil immersion objective (63× Plan-Apo/1.4 NA). For folic acid green fluorescence, excitation was at λ_{exc} = 488 nm and the emission was collected at λ_{em} = 506-550 nm (FITC filter cube). For orange-red-emitting DOX, excitation was at λ_{exc} = 543 nm, and emission was collected at λ_{em} = 545-645 nm (TRITC filter cube). Dual-color confocal fluorescence images were recorded separately in the correspondent channel and merged afterward.

.. Hyperthermia test *in vitro* with breast cancer cells

All of the cells were plated (1×10^5 cells/well) in 24-well microplates. Cell populations were synchronized in serum-free media for 24 h. After that, the media volume was suctioned and replaced with L-15 medium containing 10% FBS for 24 h. Then, membrane-receptor biofunctionalized magnetonanohybrids (MNP-CMC-ARG-FA and MNP-CMC-ARG-FA_DOX) were added to individual wells at final concentrations of 15.0 μg mL^{-1} of MNP and 18.5 μg mL^{-1} (32 μM) of electrostatically bonded DOX (1:1 v/v, nanoassembly: L-15 medium containing 10% FBS).

After 3 h of incubation in the atmosphere without CO_2 at 37 ˚C, MNP-CMC-ARG-FA and MNP-CMC-ARG-FA_DOX samples were washed with PBS, trypsinized (0.2 % trypsin, 7 min), centrifuged (1400 rpm for 5 min) and the original volume was reconstituted with L-15 supplemented with 10% FBS. In the sequence, the characterization of cancer cell death caused by ferroptosis + chemotherapy (reference) was performed using MTT protocol. Therefore, 400 μL of the MTT reagent (5 mg mL^{-1}) was added to each well with the cells and incubated for 2.5 h at 37 ˚C. Formazan crystals were dissolved using 40 μL isopropanol solution/4% HCl. Then, 100 μL of the solution was removed from each well and transferred to a 96-well plate, and the absorbance was measured (iMark™ Microplate Absorbance Reader, Bio-Rad®, with a wavelength filter at λ = 595 nm). The toxicity was calculated according to Equation 1.

For hyperthermotherapy combined with ferroptosis + chemotherapy analysis (MNP-CMC-ARG-FA_MFH and MNP-CMC-ARG-FA_DOX_MFH), after 3 h of incubation, samples were washed, trypsinized and resuspended before being exposed to the AMF (H = 19.9 kA m^{-1}, frequency = 112.6 kHz) for 1 h. After the time of exposure, the toxicity was evaluated by MTT assay, as described previously in this section.

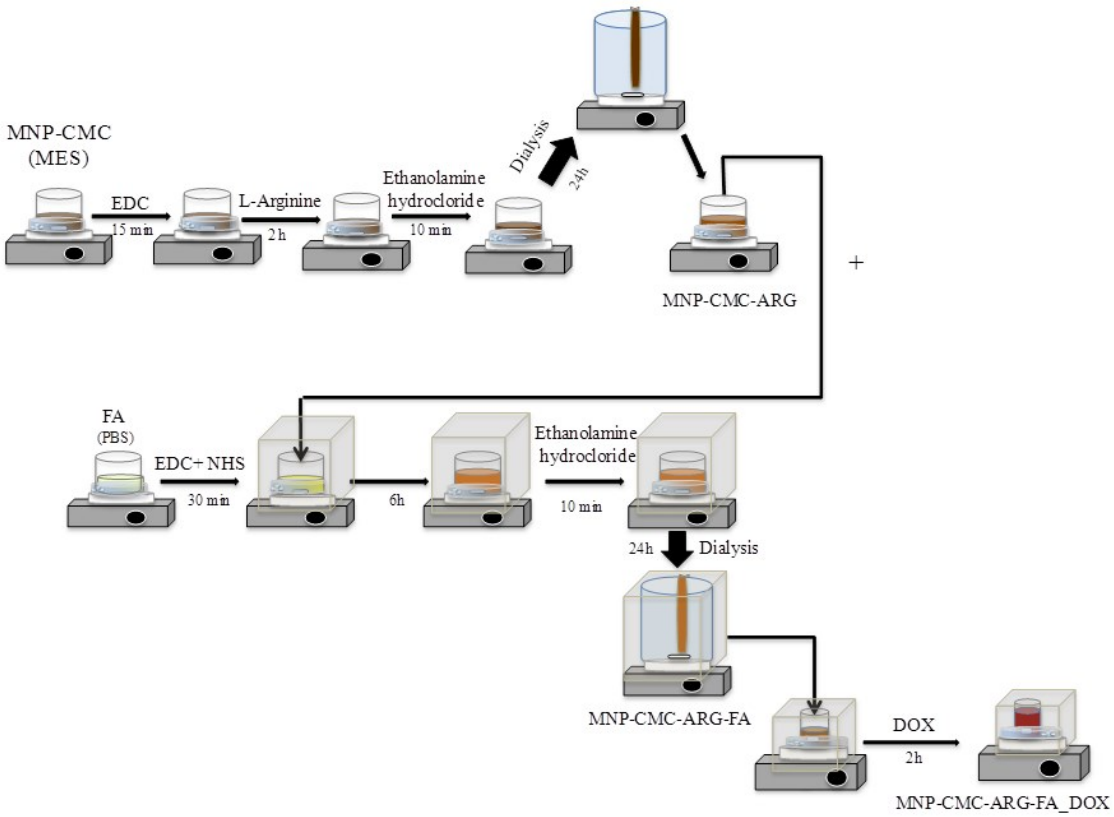

Fig. S10 Schematic illustration of the synthesis of MNP-CMC-ARG-FA_DOX and intermediary complexes.