Electronic Supplementary Material (ESI) for RSC Advances.

In vitro and in vivo applications of alginate/iron oxide nanocomposites for theranostic molecular imaging in brain tumor model

Chia-Hao Su[a] and Fong-Yu Cheng*[b]

[a] Center for Translational Research in Biomedical Sciences, Kaohsiung Chang Gung Memorial Hospital, Kaohsiung 833, and Department of Biomedical Imaging and Radiological Sciences, National Yang Ming University, Taipei 112, Taiwan.
[b] Institute of Oral Medicine, National Cheng Kung University Hospital, College of Medicine, National Cheng Kung University and Hospital, Tainan 701, Taiwan

Address correspondence to FYCheng@mail.ncku.edu.tw

Experimental Detail

Preparing NH_2-Fe_3O_4 nanoparticles

Magnetite nanoparticles (NPs) were prepared referring as previously described [1]. First, 1 M ferric chloride hexahydrate ($FeCl_3 \cdot 6H_2O$) and 2 M ferrous chloride tetrahydrate ($FeCl_2 \cdot 4H_2O$) were prepared by dissolving iron salts in 2 M HCl solutions, respectively. In a typical experimental procedure, 4 ml of 1 M $FeCl_3$ solution was mixed with 1 ml of 2 M $FeCl_2$ solution in a flask and then 1 mL of organic acid aqueous solution (0.5 g of alanine dissolved in 1 mL of deionized water) was added to the mixture solution. This solution was vigorously stirred, and then 5 M NaOH was slowly added (drop by drop) until a pH of 13 was reached and the solution turned black. The solution was vigorously stirred for another 15 minutes at room temperature. Precipitated powder in the solution was isolated using a permanent magnet. The supernatant was decanted and discarded. Deionized water was then added to wash the precipitates. This procedure was repeated 3 times to remove excess salt in the suspension. Subsequently, 3 g of alanine dissolved in 50 mL of deionized water was added to the washed precipitates and the solution was stirred for 5 minutes and then sonicated for 30 minutes. After sonication, deionized water and acetone were added (volume ratio of mixture solution:deionized water:acetone = 5:2:3) and the solution was centrifuged at 8000 rpm for 10 minutes. The supernatant was decanted and discarded, 7 mL of deionized water was added to dissolve and wash the precipitates, 3 mL of acetone was added, and the solution was centrifuged at 8000 rpm for 10 minutes. This procedure was repeated twice to remove excess organic acid in the suspension. Finally, NH_2-Fe_3O_4 NPs were obtained after the precipitates were redispersed in deionized water. A spectroanalyzer (Jobin-Yvon JY138; Horiba Taiwan, Shanhua District, Tainan 741, Taiwan) was used for an inductively coupled plasma (ICP) analysis of the Fe ion concentration for the NH_2-Fe_3O_4 NPs.

Preparing alginate-conjugated NH_2-Fe_3O_4 (alg-Fe_3O_4) NPs

Low molecular weight sodium alginate (alg) (A0682, molecular weight range: 12-80 kDa) (Sigma-Aldrich, St. Louis, MO, USA). First, 5 mL of 1% (w/v) alg solution (prepared by mixing 0.1 g of alg with 10 mL of deionized water) mixed with 5 mL of deionized water, and then 21.7 μmole of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) was added to the mixture and the solution was stirred for 10 minutes. Subsequently, 0.1 mL of NH_2-Fe_3O_4 NPs (particle concentration: 0.1 mM) was added to the mixture. The solution was then stirred for another 10 minutes. Another 10 μL of NaOH (5 M) was added, and the resulting solution was stirred for 1 hour and then centrifuged at 14000 rpm for 10 minutes. The supernatant was removed and the precipitate was washed twice with deionized water

twice. Finally, the precipitate (alg-Fe_3O_4 NPs) was redispersed and stored in deionized water for further use.

Preparing doxorubicin-encapsulated alg-Fe_3O_4 (Dox/alg-Fe_3O_4) NPs

To encapsulate doxorubicin (Dox) inside alg-Fe_3O_4 NPs, different volumes (0-750 μL) of Dox aqueous solution (5 mM) were separately incubated with 100 μL of alg-Fe_3O_4 NPs (particle concentration: 10 μM) and deionized water was added to keep the final volume at 1 mL. All samples were gently stirred at room temperature for 6 hours. Then 1 mL of the mixture was added to 2 mL of $CaCl_2$ aqueous (1 mM) solution. The mixtures were then incubated for 10 minutes. The precipitates were collected using a permanent magnet. The supernatant was collected in a separate Eppendorf tube, and then deionized water was added twice to wash the precipitates. The precipitate (Dox/alg-Fe_3O_4 NPs) was redispersed in deionized water for additional experiments. The fluorescence intensity difference in Dox between the initial amount and residue in supernatants was analyzed to estimate the amount of encapsulated Dox in Dox/alg-Fe_3O_4 NPs after a standard linear calibration curve (fluorescence intensity vs. concentration) derived based on the measurements of Dox fluorescence at 585 nm.

Stability test for Dox/alg-Fe_3O_4 NPs

To test the stabilities of Dox/alg-Fe_3O_4 NPs, 200 μL of Dox/alg-Fe_3O_4 NPs was incubated with 800 μL of deionized water and PBS (10 mM) in Eppendorf tubes at 37°C for different time periods. The released Dox was collected from the supernatants, and its concentration was calculated using a linear calibration curve by measuring the fluorescence emission of the Dox at 585 nm.

***In vitro* release test of Dox from Dox/alg-Fe_3O_4 NPs**

To test the release of Dox from Dox/alg-Fe_3O_4 NPs, 100 μL of Dox/alg-Fe_3O_4 NPs were separately incubated with 900 μL of cytoplasm mimicking (CM) buffer and phosphate-buffered saline (PBS) (10 mM, pH 5.5) in Eppendorf tubes at 37°C for different time periods. The released Dox was collected from the supernatants and its concentration using a linear calibration curve by measuring the fluorescence emission of the Dox at 585 nm.

Characterization

Electron micrographs of all as-prepared nanomaterials were obtained by placing a drop of the sample onto a copper mesh coated with an amorphous carbon film and dried in a vacuum desiccator. The mean diameter and morphology of the as-prepared

nanomaterials were characterized using transmission electron microscopy (TEM) (H-7500; Hitachi Koki Co., Tokyo, Japan). The extinction characteristics of the nanomaterials were determined using an ultraviolet-visible (UV-vis) spectrophotometer (HP8453; Agilent Technologies, Santa Clara, CA). The surface charge of the nanomaterials was measured using zeta potential measurement (Zetasizer Nano-zs90; Malvern Instruments, Malvern, Worcestershire, UK). All as-prepared nanomaterials were dissolved by adding hydrochloric acid (HCl). The Fe ion concentration for each nanomaterial was measured on the spectroanalyzer using inductively coupled plasma (ICP) analysis for elemental composition the spectroanalyzer.

Cell viability of non-tumorous human umbilical vein endothelial cells tested using an MTT assay

Non-tumorous human umbilical vein endothelial cells (HUVECs) were seeded at a density of 5×10^3 cells per well in 96-well plates for 24 hours, and then they were incubated with alg-Fe_3O_4 NPs with different iron concentrations (0, 1, 5, 10, 25, 50, and 100 μg/mL). After another 24 and 48 hours of incubation, the cytotoxicity analysis (a medium containing 10% MTT reagent) was added was added, and the cultures were incubated for 4 hours to allow formazan dye to form. The crystals were dissolved in dimethyl sulfoxide (DMSO) (200 μL), incubated in the dark for another 10 minutes, and then centrifuged at 4000 rpm for 10 minutes. The supernatant was transferred onto a new enzyme-linked immunosorbent assay (ELISA) plate, and the absorbance was measured at a wavelength of 540 nm using an ELISA reader.

Cell viability of brain cancer cells (C6) tested using an MTT assay

Brain cancer cells (C6) were seeded at a density of 5×10^3 cells per well in 96-well plates for 24 hours, and then separately incubated with Dox, alg-Fe_3O_4 NPs, or Dox/alg-Fe_3O_4 NPs. The final iron concentrations were 0, 0.052, 0.256, 0.520, 2.560, 5.200, and 10.400 μg/mL and the corresponding Dox concentrations were, respectively, 0, 0.1, 0.5, 1, 5, 10, and 20 μg/mL (1.39 mg Dox per mg Fe_3O_4 NPs and 0.72 mg Fe per mg Fe_3O_4 NPs for alg-Fe_3O_4 NPs and Dox/alg-Fe_3O_4 NPs). After another 24 and 48 hours of incubation, the cytotoxicity analysis was measured by MTT assay with same steps as above described.

Brain tumor-bearing animal model and IVIS Spectrum *in vivo* pre-clinical imaging system

Human glioblastoma cells U87MG-luc2 (a luciferase-expressing cell line) (Caliper Life Science, PerkinElmer, Hopkinton, MA, USA) maintained in Eagle's

essential minimum medium (EMEM), were incubated at 37°C with 5% CO_2 in air. The cells were trypsinized from the tissue culture flasks and washed twice with PBS, and were then counted on a hemacytometer slide and resuspended in serum-free, antibiotic-free normal saline before they were injected (27G needle) into 6-week old male B6 mice (25-30 g) (National Cheng Kung University Laboratory Animal Center, Tainan, Taiwan). All the mice were given humane care in compliance with institutional guidelines for the maintenance and use of laboratory animals in research. All of the experimental protocols involving live animals were reviewed and approved by the university's Animal Experimentation Committee. The U87MG-luc2 tumor xenografts were established using a subcutaneous dorsal flank injection of 5×10^6 tumor cells in 100 μL of normal saline by using a 27G needle. When the tumor was near 25 mm^3, the tumor-bearing mice were ready for the study.

To evaluate the anti-cancer efficacy of Dox/alg-Fe_3O_4 NPs, we used 25 tumor-bearing mice divided into 4 treatment groups: controls (PBS), free Dox, alg-Fe_3O_4 NPs, and Dox/alg-Fe_3O_4 NPs. The mice were intratumorally injected with Dox/alg-Fe_3O_4 NPs (Dox dose: 3 mg Dox/Kg body weight), with alg-Fe_3O_4 NPs (Fe dose = that in Dox/alg-Fe_3O_4 NPs), free Dox (Dox dose = that in Dox/alg-Fe_3O_4 NPs).
The whole body of each tumor-bearing mouse was imaged pre-injection, and at 3 and 7 days post-injection (n = 5 mice each). Real-time fluorescent imaging was monitored using an IVIS Spectrum *in vivo* pre-clinical imaging system (Xenogen, Alameda, CA, USA) with a cooled CCD optical system. Before imaging, the mice were anesthetized with 2% isoflurane. The field of view was 125 mm in diameter. The images were acquired for 0.5 seconds using Living Image Analysis and acquisition software (PerkinElmer Life and Analytical Sciences, Inc., Boston, MA). A pseudo color image representing the spatial distribution of photon counts was projected onto the photographic image.

Reference

1. D. B. Shieh, F. Y. Cheng, C. H. Su, C. S. Yeh, M. T. Wu, C. Y. Tsai, C. L. Wu, D. H. Chen, C. H. Chou, *Biomaterials* **2005**, *26*, 7183-7191.

FIGURES AND FIGURE LEGENDS

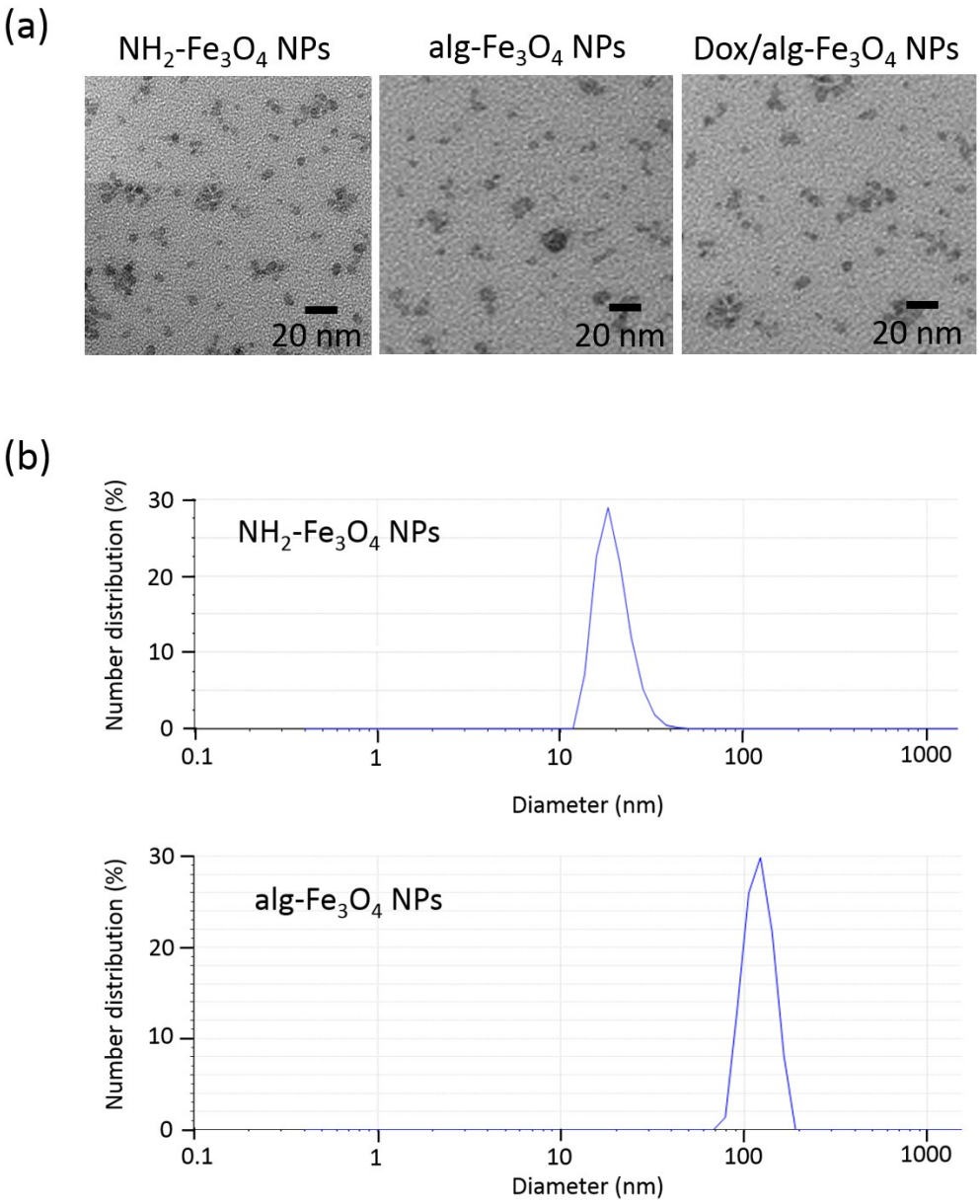

Figure S1. a) TEM images of NH_2-Fe_3O_4 NPs, alg-Fe_3O_4 NPs and Dox/alg-Fe_3O_4 NPs. b) Number distribution obtained by dynamic light scatting (DLS) of NH_2-Fe_3O_4 NPs and alg-Fe_3O_4 NPs.

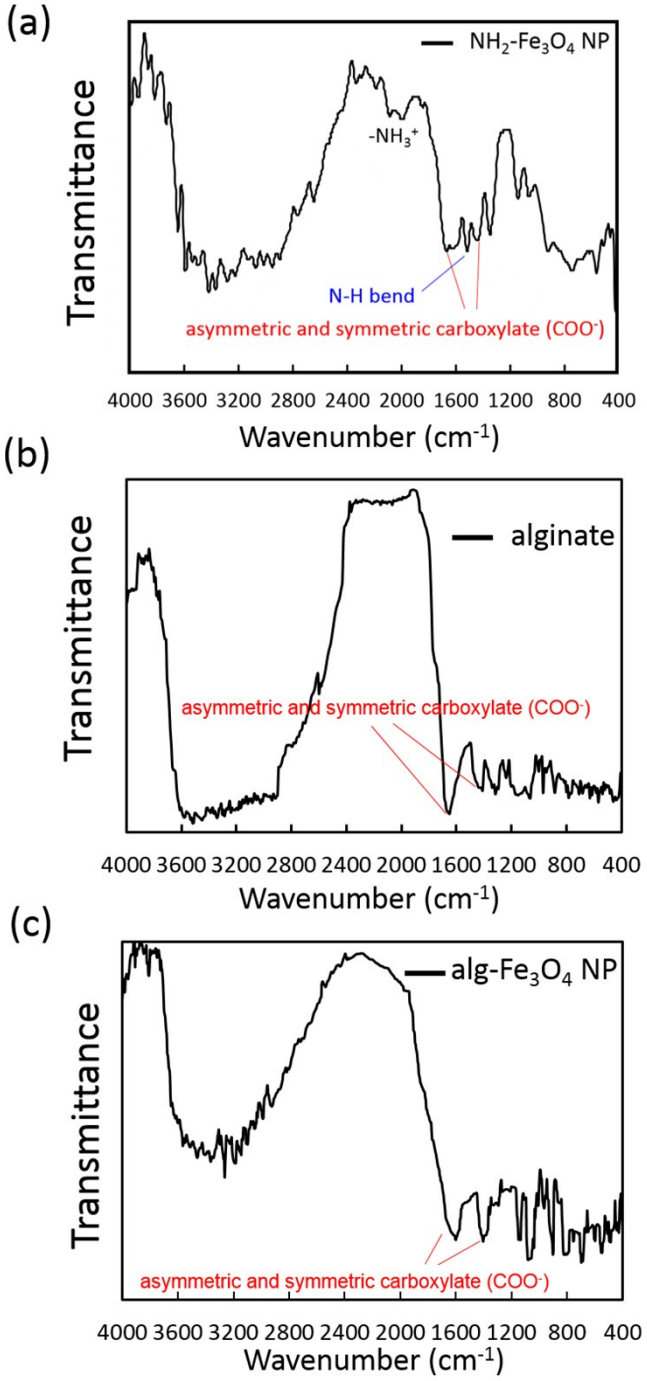

Figure S2. FT-IR spectra obtained from (a) Fe_3O_4 NPs, (b) alginate, and (c) alg-Fe_3O_4 NPs. The appearance of characteristic groups of Fe_3O_4 NPs and alginate observed in alg-Fe_3O_4 NPs. The amine groups of NH_2-Fe_3O_4 NPs formed amide bonds with carboxylate groups of alginate through carbodiimide (EDC) chemistry. The disappearance of $–NH_3^+$ and N-H bend of NH_2-Fe_3O_4 NPs and the characterized peaks of alginate and alg-Fe_3O_4 NPs in FT-IR support the successful conjugation of alginate on NH_2-Fe_3O_4 NPs.

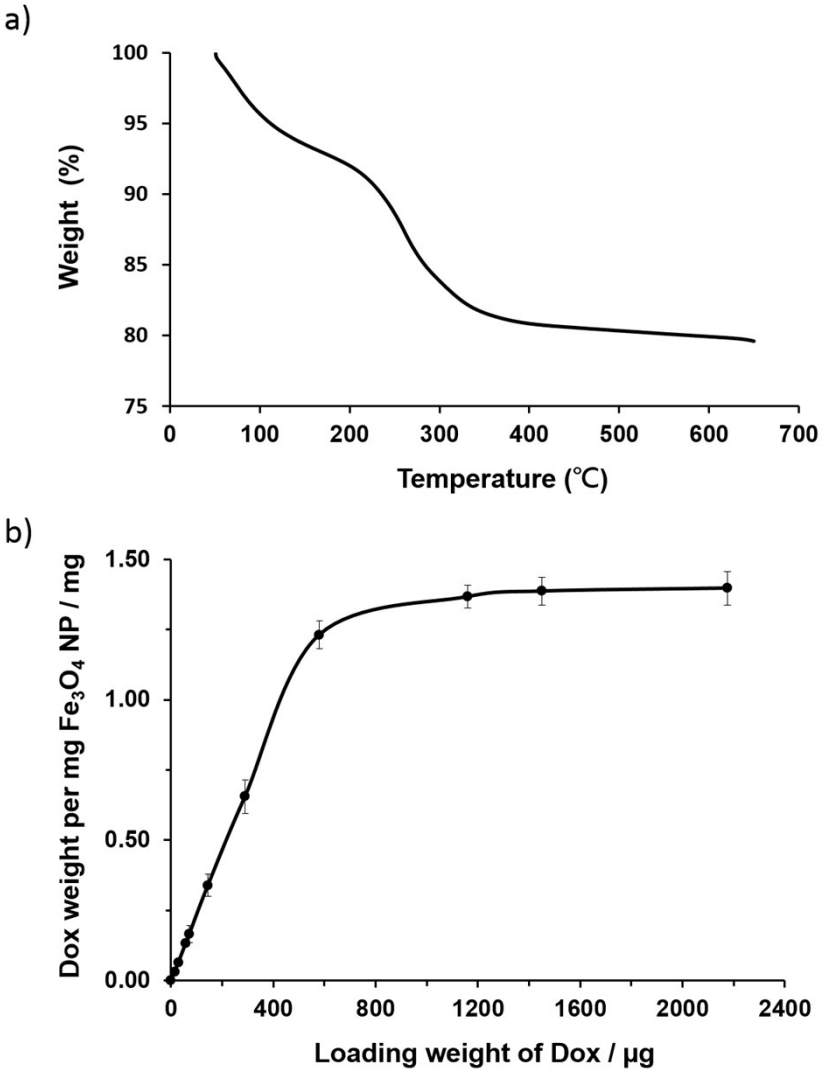

Figure S3. a) Thermogravimetric analysis (TGA) curve of alg-Fe_3O_4 NPs. The weight loss of alg-Fe_3O_4 NPs was about 12.1%. The alginate content of alg-Fe_3O_4 NPs was calculated as 0.15 mg alginate per mg of Fe3O4 NPs. b) The Dox encapsulation efficiency of alg-Fe_3O_4 NPs with expression by Dox weight per mg of Fe_3O_4 NPs. The amounts of alg-Fe_3O_4 NPs in all tests were fixed at 1 nmole of Fe_3O_4 NPs (equal to 414.12 μg of Fe_3O_4 NPs), and the crosslink of alginate in all tests used 1 mM of Ca^{2+} aqueous solution. The saturated encapsulation amounts is ~1.42 mg Dox per mg Fe_3O_4 NPs.

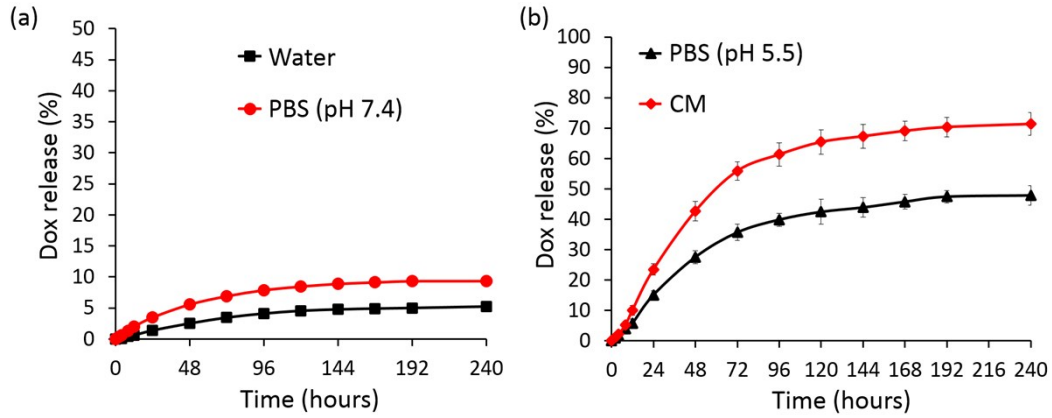

Figure S4. (a) Time-dependent stabilities of Dox in Dox/alg-Fe_3O_4 NPs. A 0.05 μM (particle concentration) of Dox/alg-Fe_3O_4 NPs were distributed in deionized water and PBS (10 mM, pH 7.4) at 37°C, and the cumulative release of Dox was monitored over time. (b) Dox release profile in Dox/alg-Fe_3O_4 NPs. The samples with 0.01 μM (particle concentration) of Dox/alg-Fe_3O_4 NPs were distributed in PBS (pH 5.5) and cytoplasm mimicking (CM) buffer at 37°C, and the cumulative release of Dox was monitored over time. All experiments were repeated in triplicate. All data are averages of three results.

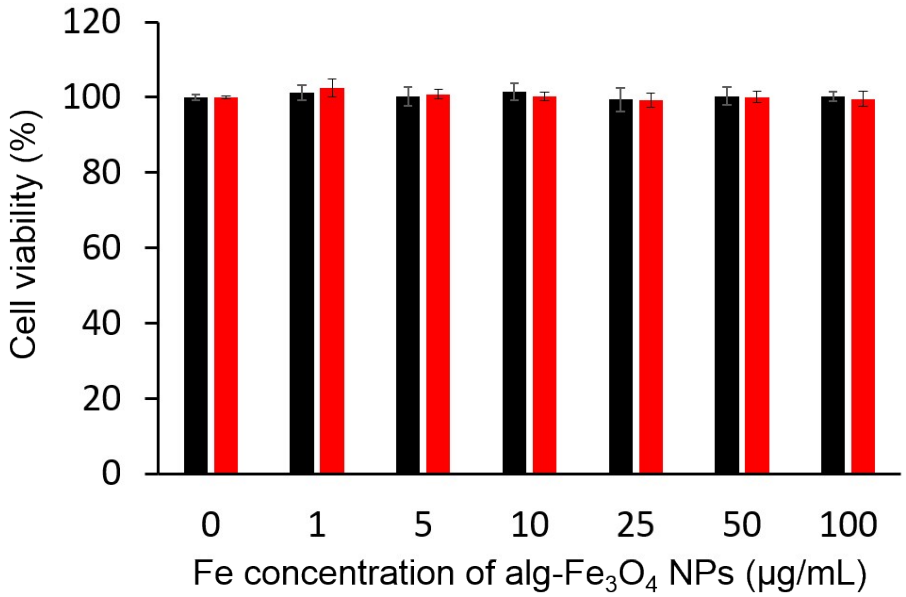

Figure S5. Iron-dose-dependent cytotoxicity of alg-Fe_3O_4 NPs after incubation with HUVEC cells at 37°C for 24 and 48 hours. All experiments were repeated in triplicate. All data are averages of three results.

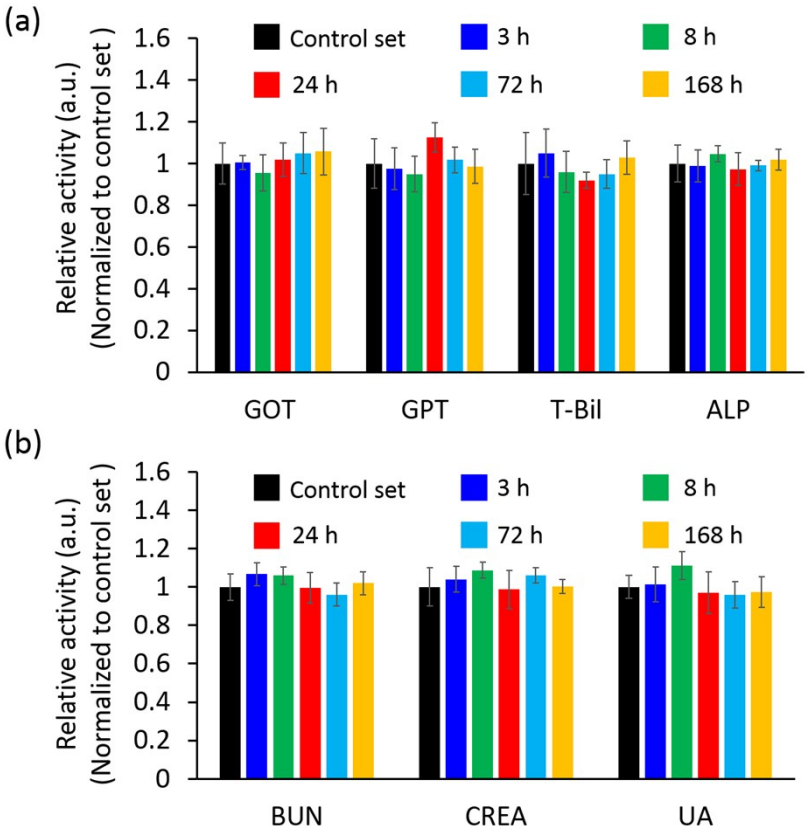

Figure S6. (a) The activity levels of liver enzymes (GOT, GPT, Ti-Bil, and ALP) of mice treated with alg-Fe_3O_4 NPs. (b) The activity levels of kidney enzymes (BUN, CREA, and UA) of mice treated with alg-Fe_3O_4 NPs. The enzyme activities of mice in were measured at 3 h, 8 h, 24 h, 72 h, and 168 h. The mice were treated with alg-Fe_3O_4 NPs by tail vein injection. The injected Fe dose was 5 mg/Kg of body weight. The mice of control set were injected with PBS. All measured enzyme activities were normalized to the enzyme activity of control set.

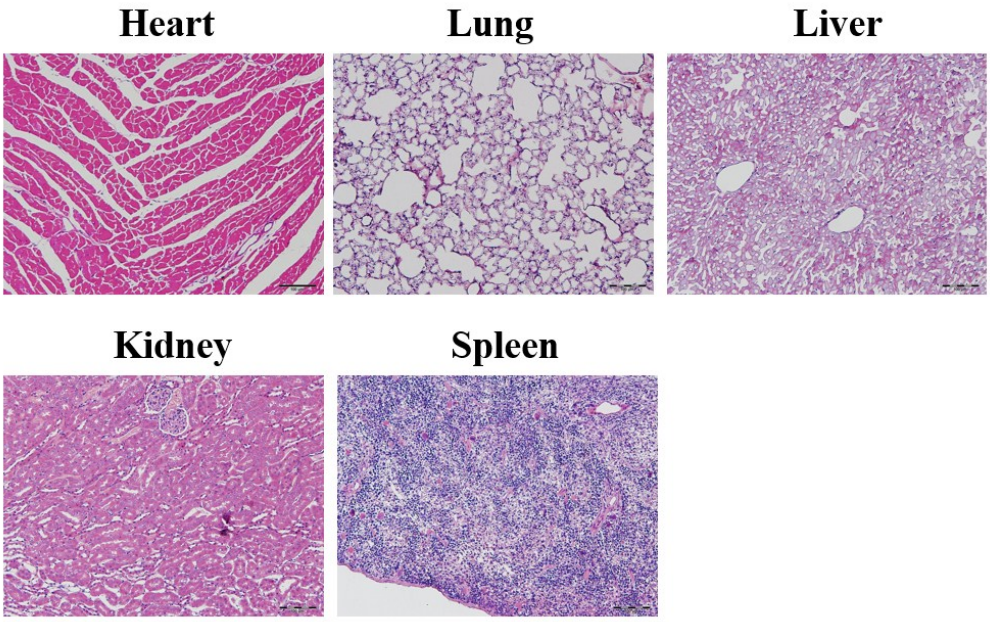

Figure S7. Histological studies of mouse heart, lung, liver, kidney, and spleen after tail vein injection of alg-Fe_3O_4 NPs for 24 hours. The injected Fe dose was 5 mg/Kg of body weight. (magnification ×20).

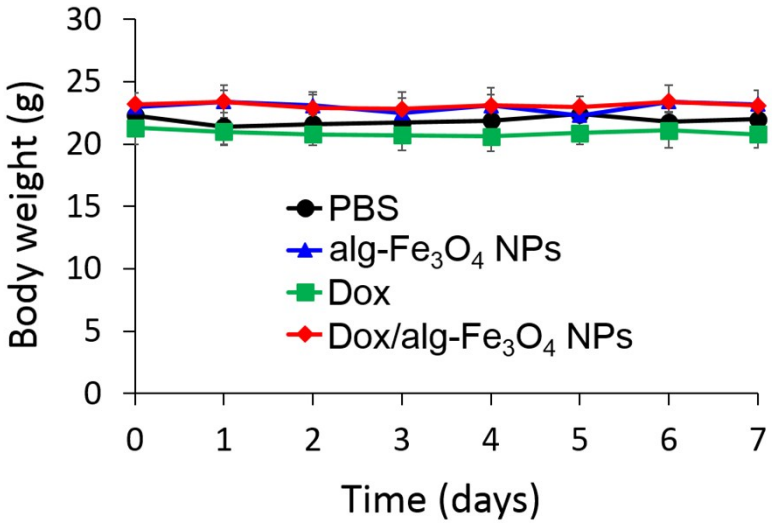

Figure S8. The body weights of C6-tumor-bearing mice during the experimental period. The tumor volume and body weight of mice in all groups were measured every day. The mice were intratumorally injected with PBS (10 mM, pH 7.4), alg-Fe_3O_4 NPs, Dox only, or Dox/alg-Fe_3O_4 NPs. The injected Dox dose was 3 mg/Kg of body weight, and the equivalently injected Fe dose was 5 mg/Kg of body weight.

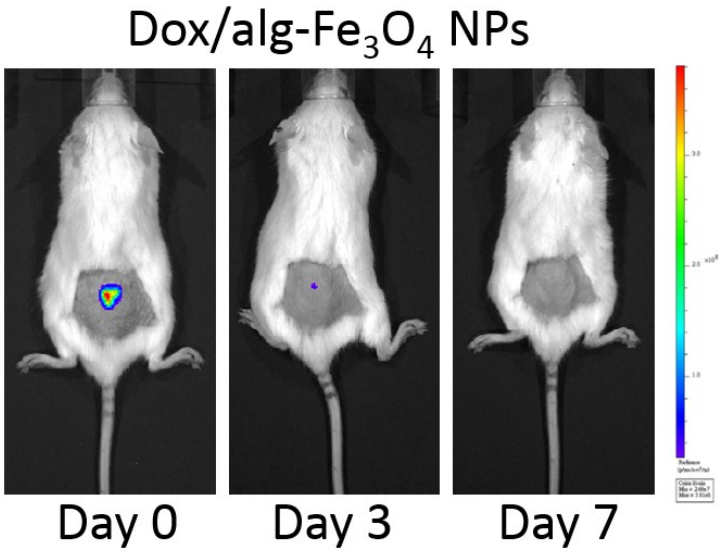

Figure S9. *In vivo* anti-tumor activity of Dox/alg-Fe_3O_4 NPs in mice with ~25 mm^3 tumor during the experimental period. All images are luminescence images of U87MG-luc2 cells monitored using the IVIS imaging system. The mice were intratumorally injected with Dox/alg-Fe_3O_4 NPs. The injected Dox dose was 3 mg/Kg of body weight and the injected Fe dose was 5 mg/Kg of body weight.