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Evaluation of Blood-Brain Barrier-Stealth Nanocomposites for *in situ* Glioblastoma Theranostic Applications

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Experimental Details

Preparing NH_2-Fe_3O_4 nanoparticles

Magnetite nanoparticles (NPs) were prepared 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 separately dissolving iron salts in 2 M HCl solutions. 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 organic acid dissolved in 1 mL of deionized water) was added to the 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 to remove excess salt in the suspension. Subsequently, 3 g of organic acid 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 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) was 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 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. 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 solution with various Ca^{2+} concentrations (0, 0.1, 1, and 10 mM). 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 the residue in the 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 from 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 treated with different concentrations of Ca^{2+} were separately 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 compositions of cytoplasm mimicking (CM) buffer are 120 mM KCl, 0.15 mM $CaCl_2$, 10 mM K_2HPO_4/KH_2PO_4, 25 mM (4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid) HEPES, 2 mM ethylene-bis(oxyethylenenitrilo) tetraacetic acid (EGTA), 5 mM $MgCl_2$, and 5 mM glutathione. The released Dox was collected from the supernatants and its concentration was measured using a linear calibration curve by measuring the fluorescence emission of the Dox at 585 nm. Based on the results of these tests, 1-mM Ca^{2+}-treated Dox/alg-Fe_3O_4 NPs were used for additional *in vitro* and *in vivo* experiments.

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 nitric acid (HNO_3) and hydrochloric acid (HCl). The Fe ion concentration for each nanomaterial was measured on the spectroanalyzer using inductively coupled plasma (ICP) analysis.

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 incubated with 1-mM Ca^{2+}-treated 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 cultures were incubated for 4 hours to allow formazan dye to form. The crystals were dissolved in dimethylsulfoxide (DMSO) (200 μL), incubated in the dark for 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, 1-mM Ca^{2+}-treated alg-Fe_3O_4 NPs, or 1-mM Ca^{2+}-treated 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 corresponding to Dox concentrations of 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 1-mM Ca^{2+}-treated alg-Fe_3O_4 NPs and Dox/alg-Fe_3O_4 NPs). After another 24 and 48 hours of incubation, cytotoxicity was measured by MTT assay following the same steps as above described.

Preparing G23 peptide-conjugated alg-Fe_3O_4 (G23-alg-Fe_3O_4) NPs

To evaluate the BBB-permeating efficacy of G23-alg-Fe_3O_4 NPs (G23 sequence: HLNILSTLWKYRC) using an *in vitro* test, G23-alg-Fe_3O_4 NPs were prepared by mixing 100 μL of 1-mM Ca^{2+}-treated alg-Fe_3O_4 NPs (particle concentration: 10 μM), 100 μL of G23 (0.5 mM), and 50 nmole of EDC at 4°C for 2 hours. The precipitates were collected using a permanent magnet. The supernatant was also collected in a different Eppendorf tube, into which deionized water was added to wash the precipitates. The precipitates (G23-alg-Fe_3O_4 NPs) were redispersed in deionized water or PBS for further use. For preparation of random sequence peptide (LTCNLHKYSRWL)-conjugated Fe_3O_4 NPs (RS-alg-Fe_3O_4 NPs), random sequence peptides were used to substitute G23 peptides and followed same processes of G23-alg-Fe_3O_4 NPs.

To calculate the number of conjugated G23 peptides on the surface of the G23-alg-Fe_3O_4 NPs, FITC-conjugated N-terminal G23 peptides (FITC-G23) were used to substitute G23 peptides in the above experimental steps. The concentration of FITC-G23 in the supernatants was calculated based on their fluorescence intensity by measuring the fluorescence emission of the fluorescein isothiocyanate (FITC) at 520 nm. The average number of FITC-G23 on a G23-alg-Fe_3O_4 NP was calculated to be about 23.6.

Cell viability of bEnd3 cells tested using an MTT assay

bEnd3 cells (mouse brain endothelial cell line) were used for our *in vitro* BBB model. Before the *in vitro* BBB-permeating experiments, the cytotoxicity of G23-alg-Fe_3O_4 NPs was tested first to prove that G23-alg-Fe_3O_4 NPs had not damaged the bEnd3 cells. The bEnd3 cells were seeded at a density of 5×10^3 cells per well in 96-well plates for 24 h, and then incubated with 1-mM Ca^{2+}-treated G23-alg-Fe_3O_4 NPs with different concentrations of iron (0, 1, 5, 10, 25, 50, and 100 μg/mL). After another 24 hours of incubation, the cytotoxicity analysis was measured by MTT assay using the same steps described above. In parallel, the same procedures were also applied for the control experiment based on the alg-Fe_3O_4 NPs with the same Fe concentrations.

***In vitro* BBB-stealth test of G23-alg-Fe_3O_4 NPs**

To construct the BBB *in vitro* model, we used the methods described by Ni [2] and Liu.[3] bEnd3 cells were seeded at a density of 2×10^5 cells/cm^2 on Transwell permeable inserts (0.4-μm pore size, 12 mm in diameter; Corning Incorporated, Corning, NY, USA) and cultured in Dulbecco's modified Eagle's medium (DMEM). After 4-5 days, the cell monolayer integrity developed and the *in vitro* transfer

experiments were performed. G23-alg-Fe_3O_4 NPs that diluted with DMEM were added into the apical (AP) side chamber and the final Fe concentration was 10 μg/mL. After incubation for another 24 hours, the mediums in the AP and basolateral (BL) side chambers were separately collected and then the Fe concentrations were determined by ICP. The transcytosis percentage could be calculated by comparing with the Fe content in AP, BL, and the loading G23-alg-Fe_3O_4 NPs. The same procedures and alg-Fe_3O_4 NPs with the same Fe concentrations were also used for the control experiment.

Middle cerebral artery occlusion (MCAO)

Age of 6-8 weeks male C57B6 mice were anesthetized by using a Small Animal Ventilator (MatrxTM VIP3000, Midmark, Inc., USA) with positive-pressure ventilation (0.2mL/sec) two percent of isoflurane and mixed with oxygen. The right eye to ear area then were shaved, and the mice were placed in a prone position on a warming pad at 37 °C and continuing incubated with two percent of isoflurane until whole procedures were done. Anesthetized mice were incised a 1 cm cut on rat scalp at the midpoint between the right eye and right ear. The temporalis muscle was separated to expose the zygoma and sequamosal bone. An approximate 25-mm^2 window was made with dental drill 1 mm rostal to the anterior junction of the zygoma and the sequamosal bone. The dura mater was carefully pierced with microsurgical tweezers. The exposed middle cerebral artery was carefully isolated and ligated by Aaron 940 High Frequency Desiccator (Bovie Medical Corp.) to induce ischemic stroke in cortex of right hemisphere.

Evans blue staining for evaluating BBB integrity

A 2% solution of evans blue in PBS was injected the tail intravenous of SCID mice (sham control (PBS), treated with G23-alg-Fe_3O_4 nanocomposites, tumor-breaing mice, and stroke mice. The stain was allowed to circulate for 1 hours, and then the brain and organ tissue was removed. Observe the staining imaging using Leica M80 and Leica DFC 295. The injection concentration of G23-alg-Fe_3O_4 NPs was 10 mg Fe/kg.

Establishment of brain tumor-bearing animal model

All animals were received humane care in compliance with the institution's guidelines for maintenance and use of laboratory animals in research. All of the experimental protocols involving live animals were reviewed and approved by National Cheng Kung University Laboratory Animal Center and the Animal Experimentation Committee of Chang Gung Memorial Hospital Laboratory Animal

Center.

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 then w erecounted on a hemacytometer slide and resuspended in serum-free, antibiotic-free normal saline before they were injected into 4-to-6-week-old NOD-SCID mice (25-30 g). A sterile environment was maintained throughout the surgery. Animals were anesthetized with 2% isoflurane and body temperature are maintained using a heating pad, and the head of the animals were fixed to a stereotaxic head holder (Kopf, Tujunga, CA). The surgical incision site was shaved. Povidine-Iodine Swab Sticks were used to wash the area. A 5μl Hamilton syringe were used, and he coordinates were used 2 mm right to the bregma, 1 mm anterior to the coronal suture, and 3 mm below the dorsal surface of cerebellum. 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, and were infused over 3 minutes to a volume of 2 μL. After injection, the syringe needle was left in place for 2 minutes to prevent leakage from the site of infusion and slowly withdrawn completely. Sterile bone wax was used to the hole and the incision was sutured using a 5-0 suture. After surgery the animals were placed in a warm (37℃) cage for recovered from the anesthesia. When the tumor was near 25~50 mm^3, the tumor-bearing mice were ready for the studies.

***In vivo* magnetic resonance imaging (MRI)**

Adult male BALB/c mice (26 ± 3 g) were purchased from the Experimental Animal Center of the National Science Council, Taiwan. The mice were housed under temperature control (24-25°C) and a 12-hour light-dark cycle (lights on at 07:00) at the Chang Gung Memorial Hospital Laboratory Animal Center which is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Standard laboratory rat chow and tap water were available *ad libitum*.

To attain the required Fe concentration for MRI, the Dox content of Dox/alg-Fe_3O_4 NPs was adjusted to 0.43 mg per mg of Fe_3O_4 NP for animal tests. The mice were anesthetized using 2% isoflurane (Abbott Laboratories, Abbott Park, IL, USA) mixed with 100% O_2 delivered using a veterinary anesthesia delivery system (ADS 1000; Engler Engineering, Hialeah, FL, USA) and then injected via the tail vein with alg-Fe_3O_4 or G23-alg-Fe_3O_4 NPs (10 mg [Fe]/kg). MRI was then performed in a 9.4T horizontal-bore animal MR scanning system (Biospec 94/20; Bruker, Ettingen, Germany) to evaluate the contrast enhancement and BBB penetration of alg-Fe_3O_4 and G23-alg-Fe_3O_4 NPs in the brain region.

The scanning system is made up of a self-shielded magnet, with a 20-cm clear bore and a gradient that offers a maximal gradient strength of 675 mT m^{-1} and a minimum slew rate of 4673 $Tm^{-1}s^{-1}$. It is equipped with a mouse brain receiver-only coil array that uses a circular polarized transmit-only coil to detect signals from the mouse head. We first used a multislice turbo rapid acquisition with a refocusing echo (Turbo-RARE) sequence to record high-resolution T_2-weighted coronal anatomical images. The parameters used were: field of view = 20.0 mm × 20.0 mm; matrix dimension = 384 × 384 pixels; spatial resolution = 52.0 μm × 52.0 μm; slice thickness = 500 μm; interslice distance = 500 μm; echo time = 9.4 milliseconds; effective echo time = 17.8 milliseconds; repetition time = 3500 ms; rare factor = 6; refocusing flip angle = 180°; number of averages = 8; total acquisition time = 29 minutes 52 seconds.

T_2-weighted axial anatomical reference imaging was performed on adjacent slices that covered the whole brain, again using Turbo-RARE sequence acquisition: field of view = 20.0 mm × 20.0 mm; matrix dimension = 384 × 384 pixels; spatial resolution/pixel = 52.0 μm × 52.0 μm; slice thickness = 500 μm; interslice distance = 500 μm; echo time = 9.4 milliseconds; effective echo time = 17.8 milliseconds; repetition time = 3500 milliseconds; rare factor = 6; refocusing flip angle = 180°; number of averages = 8; total acquisition time = 29 minutes 52 seconds. The MR imaging signal intensities were measured using Image J 1.49p software.

Iron staining the mouse brain

The paraformaldehyde-fixed paraffin-embedded brains were cut into 3-4 μm slices on positively charged slides, and the sections were dried in an oven for 1 hour at 65°C. Furthermore, all slides were de-paraffinized and the tissue was hydrated with deionized water. The working iron solution was prepared by mixing equal volumes of potassium ferrocyanide solution and hydrochloric acid solution (HT20; Sigma-Aldrich). The slides were treated with working iron solution for 10 minutes, washed with deionized water 3 times, and then treated with working pararosaniline solution for 2 minutes. A working pararosaniline solution was prepared by adding 1 ml of pararosaniline solution to 5 ml of water. The slices were then washed with deionized water for 2 minutes and rapidly dehydrated using alcohol and xylene. Finally, the slices were mounted with a coverslip and images were acquired using a microscope (Olympus BX51; Yuan Li Instrument Co., Taipei, Taiwan)

IVIS Spectrum *in vivo* pre-clinical imaging system

To evaluate the anti-cancer efficacy of Dox/alg-Fe_3O_4 NPs, we used U87MG-luc2 tumor bearing mice (~50 mm^3) divided into 5 treatment groups: controls (PBS), free Dox, Dox/alg-Fe_3O_4 NPs, G23-alg-Fe_3O_4 NPs and G23-Dox/alg-Fe_3O_4 NPs. The

mice were intravenously 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 and (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 in each group). 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

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2. D. Ni, J. Zhang, W. Bu, H. Xing, F. Han, Q. Xiao, Z. Yao, F. Chen, Q. He, J. Liu, S. Zhang, W. Fan, L. Zhou, W. Peng, J. Shi, *ACS Nano* **2014**, *8*, 1231-1242.
3. D. Liu, B. Lin, W. Shao, Z. Zhu, T. Ji, C. Yang, *ACS Appl. Mater. Interfaces* **2014**, *6*, 2131-2136.

FIGURES AND FIGURE LEGENDS

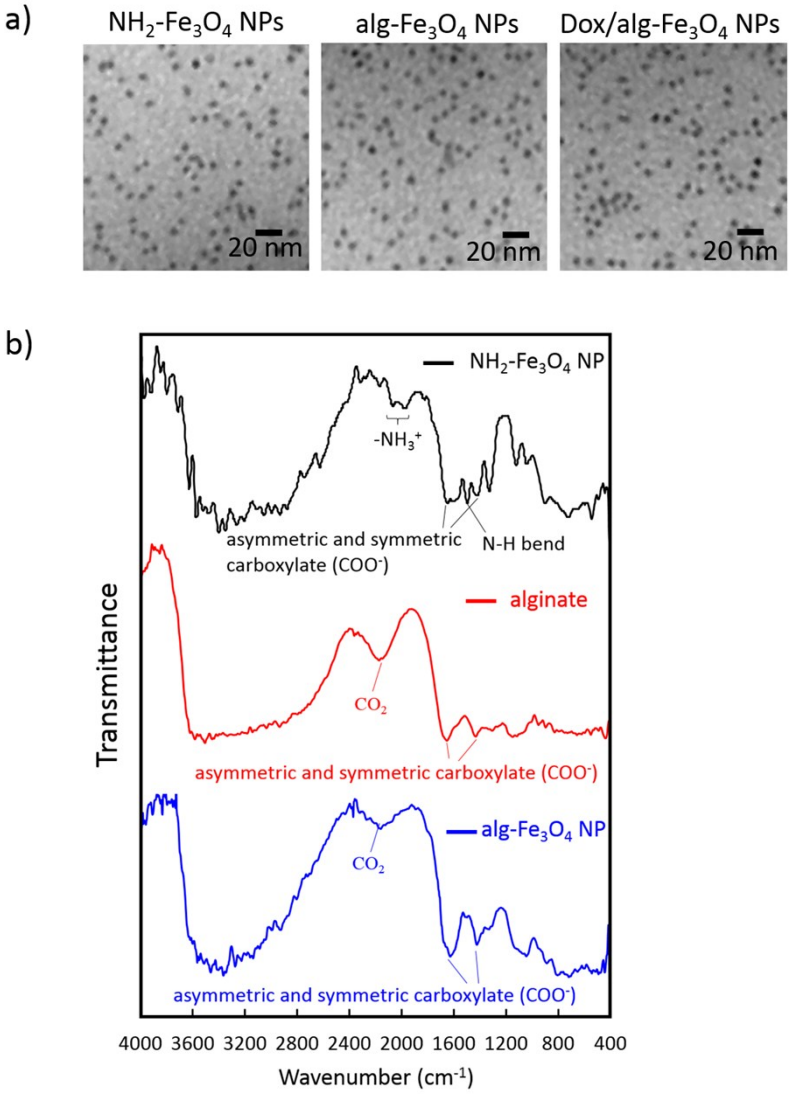

Figure S1. a) TEM images of NH_2-Fe_3O_4 NPs and alg-Fe_3O_4 NPs. b) FT-IR spectra obtained from Fe_3O_4 NPs, alginate, and alg-Fe_3O_4 NPs. The appearance of characteristic groups of Fe_3O_4 NPs and alginate observed in alg-Fe_3O_4 NPs supports the successful conjugation of alginate on NH_2-Fe_3O_4 NPs. FT-IR spectra obtained from NH_2-Fe_3O_4 NPs, alginate, and alg-Fe_3O_4 NPs. The alg-Fe_3O_4 NPs were prepared by the subsequent modification with alginate. The amine groups of NH_2-Fe_3O_4 NPs formed amide bonds with carboxylate groups of alginate through carbodiimide 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.

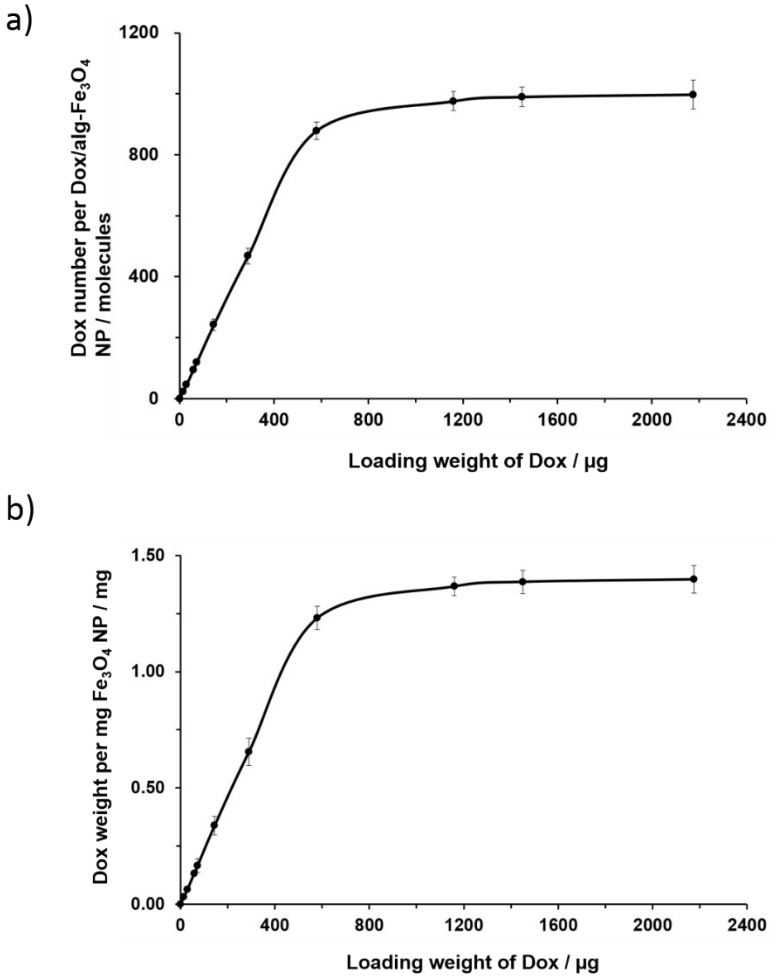

Figure S2. The Dox encapsulation efficiency of alg-Fe_3O_4 NPs with expression by a) Dox number per Dox/alg-Fe_3O_4 NP and b) Dox weight per mg of Fe_3O_4 NPs. The amounts of alg-Fe_3O_4 NPs in all tests were fixed at 1 nmole of Fe_3O_4 NPs (equal to 414.12 μg of Fe_3O_4 NPs), and the crosslink of alginate in all tests used 1 mM of Ca^{2+} aqueous solution. The saturated encapsulation amounts of Dox of alg-Fe_3O_4 NPs were ~574 Dox per Dox/alg-Fe_3O_4 NP and ~1.39 mg Dox per mg Fe_3O_4 NP.

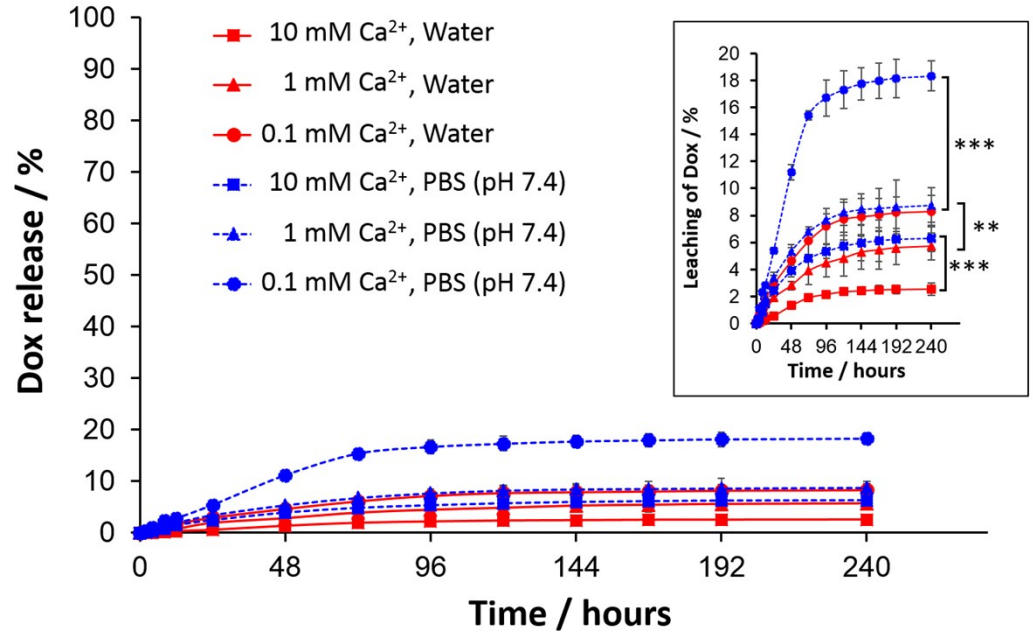

Figure S3. Time-dependent stabilities of Dox in Dox/alg-Fe_3O_4 NPs crosslinked using Ca^{2+} aqueous solutions: 0.1 (●), 1 (▲), and 10 mM (■). A 0.05 μM (Fe_3O_4 particle concentration) of Dox/alg-Fe_3O_4 NPs were distributed in deionized water (solid line, —) and PBS (10 mM, pH 7.4) (dotted line, ---) at 37°C, and the cumulative release of Dox was monitored over time. The inset is an enlargement of the release of Dox between 0 and 20%. All experiments were repeated in triplicate. (**, $p < 0.05$; ***, $p < 0.001$)

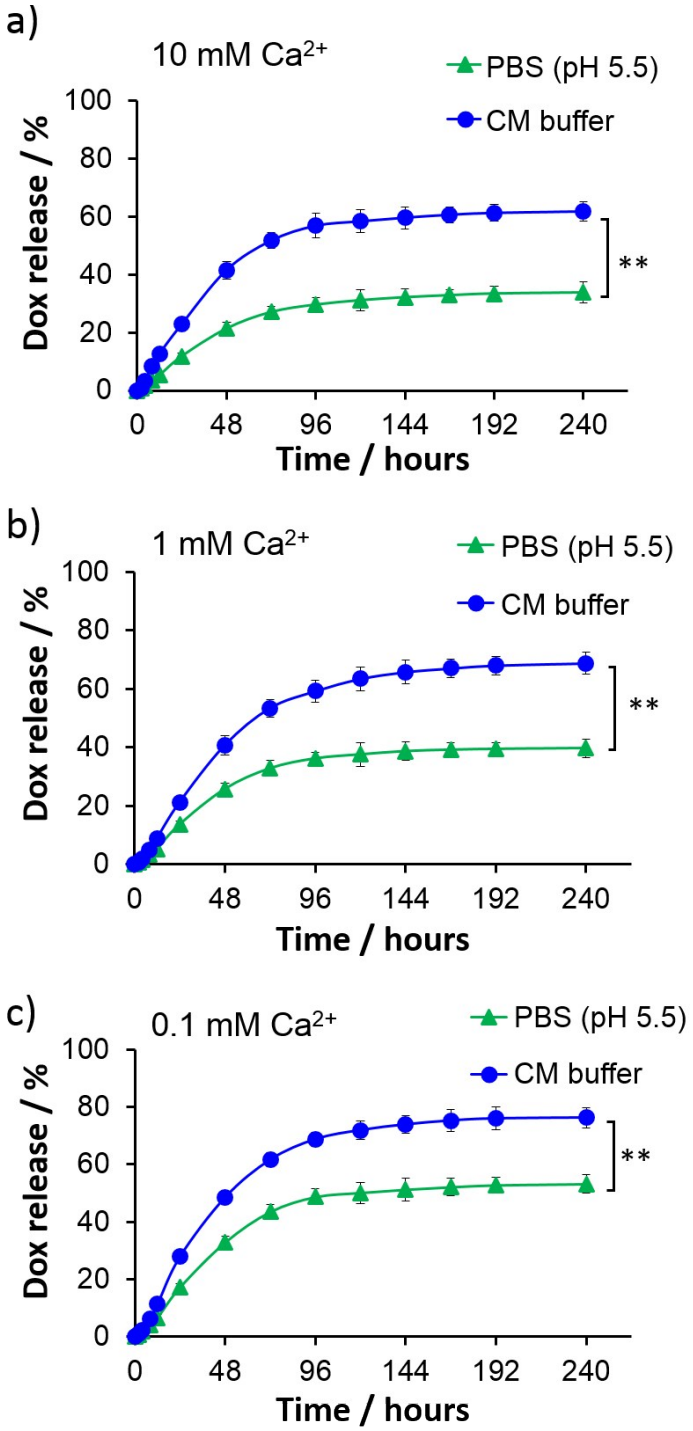

Figure S4. Dox release profile in Dox/alg-Fe_3O_4 NPs prepared under different crosslink conditions of Ca^{2+} aqueous solutions: a) 10 mM, b) 1 mM, and c) 0.1 mM. The samples with 0.01 μM (Fe_3O_4 particle concentration) of Dox/alg-Fe_3O_4 NPs were distributed in PBS (pH 5.5) and cytoplasm mimicking (CM) buffer at 37°C, and the cumulative release of Dox was monitored over time. All experiments were repeated in triplicate. (**, $p < 0.01$)

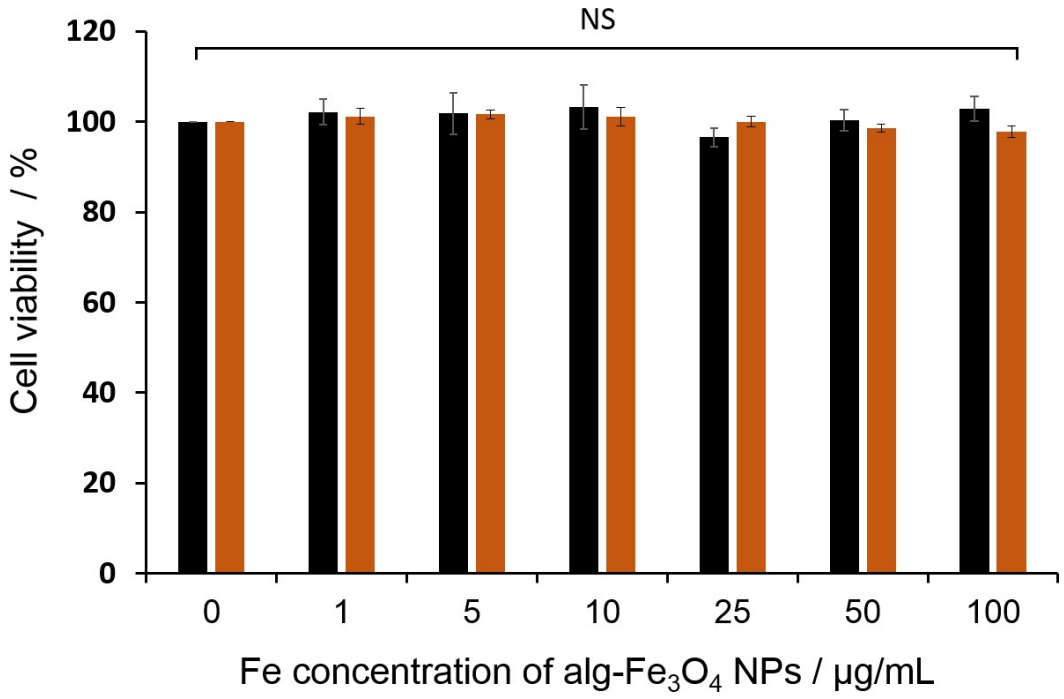

Figure S5. Iron-dose-dependent cytotoxicity of alg-Fe_3O_4 NPs crosslinked using 1 mM of Ca^{2+} after incubation with HUVEC cells at 37°C for 24 and 48 hours. All experiments were repeated in triplicate. All data are averages of three results. (NS, not significant)

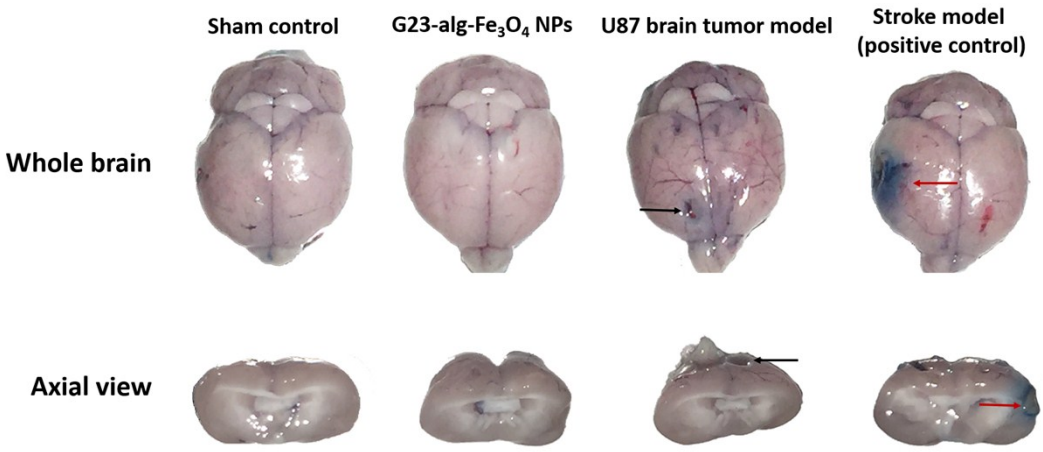

Figure S6. Evans blue staining for evaluating BBB integrity of PBS-treated mice (sham control), G23-alg-Fe_3O_4 NP-treated mice, U87MG-luc2-tumor baring mice, and stroke model (positive control). Black arrows represented the tumor site of whole brain of U87MG-luc2 tumor baring mice. Red arrows represented the infarct area of stroke of stroke model. No damage of the BBB was found in sham control, G23-alg-Fe_3O_4 NP-treated mice, or U87MG-luc2-tumor baring mice. Only brain of stroke model was stained with evans blue, representing the BBB had a damage. The injection concentration of G23-alg-Fe_3O_4 NPs was 10 mg Fe/kg.

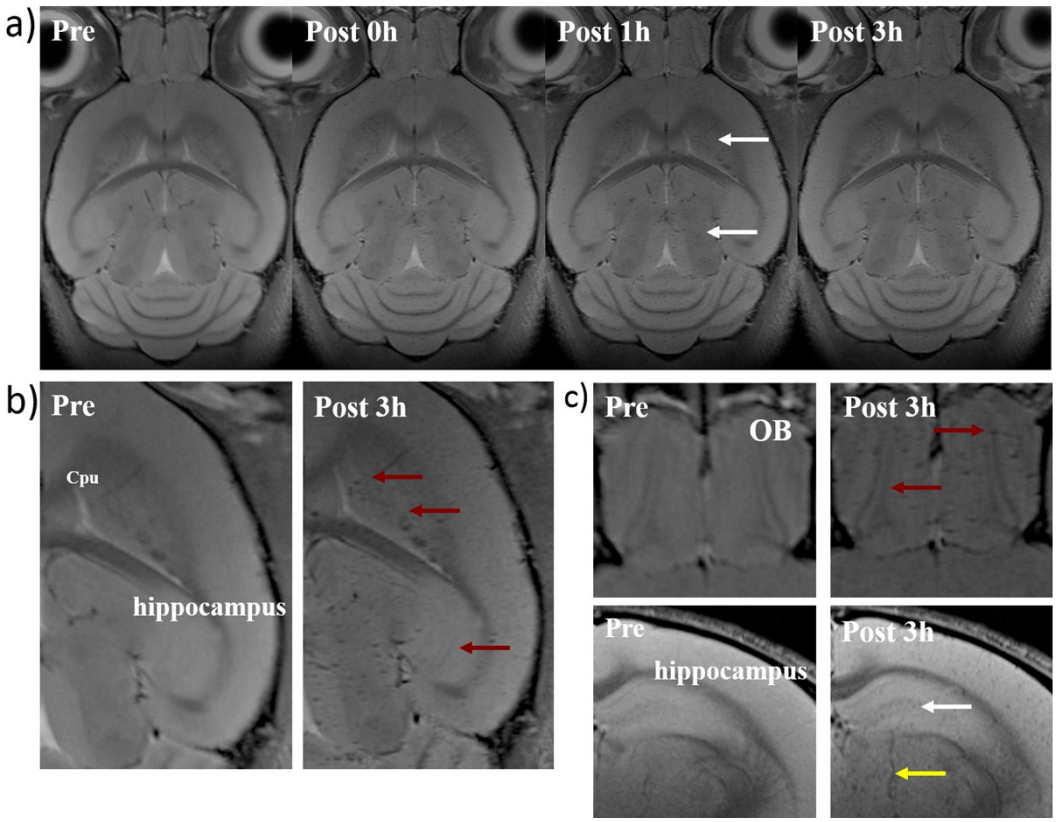

Figure S7. a) *In vivo* mouse-brain T2-weighted MRIs acquired pre-injection and 0 h, 1 h, and 3 h after they had been treated with G23-alg-Fe_3O_4 NPs with 10 mg Fe/kg. The white arrows point to where the G23-alg-Fe_3O_4 NPs enhanced the image (made it darker) of the brain regions. b) G23-alg-Fe_3O_4 NPs enhanced the image of brain regions of the nucleus 3 h post-treatment. The red arrows highlight the caudate putamen (Cpu) and hippocampus. c) G23-alg-Fe_3O_4 NPs also enhanced the image of the fine structure of the olfactory bulb (OB) (red arrows) and hippocampus (white arrow) 3 h post-treatment. In addition, the blood vessels were highlighted because of the G23-alg-Fe_3O_4 NPs (yellow arrow).

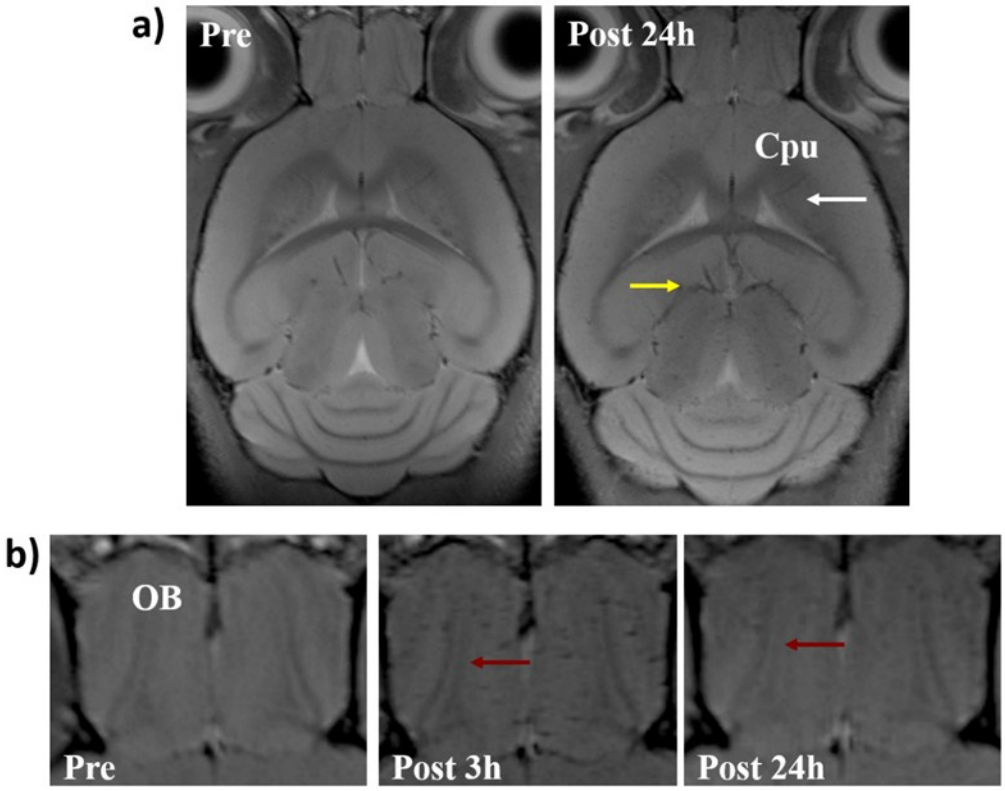

Figure S8. Coronal T2-weighted images of mouse brains captured pre- and post-injection of G23-alg-Fe_3O_4 NPs at 10 mg Fe/kg dosage. a) The caudate putamen (Cpu) was still contrast-enhanced 24 hours post-injection (white arrow), and the blood vessels were highlighted at the same time (yellow arrow). b) The fine structure of the olfactory bulb (OB) (red arrows) was contrast-enhanced at 3 hours post-injection, and the imaging contrast was slightly recovered at 24 hours post-injection.

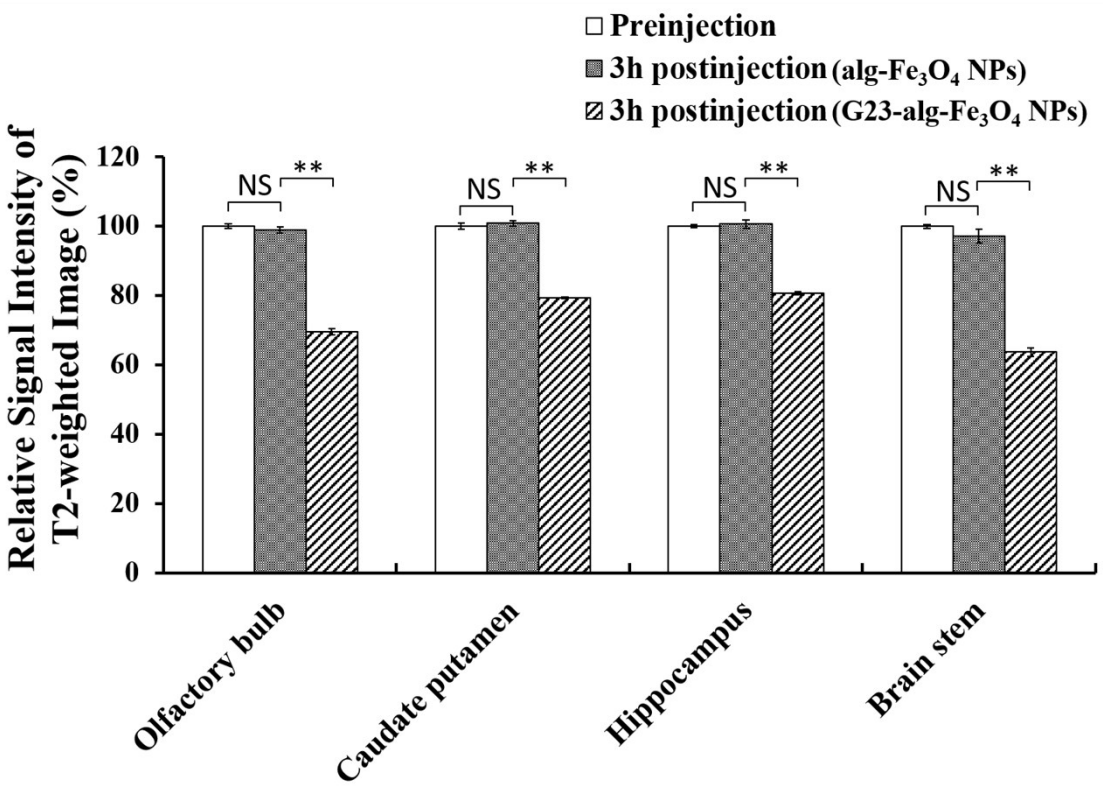

Figure S9. Relative signal intensity of T2-weighted MRIs of different brain nuclei and regions extracted from T2 images of BALB/c mouse brains pre- and post-injection of a 10 mg Fe/kg dose of alg-Fe_3O_4 or G23-alg-Fe_3O_4 NPs. (**, $p < 0.01$; NS, not significant)

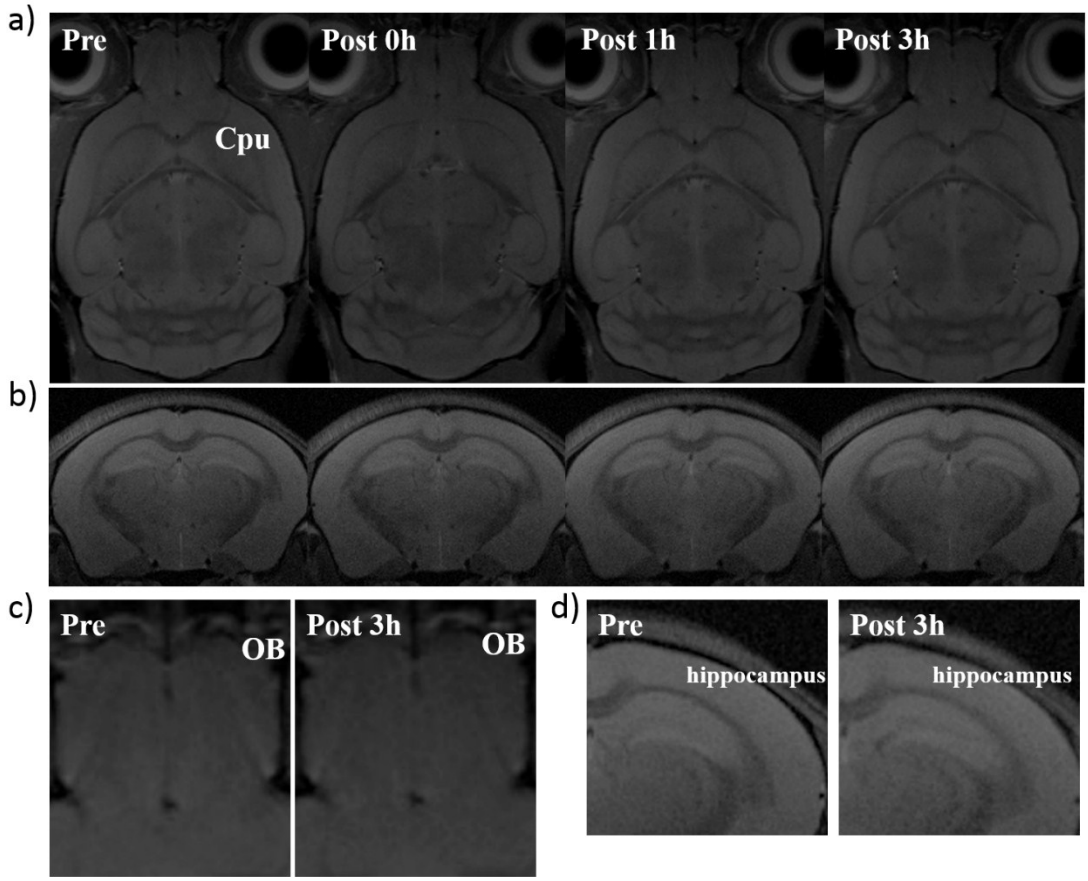

Figure S10. a) Coronal and b) axial T2-weighted images of mouse brains captured pre- and post-injection of alg-Fe_3O_4 NPs at 10 mg Fe/kg. The fine structure images of the c) olfactory bulb (OB) and d) hippocampus acquired pre- and 3 hours post-injection. Alg-Fe_3O_4 NPs did not penetrate the BBB, and the NPs did not contrast-enhance the mouse-brain T2-weighted images.

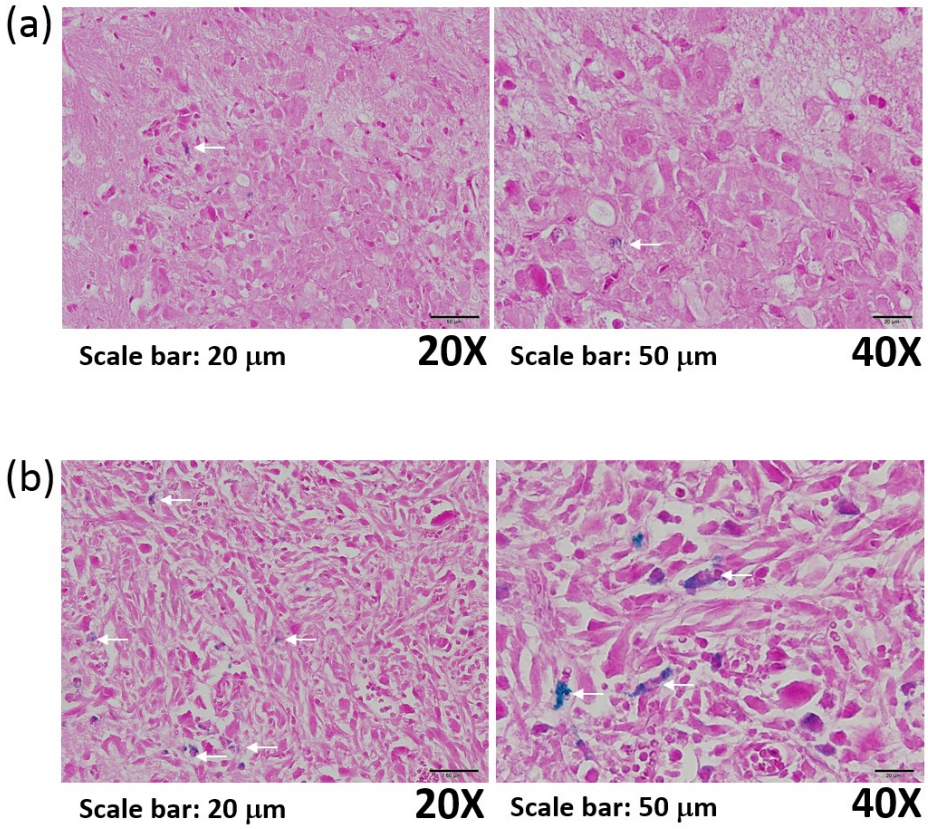

Figure S11. Mouse brain tumor tissue micrographs taken for histological and histochemical analysis 24 hours after intravenously injection of (a) alg-Fe_3O_4 or (b) G23-alg-Fe_3O_4 NPs. Pearl's iron stain of the adjacent section shows a diffuse bluish-purple in the brain tumor tissue (white arrows) with significant accumulation in G23-alg-Fe_3O_4 NPs treated brain tissue.

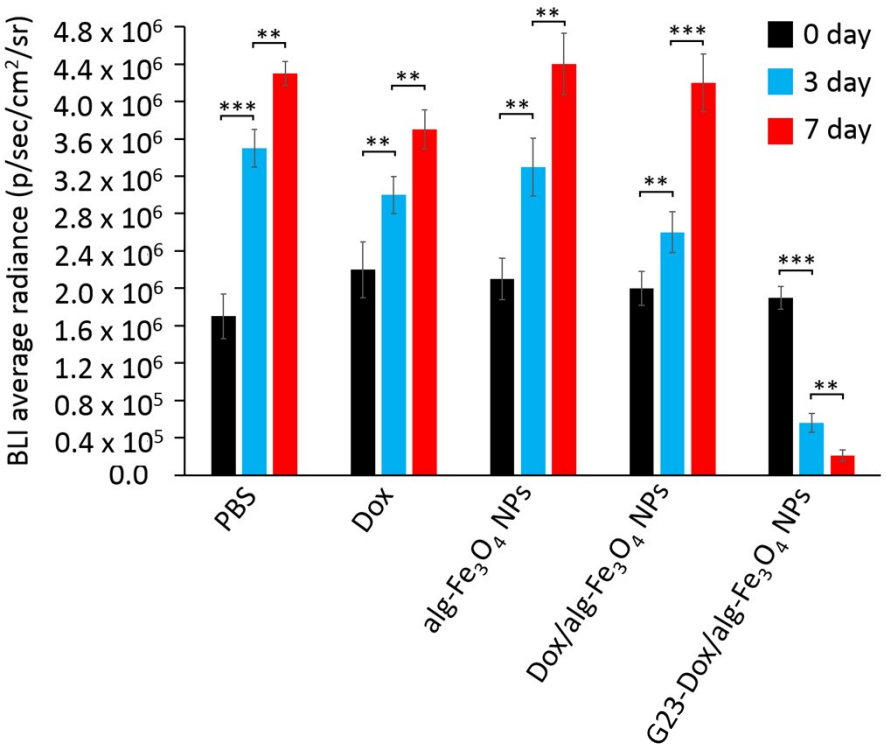

Figure S12. Bioluminescent signal quantification of U87MG-luc2 tumor bearing mice before (0 day) and after treatment with PBS, Dox, alg-Fe_3O_4 NPs, Dox/alg-Fe_3O_4 NPs, and G23-Dox/alg-Fe_3O_4 NPs for 3 and 7 days. All quantitative analyses focused on tumor area of the IVIS images in figure 4. (**, $p < 0.01$; ***, $p < 0.001$)