Supporting Information

A tailored nanosheet decorated with a metallized dendrimer for angiography and magnetic resonance imaging-guided combined chemotherapy.

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Electronic supplementary information (ESI) available: Experimental details and supporting results.

Experimental details

Materials. All chemical reagents were used as received without further purification. Graphite flakes, DOX, COLC, dendrimer (ethylenediamine core, generation 3.0, 32 amino groups), and DTPA dianhydride were obtained from Sigma-Aldrich Co. (USA). $GdCl_3$, folic acid, FITC, N-hydroxysuccinimide (NHS), N-ethyl-N′- (3-dimethylaminopropyl) carbodiimide (EDC), and amino polyethyleneglycol (PEG-NH_2) were acquired from Aladdin Co. (Shanghai, China). Triethanolamine (TEA) and dimethylsulfoxide (DMSO) were purchased from Sinopharm Co. (Shanghai, China). The CCK-8 assay was obtained from Dojindo (Japan), and an Annexin V-FITC Apoptosis Detection Kit was purchased from BD Biosciences (San Jose, CA, USA). All other analytical grade chemicals were purchased from Sinopharm Group Chemical Reagent Co., Ltd. (Shanghai, China).

Fabrication of TGO. (1) First, GO was prepared by the classical Hummers' method. Briefly, concentrated H_2SO_4 (50mL) and concentrated HNO_3 (20 mL) were mixed and added to graphite flakes (2.0 g). The mixture was then cooled in an ice bath. Subsequently, $KMnO_4$ (6.0 g) was added to resulting solution, and the reaction temperature was kept below 20 °C. Then, the reaction was slowly heated to 35 °C under magnetic stirring for 8 h. Additional $KMnO_4$ (6.0 g) needed to be added to the reaction system, which was then stirred for 12 h at

35 °C. After the reaction mixture was cooled to room temperature, ice water (300 mL) containing 30 % H_2O_2 (5 mL) was poured into the mixture. Next, the obtained mixture was purified using following steps: sifting, centrifugation, and dialysis with multiple washes. The product was tailored via ultrasonication for 1.5 h to obtain TGO nanosheets. Finally, a filtration membrane with 200 nm pores was used to separate GO from TGO.

Fabrication of FA-GCGLD. (1) Dendrimer (G 3.0, 300 mg) was dissolved in sodium bicarbonate buffer (40 mL, 0.1 M, pH 9.5) with DMSO (100 μL) and reacted with DTPA dianhydride (1.0 g) at room temperature for 10 h. To keep the solution pH at 9.5, NaOH solution (0.1 M) was added as needed. The DTPA-grafted dendrimer (dendrimer-DTPA) was dialyzed with multiple washes (3500 Dalton, Millipore Corp.). Purified dendrimer-DTPA was added to citrate buffer (10 mL, 0.1 M, pH 5.6) containing $GdCl_3$ (300 mg) and reacted overnight at 45 °C. Subsequently, unreacted Gd^{3+} was removed by dialysis with multiple washes (3500 Dalton, Millipore Corp). To ensure the thorough removal of free Gd^{3+}, the Gd content of the dialysis fluid was monitored via ICP-OES until no Gd^{3+} was detectable. Next, folic acid was conjugated to the Gd-DTPA-labeled dendrimer via its surface residue amine groups. For this reaction, folic acid was first activated to form NHS-folate as previously described.[1-3] Then, 50 μL of NHS-folate (10 mg/mL in DMSO) was added to sodium bicarbonate buffer (10 mL, 0.1 M, pH 9.5) containing the Gd-DTPA-labeled dendrimer and reacted for 2 h to form FA-GLD. The unreacted NHS-folate was removed using the method described above. (2) GO (10 mg) was dispersed into DMSO (30 mL), and then equivalent amounts of NHS (10 mg) and EDC (10 mg) were added to the above solution at 25 °C for 2 hours to activate the carboxylic groups on the TGO nanosheet. Subsequently, sodium

bicarbonate buffer (35 mL, 0.1 M, pH 9.5) containing different masses of FA-GLD was added to the resulting solution, which was maintained at pH 9.5 with NaOH throughout the reaction time of 10 h. Then, PEG (5 mg/mL, 5 mL) was added into the mixed solution under magnetic stirring for 4 hours. The unreacted FA-GLD and PEG was removed by dialysis with multiple washes (16000 Dalton, Millipore Corp.).

Loading and Release of Drugs. (1) FA-GCGLD (5 mg/mL, 2 mL) was added into a mixed solution (5 mL) of DOX (25 mg) and COLC (25 mg) with shaking (200 rpm) for 24 h. Subsequently, the mixed solution was centrifuged at 15000 rpm for 10 min. The supernatant was collected, and the corresponding concentration was measured via UV-Vis spectroscopy. The drug capacity could be calculated via the following equation: $RR(\%)=(C_{pre}-C_{sup})\times V/M_0\times 100$ %. C_{pre} and C_{sup} were the concentration of the drugs in the original and supernatant solutions, respectively. V was the volume of the solution, and M_0 was the amount of TGO. (2) FA-GCGLD-DOX/COLC (2 mg) was uniformly dispersed into phosphate-buffered saline (PBS, pH 7.4, 10 mL). Subsequently, the mixed solutions were poured into dialysis bags, and the dialysis bags were rapidly immersed in PBS (100 mL) for 40 h. Similarly, for other groups, the pH of medium was adjusted to 5.5, 4.5 or 3.0. Medium was collected at defined time intervals, and the drug content was determined using UV-Vis spectroscopy.

Release Kinetics of Gd ions from FA-GCGLD. FA-GCGLD solution (1 mg/mL, 10 mL) was poured into dialysis bags. Then, the dialysis bags were rapidly immersed into simulated body fluid (pH 7.4, 200 mL) at room temperature with magnetic stirring for 24 h. Subsequently, the pH of the medium was adjusted to 6.5 or 5.5 by adding HCl solution (0.05

M). Medium was collected at defined time intervals, and the Gd content was determined via ICP-OES (ThermoFisher 7200, USA).

Confocal Laser Scanning Microscopy. FITC-labeled FA-GCGLD-DOX/COLC was fabricated by previously described methods.[4] HepG2 cell lines, provided by the Institute of Biochemistry and Cell Biology of Chinese Academy of Sciences, were cultured in DMEM/high glucose medium containing 10% fetal bovine serum, 100 U/mL penicillin G sodium, and 100 μg/mL streptomycin sulfate at 37 ^{o}C and 5% CO_2 in a humidified incubator. HepG2 cells were then seeded on 15 mm glass coverslips, placed in 12-well plates (8×10^4 cells per well), and incubated with FA-GCGLD-DOX/COLC at 20 μg/mL for 1, 6, and 12 h. The cells were washed three times with PBS and fixed in freshly prepared 4 % paraformaldehyde. The cells were imaged using CLSM (Zeiss LSM710 NLO, Germany) and oil immersion lenses at 37 °C under an atmosphere of 5 % CO_2 in a Zeiss image chamber. An excitation energy of 488 nm was used, and the fluorescence emission measured at 500-550 nm. Image data acquisition and processing was performed using Zeiss LSM Image Browser and ImageJ.

Cell TEM Observations. HepG2 cells were seeded on 15 mm glass coverslips, placed in 12-well plates (8×10^4 cells per well) and incubated with FA-GCGLD at 20 μg/mL for 1, 6, and 12 h. Subsequently, the cells were collected and pelleted in tubes. Fresh 3 % glutaraldehyde in 0.1 M phosphate buffer was added to re-suspend the pellet in order to ensure optimal fixation and was left overnight at 4 °C. The specimens were then washed with 0.1 M phosphate buffer at 4 °C twice at 30 min intervals. Secondary fixation was performed in 2 % aqueous osmium tetroxide for 2 hours at room temperature, followed by washing with buffer as above. The

specimens were then dehydrated through a graded series of ethanol at room temperature as follows: 75 % (15 min), 95 % (15 min), 100 % (15 min) and 100 % (15 min). The 100 % ethanol was prepared by drying over anhydrous copper sulfate for 15 min. The specimens were then placed in an intermediate solvent, propylene oxide, for two changes with a duration of 15 min. Resin infiltration was accomplished by placing the specimens in a 50/50 mixture of propylene oxide/Araldite resin and allowing them to sit overnight at room temperature. After being embedded in fresh Araldite resin for 48-72 h at 60 °C, the specimens were left in full strength Araldite resin for 6-8 h at room temperature (with a resin change after 3-4 h). Semi-thin sections with a thickness of approximately 0.5 μm were cut on a Leica ultramicrotome and stained with 1 % toluidine blue in borax. Ultra-thin sections, approximately 70-90 nm thick, were cut on a Leica ultramicrotome and stained for 25 min with saturated aqueous uranyl acetate followed by staining with Reynold's lead citrate for 5 min. The sections were examined using a TEM (FEI, Tecnai, USA) at an accelerating voltage of 80 kV.

Biodistribution of FA-GCGLD In Vivo. The bio-distribution analysis in mice was performed 30 min after the injection of Gd-DTPA, GCGLD, or FA-GCGLD at a dose of 2.0 mg/kg via tail vein. The dissected organs were weighed, homogenized, and treated with a mixture of concentrated nitric acid (70 %) and concentrated hydrochloric acid (30 %) at 45 °C for 24 h. Then, the solutions were centrifuged at 9000 rpm for 10 min, and the supernatant was subjected to ICP-OES. The gadolinium element distribution in different organs was calculated.

Anticancer Activity Investigation. Nude mice bearing liver cancer xenografts were used for the *in vivo* therapeutic studies. When the tumor volumes reached 50 mm^3, the mice were

injected with DOX, GCGLD-DOX/COLC, FA-GCGLD-DOX, FA-GCGLD-COLC, or FA-GCGLD-DOX/COLC at a dose of 2 mg/kg via tail vein three times per week. Control mice were injected with saline buffer. The animal study was performed three times under the same conditions, and the results of three separate experiments were combined to analyze values. Tumor sizes and body weight were measured and recorded three time a week, and mice were monitored daily for potential signs of toxicity. In addition, the volume (V) of the tumor was calculated as $V=a\times b^2/2$, where "a" and "b" were the longest and shortest diameter of the tumor, respectively. After 50 days, one mouse from each group was killed, and the major organs were harvested for histopathology evaluation.

In Vitro and In Vivo MRI. All MR studies were performed on a 9.4 T/400 mm wide bore scanner (Agilent Technologies, Inc., Santa Clara, CA, USA) using a volume RF coil (inner diameter 40 mm for mice; 72 mm for rats). For *in vitro* or in cell phantom MR experiments, the scanning procedure began with a localizer, and then a series of inversion-prepared fast spin-echo images were acquired for longitudinal relaxation time (T_1) measurement. This series identical in all aspects (TR 6000 ms, effective TE 5.6 ms, BW 25 kHz, slice thickness 1 mm, matrix 96×96, 1 average) except for the 20 different inversion times (TIs) that were varied linearly from 10 to 2500 ms. Signal intensity (SI) versus TI relationships were fit to the following exponential T_1 decay model by nonlinear least-squares regression: $SI(TI) = A1*\exp(-TI/T_1)+SI(0)$.

For the *in vivo* MR studies, the mice used in the experiment were treated in accordance with the Ethics Committee Guidelines of the University of Science and Technology of China. The hepatic cancer model was established through direct subcutaneous injection of

5×10^7 HepG2 cells into the left hind leg of BALB/c nude mice. Three to four weeks following the injection of cells, hepatic tumors were apparent. Next, nude mice or rats with tumors were anesthetized with isoflurane (3.5 % induction, 1.0-1.5 % maintenance) in air/O_2 (2:1) for the duration of the scan. The animals were placed in a prone position on a specially designed cradle and inserted into the magnet. The respiratory rate and rectal temperature were monitored throughout the experiment with a physiologic monitoring unit (model 1030; SA Instruments, Inc., Stony Brook, NY). For the duration of the experiment, the animal's body temperature was maintained at 36.5 °C with a homemade heating pad.

Following the acquisition of a tripilot scan, T_1-weighted MR images were acquired, typically along the coronal orientation, using a spin-echo sequence. For the mouse studies, the following acquisition parameters were chosen: repetition time (TR) = 370 ms, echo time (TE) = 11.6 ms, field of view (FOV) = 40 mm × 40 mm, matrix size = 192 × 192, slice thickness = 1 mm (12 slices, gap = 0), 1 average, and bandwidth (BW) = 50 kHz. For the rat studies, the following acquisition parameters were chosen: TR = 590 ms, TE = 13 ms, FOV = 40 mm × 60 mm, matrix size = 256 × 256, slice thickness = 1 mm (30 slices, gap = 0), 1 average, and bandwidth (BW) = 50 kHz. We obtained a series of pre-injection baseline T_1-weighted MR images prior to CA injections via tail vein. Post-injection scans were obtained 15 min, 30 min, 60 min, 90 min and 120 min after the injection. Throughout the scanning sessions, precise measurements and markers were used to ensure consistent placement of the animal's tumor in the animal holder and of the animal's tumor within the magnet. Pulse oximeter triggering was used for MRI acquisition to reduce artifacts arising from respiratory movement. Dynamic MRA images of rats were acquired using a 3D-CEMRA

sequence with parameters as follows: T_1-weighted fast field echo (T1FFE), TR = 7 ms, TE = 3 ms, FOV = 100 mm×100 mm, slices = 60, slice thickness = 1 mm, and flip angle = 30°.

ImageJ software was used to analyze images. Briefly, for each mouse or rat, ROIs were manually drawn around the tumor on the coronal MR images, and the signal intensities were measured and normalized to the noise values in the corresponding MR slices for comparison between pre-injection and post-injection.

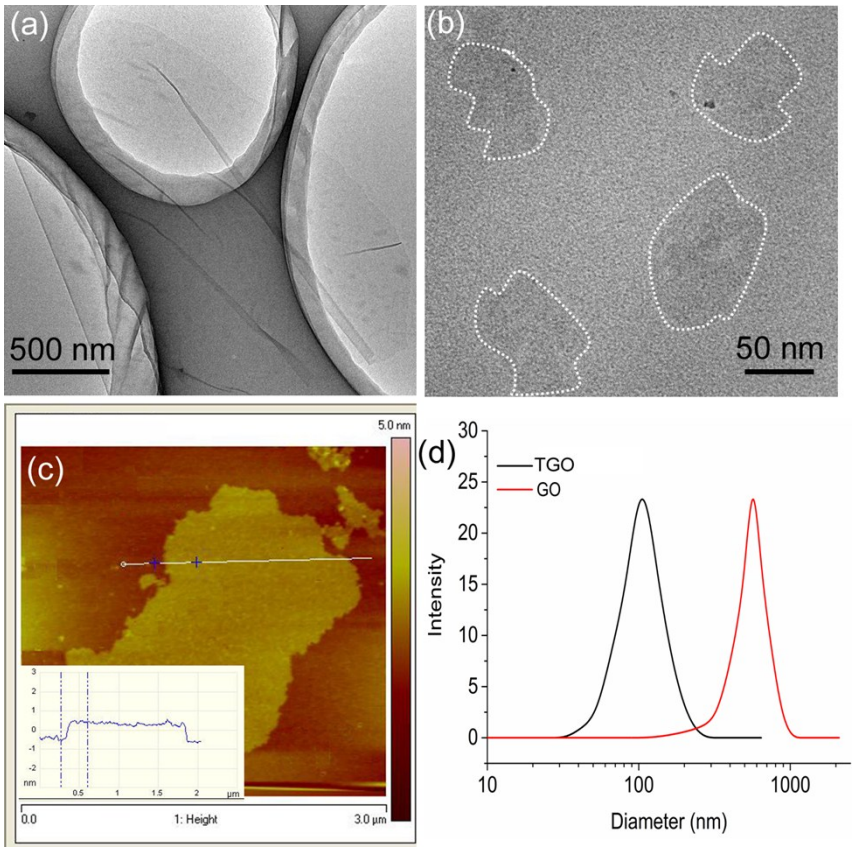

Figure S1. (a) TEM and (c) AFM images of GO. (b) TEM image of TGO. (d) Particle size of GO and TGO.

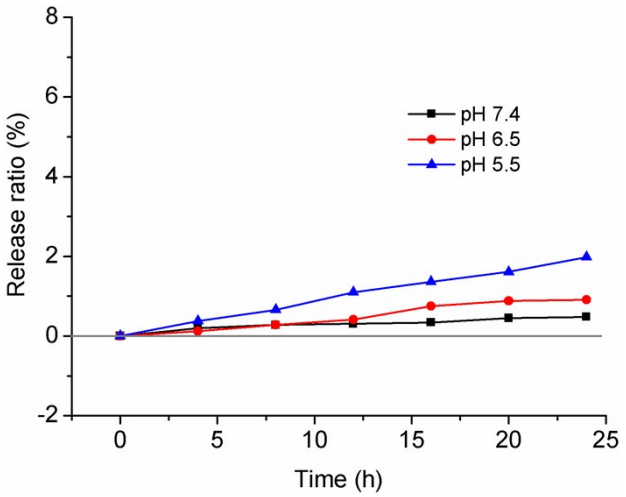

Figure S2. The release rate of Gd ions from FA-GCGLD nanosheet with time under different pH conditions at 37 °C.

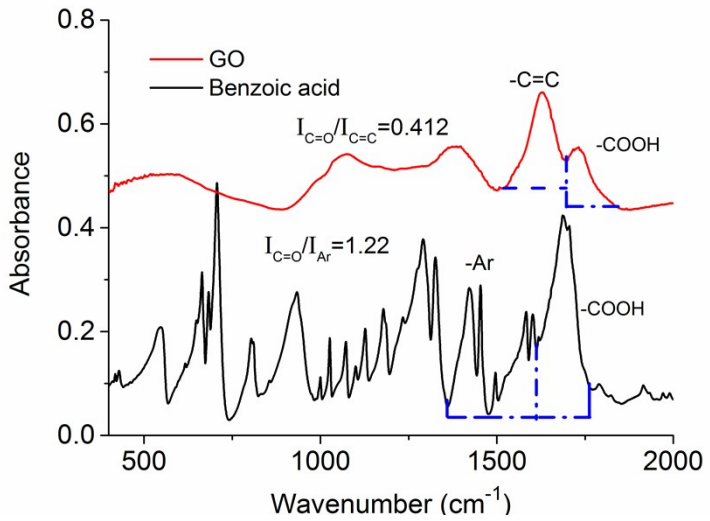

Figure S3. FT-IR spectrum of TGO and benzoic acid was used to evaluate carboxyl group content.

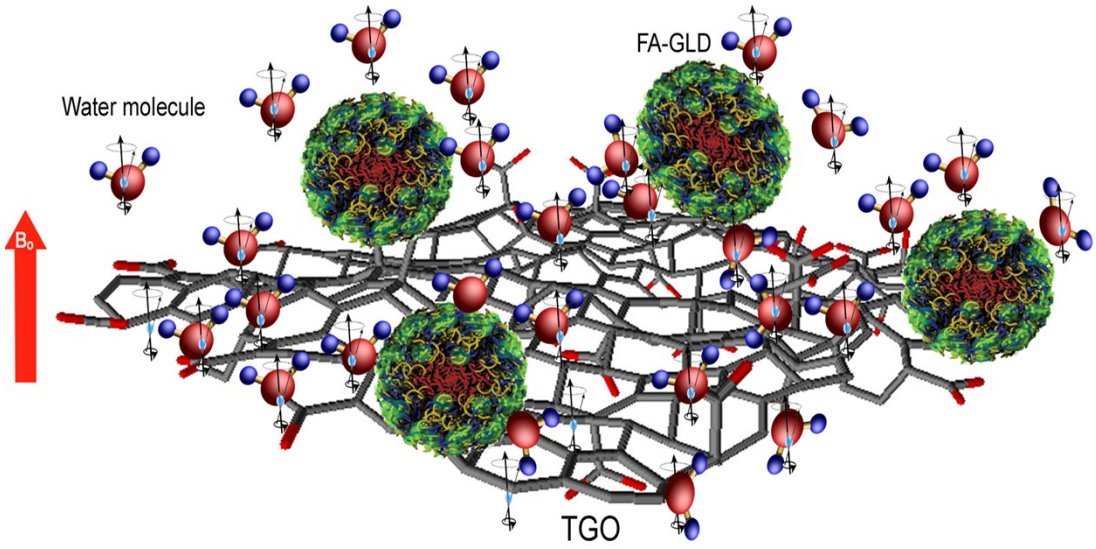

Figure S4. The mechanism schematic of TGO enhancing the exchange efficiency between gadolinium centers and water protons.

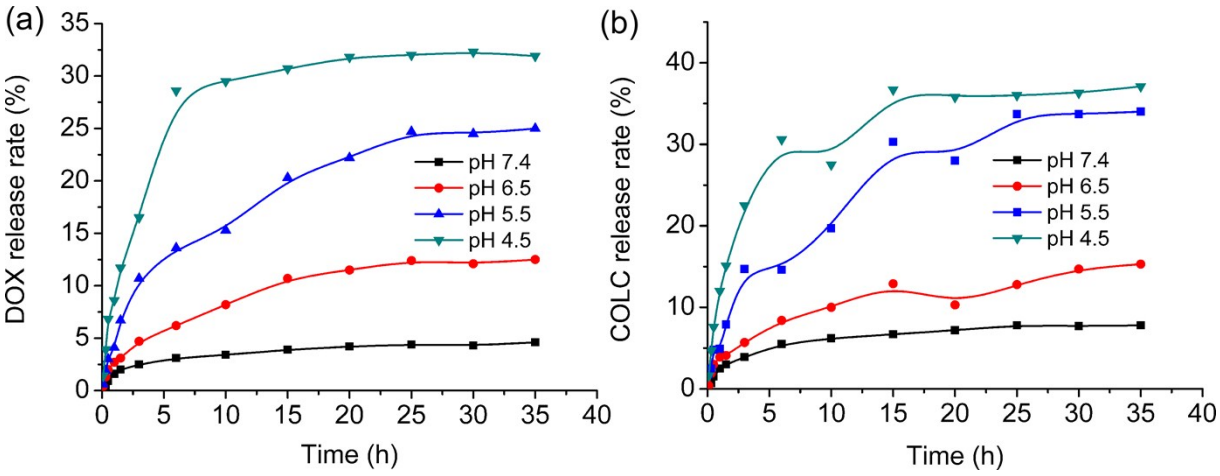

Figure S5. Release curves of DOX (a) and COLC (b) from FA-GCGLD-DOX/COLC under different pH conditions.

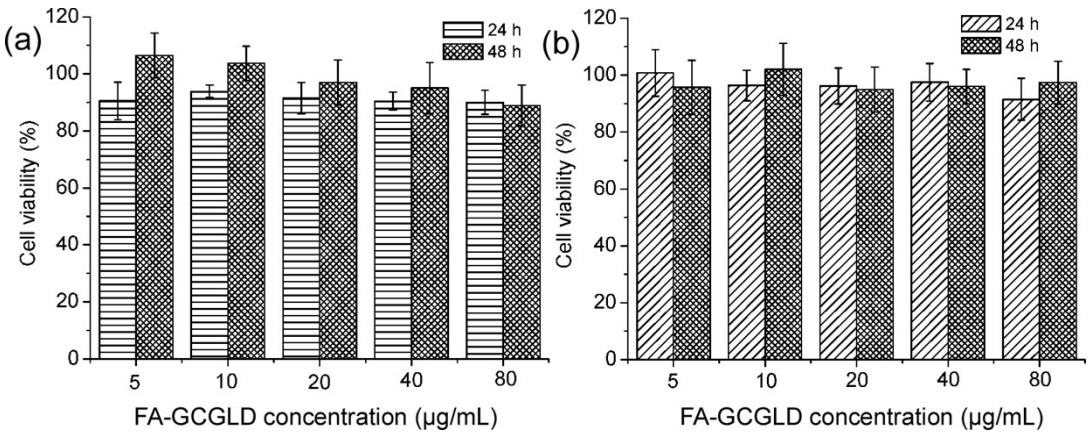

Figure S6. MTT assay for HepG2 (e) and HeLa cells (f) viabilities after incubation with different concentrations of FA-GCGLD nanosheet for 24 h and 48 h.

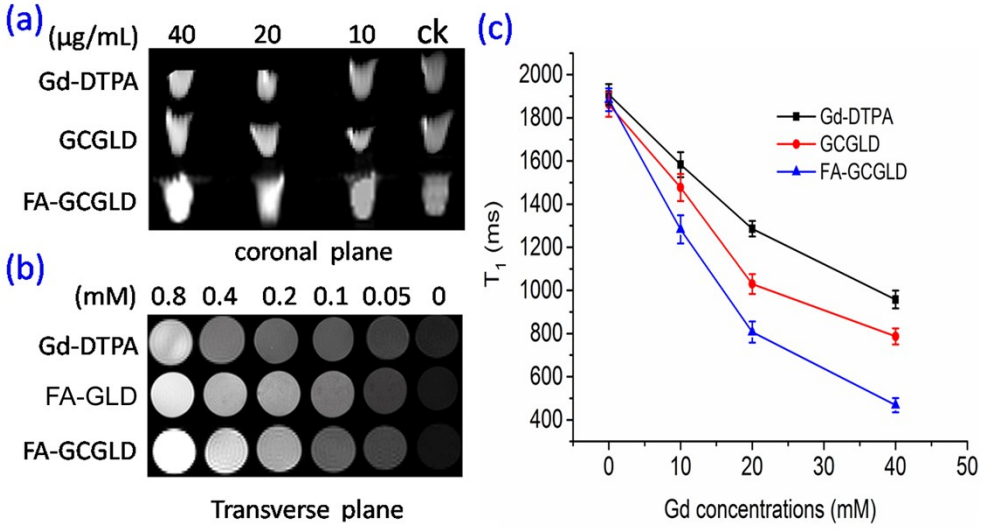

Figure S7. T_1-weighted images (a) and the corresponding T_1 values (c) of HepG2 cells treated with different concentrations of Gd-DTPA, GCGLD, and FA-GCGLD. (b) T_1-weighted phantom imaging of Gd-DTPA, FA-GLD, and FA-GCGLD using 9.4 T scanner.

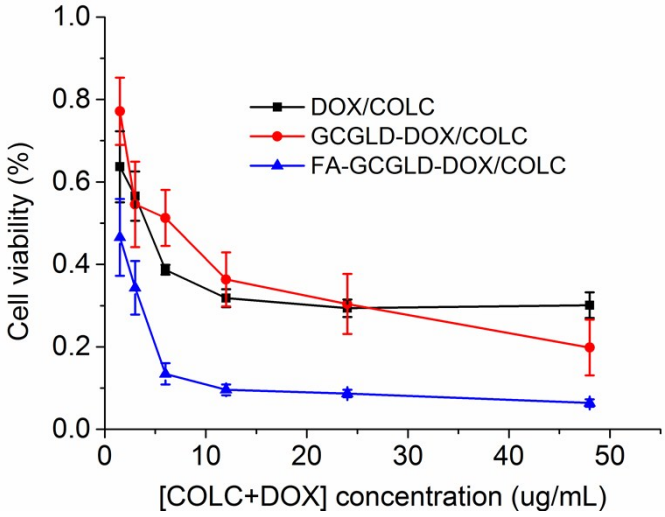

Figure S8. Viability of HepG2 cells incubated with FA-GCGLD for 48 h.

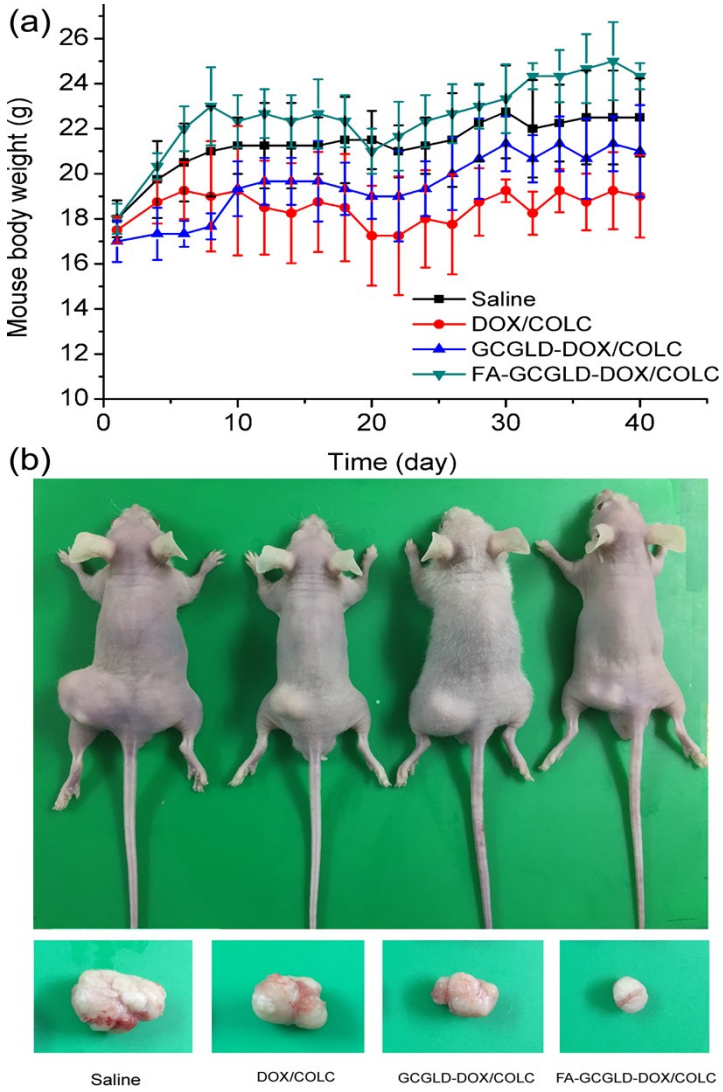

Figure S9. (a) The body weight changes of mice injected with saline, DOX/COLC, GCGLD-

DOX/COLC, and FA-GCGLD-DOX/COLC. (b) Photographs of mice and excised tumors treated with different samples.

References

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