Electronic Supplementary Information

Albumin-based nanoparticles loaded with hydrophobic gadolinium chelates as T_1-T_2 dual-modal contrast agents for accurate liver tumor imaging

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Experimental details

Synthesis of tri-*tert*-butyl 2,2',2''-(1,4,7,10-tertraazacyclododecane-1,4,7-triyl)triacetate (2)

tert-Butyl bromoacetate (1.3 g, 7.6 mmol, 3.3 equiv) dissolved in 10.0 mL of anhydrous chloroform was added dropwise to a mixture of 1,4,7,10-tetraazacyclododecane (cyclen) (**1**) (400 mg, 2.32 mmol) and triethylamine (2.3 g, 23.2 mmol, 10.0 equiv) in 40 mL of anhydrous chloroform under an argon atmosphere over half an hour. The reaction mixture was stirred for another 2 hours, and anhydrous K_2CO_3 (0.16 g, 1.16 mmol, 0.5 equiv) was added. After 24 hours, the resulting solution was washed with water (3 × 40 mL). The organic phase was dried with $MgSO_4$ and concentrated to give a transparent oil. This crude product was purified by flash chromatography (15% CH_3OH/CH_2Cl_2) on silica gel to give **2** as a white powder (3.0 g, 5.8 mmol, 77%). ^{1}H NMR (400 MHz, $CDCl_3$): δ 3.34 (4 H, s), 3.26 (2 H, s), 3.05 (4 H, s), 2.89-2.85 (12 H, m), 1.47 (27 H, s); ^{13}C NMR (100 MHz, $CDCl_3$): δ 171.02, 169.5, 81.7, 58.4, 51.0, 49.3, 48.5, 47.5, 28.1; ESI-MS (m/z) calcd for $C_{26}H_{51}N_4O_6$ [M + H^+]: 515.4, found: 515.4.

Synthesis of tri-*tert*-butyl 2,2',2''-(10-(2-methoxy-2-oxoethyl)-1,4,7, 10-tertraazacyclododecane-1, 4, 7-triyl)triacetate (3)

To a 100 mL round-bottom flask was added **2** (129.5 mg, 0.25 mmol), K_2CO_3 (82.3 mg, 0.6 mmol, 2 equiv) and 20 mL of anhydrous acetonitrile under nitrogen atmosphere. Methyl chloroacetate (70 mg, 0.65 mmol, 1.1 equiv) was added to the mixture. The suspension was vigorously stirred at room temperature for 4 h. After filtration, the filtrate was concentrated. The residue was purified by flash chromatography (100% CH_2Cl_2 - 20% CH_3OH/CH_2Cl_2) to give **3** (131.8 mg, 0.22 mmol, 90%). ^{1}H NMR (400 MHz, CD_3OD): δ 3.70-1.80 (m, 24 H), 1.52 (s, 27 H, *t*Bu), ESI-MS (m/z) calcd for $C_{29}H_{54}N_4NaO_8$ [M + Na^+]: 609.4, found: 609.3.

Synthesis of tri-*tert*-butyl 2,2',2''-(10-(2-((2-aminoethyl) amino)-2-oxoethyl)-1,4,7, 10-tertraazacyclododecane-1, 4, 7-triyl)triacetate (4)

3 (0.43 g, 0.72 mmol) was dissolved in neat ethylenediamine (0.25 mL, 0.23 g,

3.74 mmol) and the resulting solution was stirred at room temperature for 72 h. The final solution was concentrated to give a light yellow foam. The residue was purified by flash chromatography (100% CH_2Cl_2 to 50% MeOH/CH_2Cl_2) to give **4** as a white foam (0.4 g, 0.65 mmol, 93%). ^{1}H NMR (400 MHz, CD_3OD): δ 3.67–1.94 (m, 28 H), 1.50 (s, 27 H; *t*Bu); ^{13}C NMR (100 MHz, CD_3OD): δ 172.85, 170.3, 81.42, 56.58, 55.51, 50.89, 51.27, 40.37, 39.54, 27.13. ESI-MS (m/z) calcd for $C_{30}H_{58}N_6NaO_7$ [M + Na^+]: 637.4, found: 637.4.

Synthesis of compound 5

A certain amount of DIC / HOBt were added in the solution of GA (141 mg, 0.3 mmol) in 10 mL THF at 0 °C ($mol_{Ibuprofen}$: mol_{DIC} : mol_{HOBt} =1 : 1.2 : 1.2). The solution was stirred for 1 h at 0 °C. **4** (DO3A-*t*-Bu-NH_2) (200 mg, 0.3 mmol, 1.0 equiv) and triethylamine (50 μL, 0.35 mmol) were added, and the mixture was stirred at room temperature for 6 ~ 10 h. The solution was evaporated, and purified by silica column chromatography [V(CH_2Cl_2) : V(CH_3OH) = 1 : 20]. ^{1}H NMR (500 MHz, CD_3OD): δ 5.62 (s, 1 H, 12-CH), 4.32-3.97 (m, 4 H, $NHCH_2CH_2NH$), 3.76-2.94 (m, 24 H), 2.72 (m, 1 H, 3-CH), 2.46 (s, 1 H, 9-CH), 1.49 (s, 27 H, -C(CH_3)3) 1.31, 1.15, 1.07, 1.04, 1.01, 0.85, 0.82 (7 × s, 21 H, 29, 28, 27, 26, 25, 24, 23-CH_3); ^{13}C NMR (125 MHz, CD_3OD): δ 202.0, 177.8, 171.1, 170.9, 163.3, 162.9, 161.0, 160.7, 127.7, 81.7, 77.9, 61.6, 54.9, 53.8, 51.1, 48.22, 45.6, 43.6, 43.2, 41.2, 41.1,41.0, 39.3, 38.9, 36.9, 35.6, 32.4, 31.6, 30.7, 28.4, 28.0, 27.4, 27.2, 26.4, 26.2, 26.0, 22.5, 18.0, 17.2, 15.7, 15.0. ESI-MS (m/z) calcd for $C_{60}H_{102}N_6O_{10}$ [M + H^+]: 1067.7, found: 1067.7.

Synthesis of compound 6

The product of compound **5** was dissolved in 0.5 mL CH_2Cl_2, and 3 mL trifluoroacetic acid (TFA) was added. The reaction was carried out overnight at room temperature. TLC were used to observe the remove of *t*-butyl protecting groups; once complete, unreacted TFA was removed by evaporation. The solution was concentrated to give **S1** as a brown glassy solid. ^{1}H NMR (500 MHz, CD_3OD): δ 5.53 (s, 1 H, 12-CH), 4.1-3.77 (m, 4 H, $NHCH_2CH_2NH$), 3.71-3.21 (m, 24 H), 2.63 (m, 1

H, 3-CH), 2.0 (s, 1 H, 9-CH), 1.32, 3 × 1.04, 0.89, 0.73, 0.70 (7 × s, 21 H, 29, 28, 27, 26, 25, 24, 23-CH_3); ^{13}C NMR (125 M, CD_3OD): δ 200.6, 177.2, 170.7, 170.9, 161.1, 160.7, 160.4, 160.1, 126.7, 77.5, 61.4, 54.1, 52.5, 49.1, 47.5, 44.5, 42.7, 42.4, 40.3, 38.5, 38.2, 38.0, 37.5, 36.5, 36.1, 31.6, 30.7, 29.7, 27.5, 27.1, 26.5, 25.6, 25.4, 25.3, 21.6, 17.1, 16.4, 14.9, 14.2. ESI-MS of compound **S1** (m/z) calcd for $C_{48}H_{78}N_6O_{10}$ [M + H^+]: 899.2, found: 899.6.

S1 (172 mg, 0.27mmol) was dissolved in 10 mL of DI H_2O and $GdCl_3 \cdot 6H_2O$ (194.7 mg, 0.3 mmol) was added. The pH was adjusted to ~ 6 with 0.1 M NaOH and the reaction was stirred overnight at 45 °C. Lyophilization of the solution yielded a while solid, which was dissolved in CH_3OH and purified by HPLC (ZORBAX SB-18 Column. mobile phase: 0-20 min: 90% CH_3OH /10% H_2O (0.1% CF_3COOH), 20-40 min: 100% CH_3OH; 1.0 mL/min; R_t = 32 min). ESI-MS of **6** (m/z) calcd for $C_{48}H_{75}GdN_6O_{10}$ [M + H^+]: 1054.4, found: 1054.5.

Binding constant of GGD to BSA

The binding affinity of GGD to BSA was assessed by Proton Relaxation Enhancement (PRE) measurements, which is commonly used to determine affinity constants of Gd^{3+} complexes to BSA (Fig. S1).[1-3] The water proton relaxation rates were measured at increasing concentrations of BSA in 1 × PBS buffer while the concentration of GGD was maintained unchanged (0.1 mM). The binding constant was determined according to the following equilibrium and equations **1** to **3**:

GGD + BSA ⇆ GGD-BSA

$$K_D = \frac{[GGD][BSA]}{[GGD - BSA]} \qquad \textbf{Eq 1}$$

$$\varepsilon^* = \frac{(1/T_1)_{GGD-BSA} - (1/T_1)_{BSA}}{(1/T_1)_{GGD-PBS} - (1/T_1)_{PBS}} \qquad \textbf{Eq 2}$$

$$\varepsilon^* = \frac{\varepsilon_{max} [BSA]_t + K_D}{K_D + [BSA]_t} \qquad \textbf{Eq 3}$$

where:

K_D is the binding dissociation constant.

[GGD-BSA] is the concentration of GGD bound to BSA.

[GGD] is the concentration of unbound GGD in solution.

[BSA] is the concentration of free BSA in solution.

$[BSA]_t$ is the total concentration of BSA.

ε* is the enhancement factor.

ε_{max} is the maximum value that the enhancement factor can reach extrapolated to the scenario where all the contrast agent molecular are bound to HSA.

$(1/T_1)_{GGD\text{-}BSA}$, $(1/T_1)_{BSA}$, $(1/T_1)_{GGD\text{-}PBS}$, $(1/T_1)_{PBS}$ are the relaxation rates of solvent protons in solutions containing GGD plus BSA, BSA alone, GGD plus 1 × PBS, and 1 × PBS alone, respectively.

Curve fitting was carried out with Origin 8.5.

Cytotoxicity assay

All cell lines (RAW264.7 and HepG2) were purchased from Cell Bank of Chinese Academy of Sciences (Shanghai, China).

The cytotoxicity of Gd-DOTA, GGD, GGD-BSA NPs, BSA aqueous solution and BSA NPs was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. RAW264.7 and HepG2 cells were firstly seeded into a 96-well plate with a density of 1×10^4 cells/well in DMEM (containing 10% FBS and antibiotics), and incubated under the atmosphere of 5% CO_2 at 37 ^oC for 24 h, respectively. The cells were then incubated with Gd-DOTA, GGD and GGD-BSA NPs at various Gd concentrations, BSA aqueous solution and BSA NPs at various BSA concentrations for another 24 h. Subsequently, the culture media of each well were replaced by 100 μL of fresh culture media containing MTT (0.5 mg/mL) and the plate was further incubated for 4 h at 37 ^oC. Then the media of each well were replaced by 200 μL of DMSO. The OD_{490} value (Abs.) of each well was measured by MultiSkan FC microplate reader immediately. Cell viabilities were calculated from OD_{490} values.

Biodistribution in Tumor-Bearing Mice

H22 tumor bearing mice were injected with GGD-BSA NPs at a dose of 0.03 mmol Gd^{3+}/kg body weight and were sacrificed at 0.5, 1, and 2 h p.i. time points (n = 3/group). Tumors and major organs were collected and weighed, and chemically digested using trace metal grade 70% nitric acid (HNO_3), followed by trace metal grade 30% hydrogen peroxide (H_2O_2) for 3 days at room temperature. A portion of each digested sample was diluted with trace metal grade 2% HNO_3 and filtered through a 0.22 μm pore filter membrane. The Gd^{3+} concentrations in these samples were measured using ICP-MS. The final Gd^{3+} concentrations were reported as percentage of injected dose per gram tissue (% ID/g).

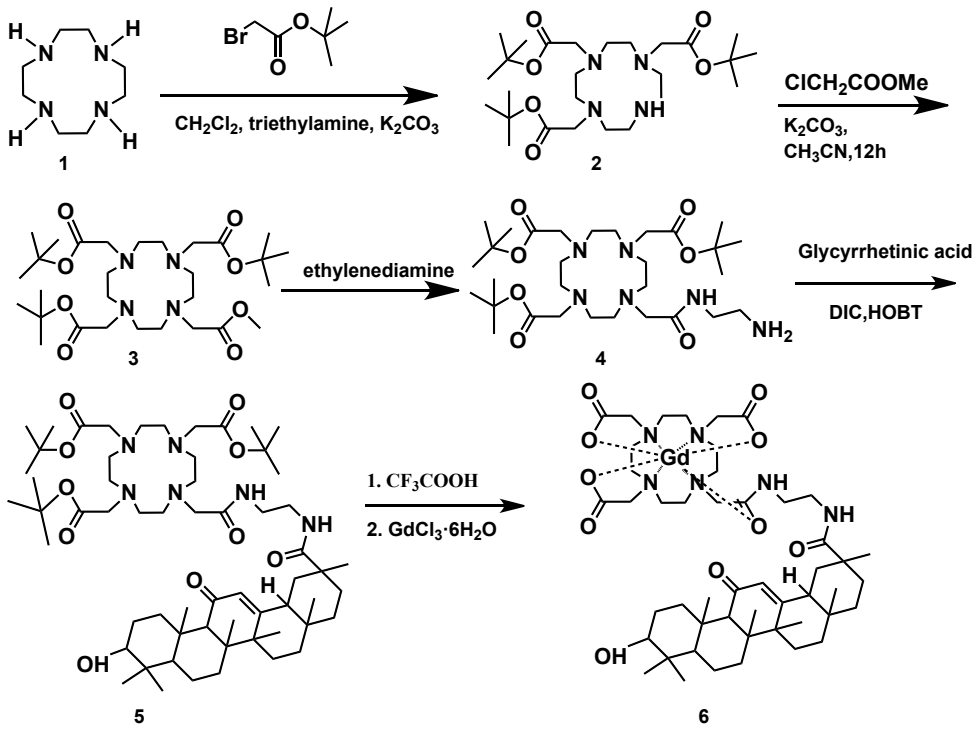
Hematology and histological examination

Healthy female ICR mice were randomly separated as control group (*n* = 3) and experimental group (*n* = 3). The dose for the experimental group was 0.03 mmol Gd^{3+}/kg mouse body weight. The mice of the control group were injected with the same volume of PBS solution. On the seventh day, blood samples and tissues from the mice were collected. Blood was collected from the orbital sinus by quickly removing the eyeball from the socket with a pair of tissue forceps. Upon completion of the blood collection, the mice were sacrificed. Then, the heart, liver, spleen, lung, and kidneys were removed, weighed, and fixed in 4% paraformaldehyde solution, and embedded in paraffin; they were then sectioned and stained with hematoxylin and eosin. The histological sections were analyzed for study of *in vivo* toxicity.

F4/80 staining

The frozen orthotropic liver tumor was sectioned into 5 mm thick slice. Slides were fixed with ice-cold acetone for 10 min. After fixation, slides were incubated with 3% H_2O_2 in PBS solution for 10 min to block endogenous peroxidase activity. Then slides were rinsed 3 times with PBS (5 min each) and incubated with phycoerythrin (PE)-conjugated F4/80 antibody (eBioscience) at 37 ^oC for 1 h in a humidified chamber. Cell nuclei were stained with DAPI.

Scheme S1 Synthetic route for GGD.



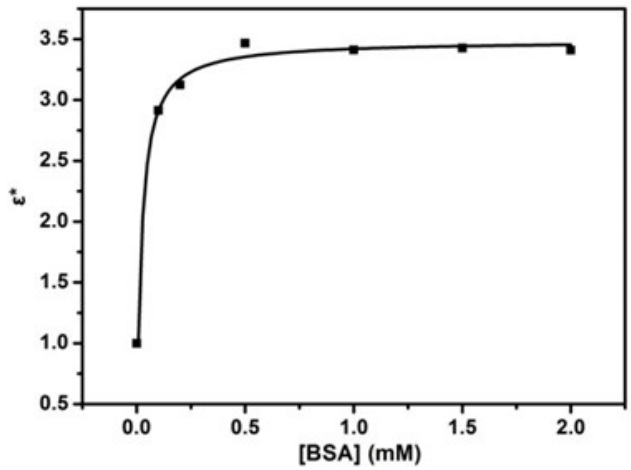


Fig. S1 ε* versus [BSA] in 1 × PBS buffer for a 0.1 mM solutions of GGD at 20 MHz. The solid curve represents the best fit according to **Eq 3**.

Table S1 The nanoparticle yields and the Gd encapsulation efficiencies of different molar ratios are shown. The molecular weight of BSA is about 67,000 Da. GGD molecules were dissolved in DMSO as the stock solution with a concentration of 79.7 mM. Both of volumes of the initial BSA solution and the final GGD-BSA NPs solution were 1 mL. The concentrations of Gd^{3+} and BSA in the final GGD-BSA NPs solution were measured by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) and Bradford protein assay, respectively.

Added BSA molecule (mg)	BSA NPs (mg)	Yield of NPs (%)	Dropping rate (mL/min)[a]	Added V_{GGD} (μL)	Final C_{Gd} (mM)	EE of Gd (%)	Molar ratios of GGD:BSA
90	87.1	96.8	0.5	90	5.2	72.5	4:1
90	86.7	96.3	0.5	45	2.6	71.9	2:1
90	86.9	96.5	0.5	22	1.3	74.1	1:1
90	85.6	95.1	1	50	0.54	13.6	0.5:1
90	86.4	96	1	30	0.39	16.2	0.3:1

[a]The dropping rate of acetone for desolvation. It was used as an indicator for desolvation rate. See experimental sections for details.

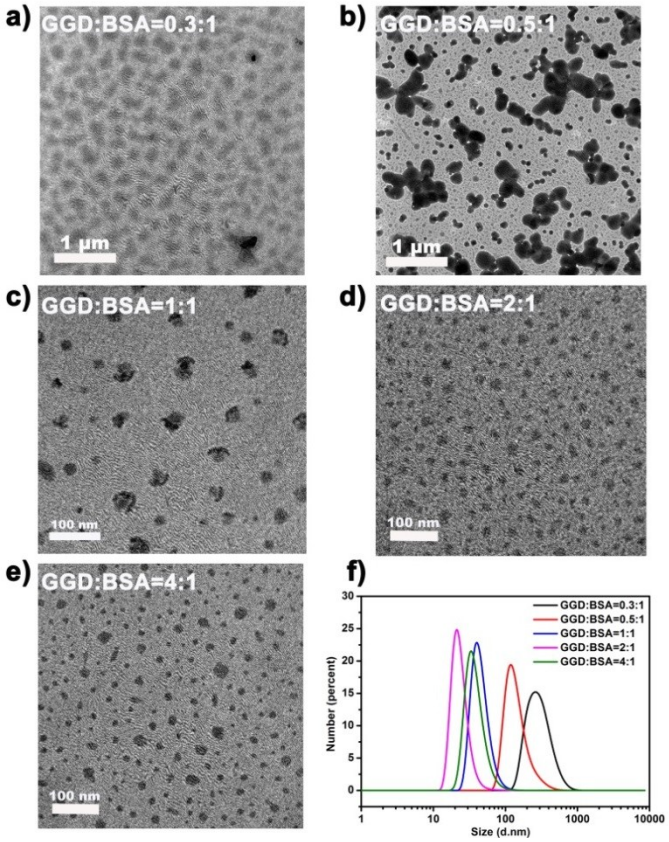


Fig. S2 TEM images of GGD-BSA NPs of different GGD to BSA ratios. (a, b, c, d, e) TEM images of GGD-BSA NPs with GGD to BSA ratios at 0.3:1, 0.5:1, 1:1, 2:1, 4:1, respectively; (f) DLS of GGD-BSA NPs with GGD to BSA ratios at 0.3:1, 0.5:1, 1:1, 2:1 and 4:1, respectively.

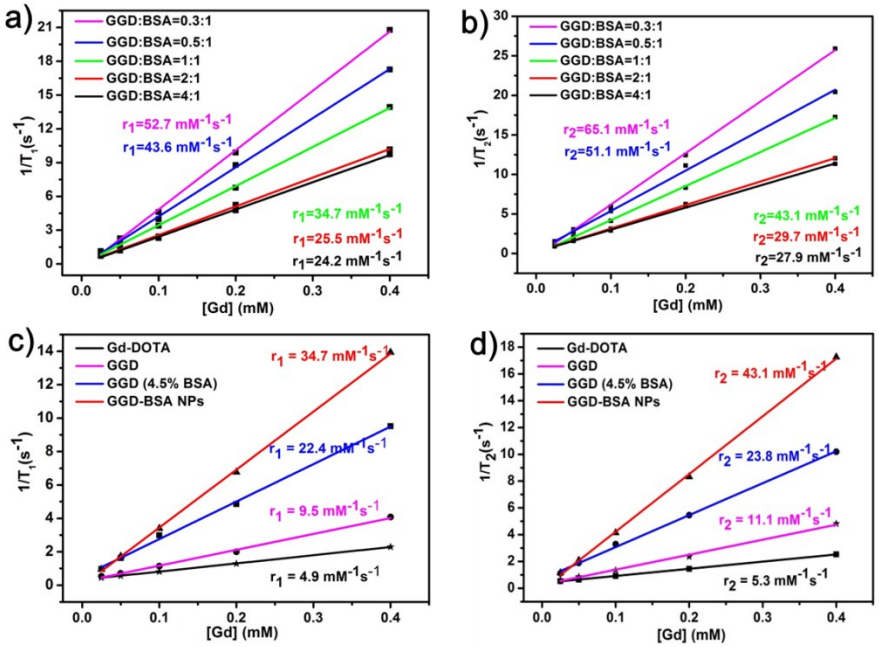


Fig. S3 Relaxivities r_1 and r_2 of GGD-BSA NPs with different GGD to BSA molar ratios. As GGD to BSA molar ratio increased, r_1 (a) and r_2 (b) decreased. (c, d) relaxivities r_1 and r_2 of Gd-DOTA, GGD, GGD (4.5% w/v BSA), GGD-BSA NPs (GGD: BSA at 1:1) at 0.5 T.

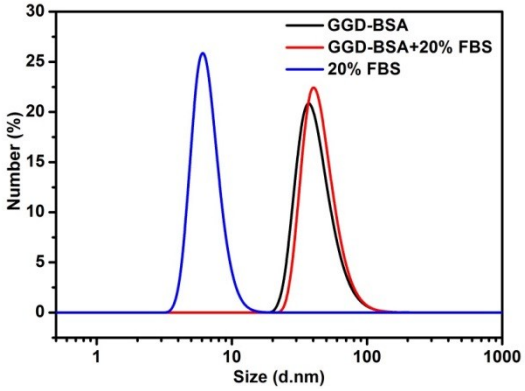


Fig. S4 Hydrodynamic diameters (HDs) of GGD-BSA NPs in PBS, GGD-BSA NPs in 20% FBS, and 20% FBS measured by Dynamic light scattering (DLS).

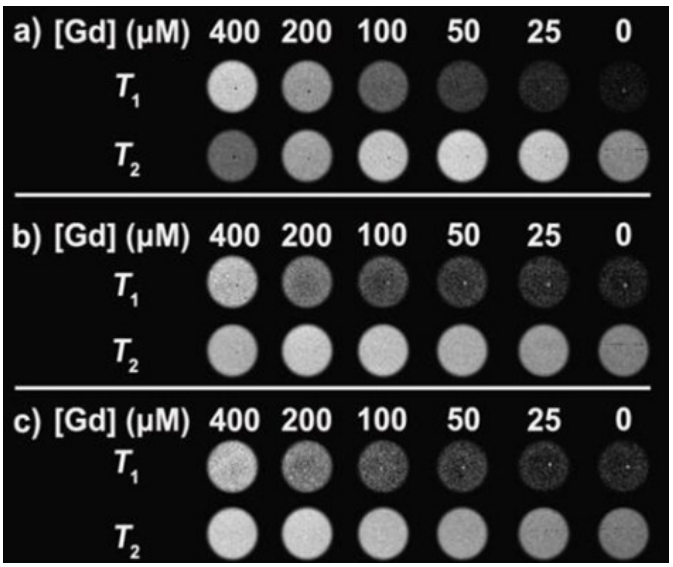


Fig. S5 MR phantom images at 0.5 T. (a) GGD-BSA NPs (GGD: BSA at 1:1); (b) GGD; (c) Gd-DOTA.

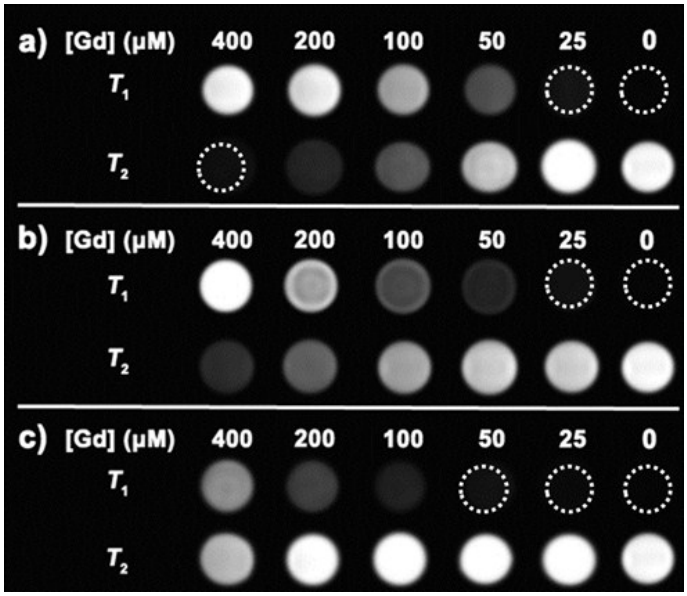


Fig. S6 MR phantom images at 1.5 T. (a) GGD-BSA NPs (GGD: BSA at 1:1); (b) GGD; (c) Gd-DOTA.

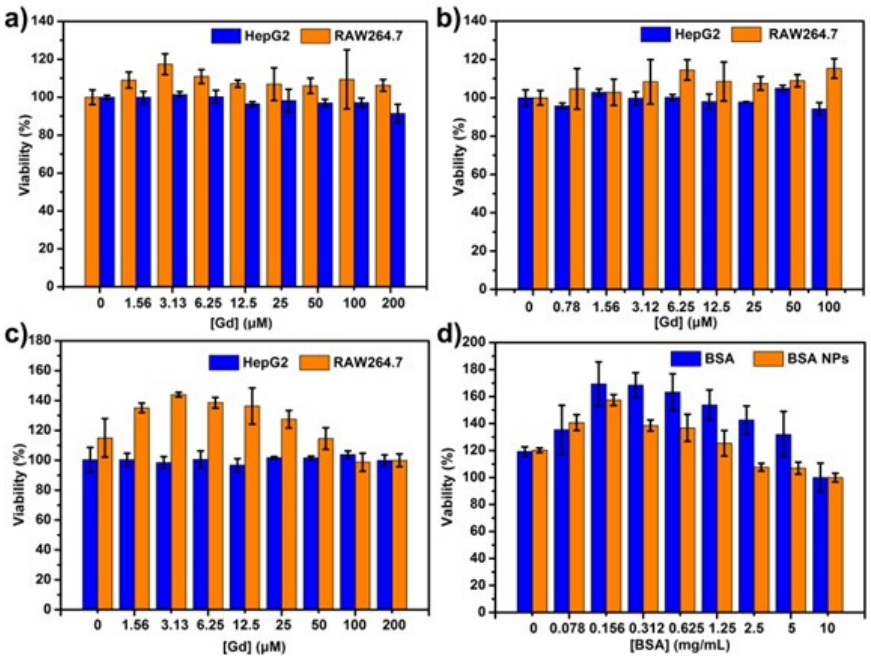


Fig. S7 MTT assay of HepG2 and RAW264.7 cells incubated with (a) Gd-DOTA, (b) GGD, (c) GGD-BSA NPs (GGD: BSA at 1:1) at different Gd^{3+} concentrations for 24 h. (d) MTT of RAW264.7 cells incubated with BSA and BSA nanoparticles at different BSA concentrations for 24 h.

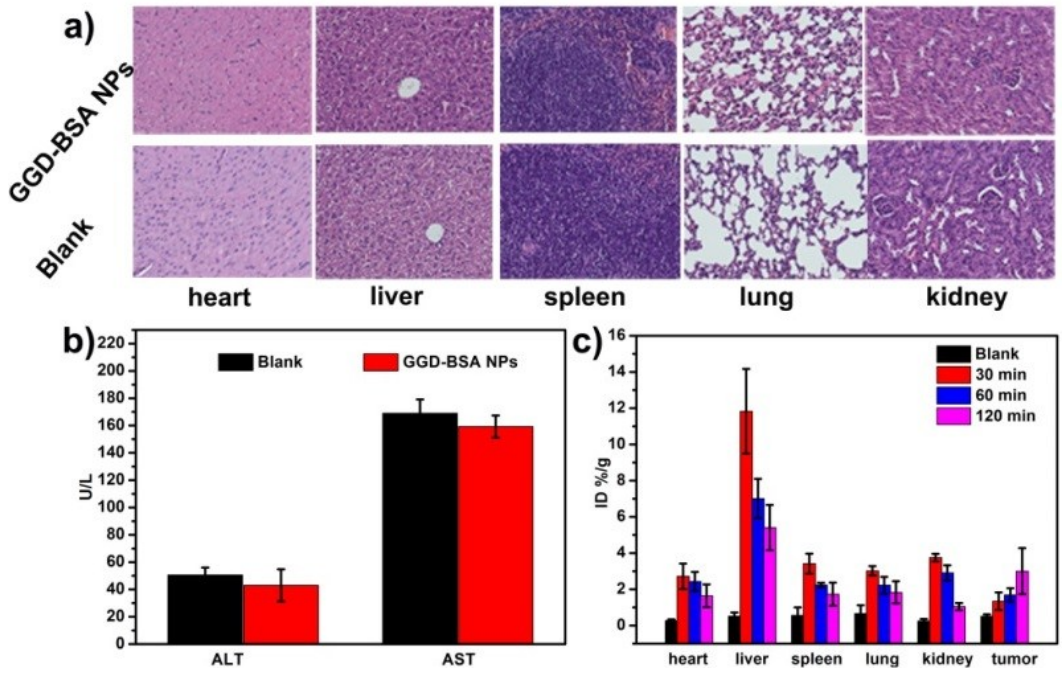


Fig. S8 Biocompatibility of GGD-BSA NPs. (a) H&E stained tissue sections from mice 7 days after injection with PBS and GGD-BSA NPs. The mice were all healthy throughout the time. Scale bar: 200 μm for all images. (b) Serological test results of the mice injected with GGD-BSA NPs and PBS (Blank). (c) Bio-distribution of GGD-BSA NPs in mouse organs at different time points after intravenous injection (0.03 mmol Gd/kg mouse body weight, $n = 3$).

Table S2 MR contrast-to-noise ratio (CNR) changes of tumor-to-liver contrast pre- and post-injection of GGD-BSA nanoparticles.

	T_1 image (%)	T_2 image (%)
CNR_{pre}	8 ± 1	7 ± 2
CNR_{post}	99 ± 2	89 ± 3
ΔCNR^a	113 ± 4	114 ± 4

[a]$CNR = |SNR_{tumor} - SNR_{liver}|/SNR_{tumor}$; $\Delta CNR = |CNR_{post} - CNR_{pre}|/CNR_{pre}$

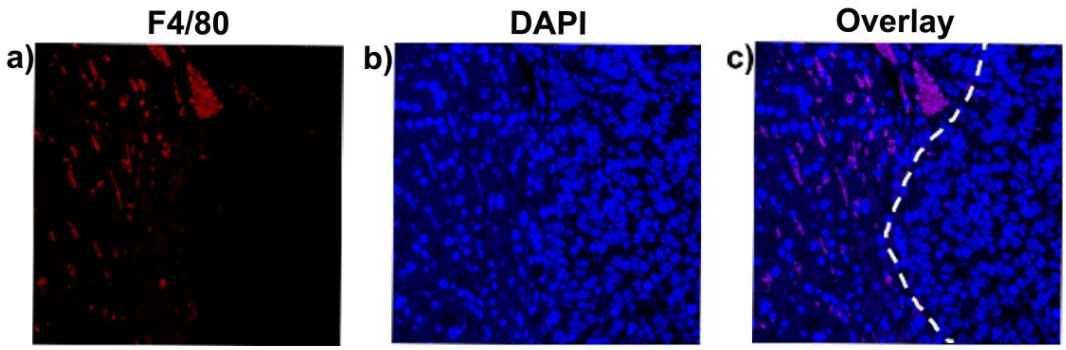


Fig. S9 Histological study of liver and orthotropic liver tumor tissue. The tissues were stained with (a) F4/80 antibody and (b) DAPI. (c) The overlay image of F4/80 staining and DAPI staining. The boundary between normal liver tissue (left) and hepatic tumor (right) was indicated by dashed line.

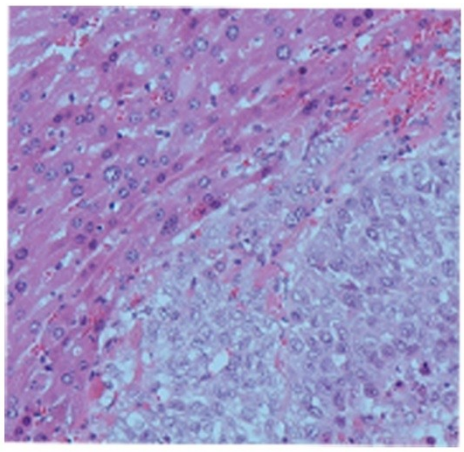


Fig. S10 Hematoxylin and eosin (H&E)-stained liver and orthotropic liver tumor issue.

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3. S. Aime, E. Gianolio, D. Longo, R. Pagliarin, C. Lovazzano and M. Sisti, *Chembiochem.*, 2005, **6**, 818-820.