Supporting Information

for

Lactose modified enzyme sensitive branched polymers as nanoscale liver cancer targeting MRI contrast agents

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Materials and Methods

The chemical compounds for preparation and characterizations were purchased and used without further purification. The RAFT reaction initiator VA044 and Dithiothreitol (DTT) were purchased from Aladdin Reagent Inc. (Shanghai, China). $GdCl_3 \cdot 6H_2O$ was obtained from Tokyo Chemical Industry (TCI, Japan), and mal-Cy5.5 from Xi'an Confluore Biological Technology Co., Ltd. (Xi'an, China). The cross-linker (MA-GFLGK-MA), RAFT polymerization monomers (MA-GFLG-CTA, Lactose-MA and PTEMA) and mal-DOTA were synthesized via the methods previously reported.[1-5] The synthesized polymers were purified by fast protein liquid chromatography (FPLC), and characterized by size exclusion chromatography (SEC), ^1H NMR analysis (400 MHz Bruker Advanced Spectrometer), inductively coupled plasma mass spectrometry (ICP-MS), dynamic light scattering (DLS) (Brookhaven Instruments, New York, USA), and field emission transmission electron microscopy (TEM) (Tecnai G2 F20 S-TWIN, FEI, USA). The mal-Cy5.5 loading content in the final product was measured via UV–vis spectrometry (UV1800ENG240 V, SOFT, Shimadzu, Japan). The fluorescent intensity was measured via a fluorescence spectroscope (Horiba Fluoromax-4, Japan) .

Preparation of Branched-LAMA-PTEMA

VA044 (4 mg, 12.2 μmol) was dissolved in 10 mL of a mixture of methanol and RO-water (v/v = 1/2). Under Ar, the above solution was added to a 15 mL sealed tube containing LAMA (1490 mg, 3.19 mmol), PTEMA (648 mg, 2.55 mmol), MA-GFLGK-MA (41.8 mg, 63.8 μmol) and MA-GFLG-CTA (23.8 mg, 31.2 μmol) at 0 ºC. The reaction mixture was stirred at 45 ºC for 12 h. After cooling for 10 min at 0

°C, the reaction mixture was drop-wise added into 300 mL acetone, and a pink solid was precipitated. The solid was dialyzed against RO-water (2 kDa, MWCO) for 2 days. After freeze-drying, 1.25 g of Branched-LAMA-PTEMA (pink solid) was obtained with a yield of 58%.

Preparation of Branched-LAMA-SH

Branched-LAMA-PTEMA (1.2 g) was dissolved in 20 mL RO-water. DTT (1.2 g) was added to the above solution. The reaction mixture was stirred overnight at room temperature, and then dialyzed against RO-water (2 kDa, MWCO) for 48 h. After freeze-drying, 1.0 g of Branched-LAMA-SH (pink solid) was obtained with a yield of 83%.

Preparation of Branched-LAMA-DOTA-Cy5.5

Branched-LAMA-SH (150 mg) was dissolved in 5 mL RO-water, and 10 mL DMSO was then slowly added in an ice-water bath. mal-Cy5.5 (2 mg) was dissolved in 1 mL DMSO and added into the above solution. After stirring for 4 h under dark, mal-DOTA (250 mg) was added to the reaction mixture, and the reaction continued for 24 h. The reaction mixture was dialyzed against RO-water (2 kDa, MWCO) for 48 h. After freeze-drying, 190 mg of Branched-LAMA-DOTA-Cy5.5 (blue solid) was obtained with a yield of 48%.

Preparation of Branched-LAMA-DOTA-Cy5.5-Gd (BLDCGd)

Branched-LAMA-DOTA-Cy5.5 (170 mg) was dissolved in 15 mL RO-water, $GdCl_3 \cdot 6H_2O$ (170 mg) was added to the above solution. 0.2 N NaOH was used to adjust the pH value between 5.2 to 5.4. The reaction mixture was stirred for 24 h

under dark, dialyzed against RO-water (2 kDa, MWCO) for 24 h, and lyophilized. 148 mg Branched-LAMA-DOTA-Cy5.5-Gd (BLDCGd) was obtained as a blue solid with a yield of 87%. BLDCGd contains 5.65 wt% Gd(III) via ICP-MS analysis, and 1.5 wt% of mal-Cy5.5 loading content via UV–vis spectrometry.

In vitro Gd release from BLDCGd

In vitro Gd release from BLDCGd was performed by dialysis of BLDCGd in a physiological environment. BLDCGd was dissolved in PBS (pH = 7.4), PBS (pH = 5.4), and PBS (pH = 5.4) with 2 μM papain to prepare a 3 mL solution for each medium at an initial concentration of 10.0 μg Gd(III)/mL. Each solution was placed in a dialysis bag (2 kDa, MWCO) which was then immersed in 50 mL of the corresponding blank PBS solution, and incubated in a shaking table at 37℃. 3×100 μL of the dialysis solution in each dialysis bag were withdrawn at a predetermine time point (0 h, 1 h, 3 h, 6 h, 12 h, 24 h, 36 h, 48 h). Each sample was diluted to 4 mL with water, and the gadolinium concentration was measured via ICP-MS.

Degradation and Stability of BLDCGd in different medium

To detect the degradation of BLDCGd, BLDCGd (350 μg/mL) with papain (300 μg/mL), BLDCGd without papain, and papain only were incubated in PBS (pH = 5.4) at 37℃, and measured the particle size at 1 h, 3 h, 6 h, 9 h, 24 h by DLS. The stability of BLDCGd (350 μg/mL) were detected in PBS pH = 7.4, and in serum pH = 7.4 incubated at 37℃, and also measured the particle size at 1 h, 3 h, 6 h, 9 h, 24 h by DLS.

Fluorescence intensity of BLDCGd incubated with and without papain

To detect the aggregation-caused quenching (ACQ) effect of BLDCGd, the fluorescence intensity of the BLDCGd (0.0125 mg/mL) was measured via a fluorescence spectroscope, then added 2 μM papain[6] and incubated at 37℃ for 24 h, and detected the fluorescence intensity (excitation: 675 nm, emission 680-800 nm, slit width: 5 nm).

Relaxivities of BLDCGd and BDGd after incubation with papain

The relaxation time of the samples after incubation of BLDCGd and BDGd in PBS (pH 5.4, 10 u/mg papain) at 37 °C for 24 h was measured on a clinical 3.0 T imaging unit (Siemens Trio Tim). The relaxivities were calculated from the slope of the best fitting line of the relaxation time versus different Gd^{3+} concentrations. The T1-weighted MR images were collected using spin-echo (SE) sequence image acquisition parameters including an echo time of 6.9 ms, a repetition time of 20, 30, 50, 70, 90, 125, 150, 175, 200, 300, 400, 500, 700, 850, and 1000 ms, a field of view of 200 mm, a section thickness of 1.0 mm, and a matrix dimension of 256 × 256.

Fluorescence imaging of BLDCGd and BDGd in tumor

Ten mice with liver cancer were randomly selected into two groups for injection of BLDCGd and BDGd at a dose of 0.08 mmol Gd^{3+}/kg, respectively. After 2 h, the mice were sacrificed and tumor tissues were harvested for frozen sectioning. Fluorescence images were obtained from automatic scanning of these frozen sections under an inverted fluorescence microscope (Olympus, IX83), and these images were used to assess the fluorescence distribution of BLDCGd and BDGd in the tumor tissue.

In vivo pharmacokinetics of BLDCGd

BLDCGd at a dose of 0.08 mmol Gd^{3+}/kg was injected into five healthy BALB/c mice (8-10 weeks, 20 ± 2 g) through the tail vein, and 20 μL of blood samples were acquired from fundus venous plexus at different time points. The acquired blood samples at each time point were digested with HNO_3 and then H_2O_2 to measure the Gd^{3+} concentration by ICP-MS.

Biodistribution of the BLDCGd and BDGd in vivo

Twenty mice with liver cancer were randomly divided into two groups (n = 10). BLDCGd and BDGd at a dose of 0.08 mmol Gd^{3+}/kg were injected into the mice through their tail vein. On day 1 after injection, the experimental mice were sacrificed and the major organs (heart, liver, spleen, lung and kidney) and tumor tissues were collected, lyophilized and weighed. They were digested with HNO_3 and H_2O_2 and the solutions were analyzed to determine the residual concentrations of gadolinium by ICP-MS.

In vitro toxicity evaluation

H22 and HUVEC cells were selected to evaluate in vitro cytotoxicity of BLDCGd. BDGd was used as a control. In addition, DTPA-Gd at the same Gd concentration was used as a control. H22 cells and HUVECs were seeded into 96-well plates at a density of 1×10^4 cells/well. BLDCGd and BDGd at different concentrations (25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL, 400 μg/mL) were added to the modified eagle medium (MEM medium), and the media were used to replace the initial cell culture media. Cells were further incubated for 24 h and washed three times with PBS after

discarding the media. The cytotoxicity evaluation kit CCK-8 (Dojindo, Japan) was added to each well. After 2 h incubation, the absorbance at 450 nm was read by a multifunctional enzyme labeler (Thermo Fisher SCIENTIFIC).

Blood compatibility test

The anticoagulant (containing anti-citrate sodium) was added into 2 mL of fresh blood from a healthy human body. The sample was centrifuged at 1000 g for 5 min, and washed 3 times with PBS. Finally, the upper supernatant was aspirated and red blood cells (RBCs) was mixed with PBS to prepare a 20% RBC suspension. The concentrations of BLDCGd and BDGd were set to 1 mg/mL, 2 mg/mL, and 5 mg/mL. PBS and deionized water were used as control groups. 1 mL of the prepared solution was transferred into an EP tube, followed by addition of 50 μL of RBC suspension. After incubation at 37 °C for 24 h, the samples were centrifuged (1000 g × 5 min). 200 μL of the supernatant was pipetted into a 96-well plate. The absorbance of the sample at 540 nm was read by a microplate reader. The above procedure was repeated three times. The percentage of hemolysis rate was calculated by a method from a previous study.[4]

In vivo toxicity evaluation

Fifteen healthy female BALB/c mice (8-10 weeks, 20±2 g) were randomly divided into 3 groups (n = 5). These mice were injection with BLDCGd and BDGd at a dose of 0.08 mmol Gd^{3+}/kg, and saline as a a control group via tail vein. All mice were sacrificed after 1 day. The main organs (heart, liver, spleen, lung, kidney) were collected, fixed with a 4% paraformaldehyde solution for 48 h, and embedded in paraffin. Tissue sections were analyzed after HE staining.

Complement activation and Contact activation

The latelet activating factor (human platelet factor 4 (PF4), Cusabio Biotech, China), complement activation (human complement fragment 3a (C3a) and human complement fragment 5a (C5a), Cusabio Biotech, China) were measured via commercial enzyme-linked immunosorbent assays (ELISA). The BLDCGd and BDGd solutions were prepared at a concentration of 1.0 mg/mL. Cellulose was used as a positive control group and the whole blood as a blank control group.

10 μL of the above prepared solution was added to 500 μL of human whole blood. After incubation for 1 h at 37°C, the whole blood was centrifuged (2500g, 2-8°C) for 10 min to obtain the plasma. To measure the C3a concentration, 5 μL of the obtained plasma was diluted 500 times with C3a-Sample Diluent, and 100 μL of the diluted plasma was added into an Antibody Coated Well (provided by C3a kit). To measure the C5a concentration, 10 μL of the obtained plasma was diluted 10 times with C5a-Sample Diluent, and the diluted plasma was added into the corresponding Antibody Coated Well (provided by C5a kit). To measure the PF4 concentration, 40 μL of the obtained plasma was diluted 10 times with PF4-Sample Diluent; and 200 μL of the diluted plasma was added into the corresponding Antibody Coated Well (provided by the PF4 kit). The detections were conducted via ELISA according to the respective instruction manuals. At least 5 parallel samples were applied to obtain a reliable value, and the result was expressed as mean ± SD (n = 5).

Results and discussion

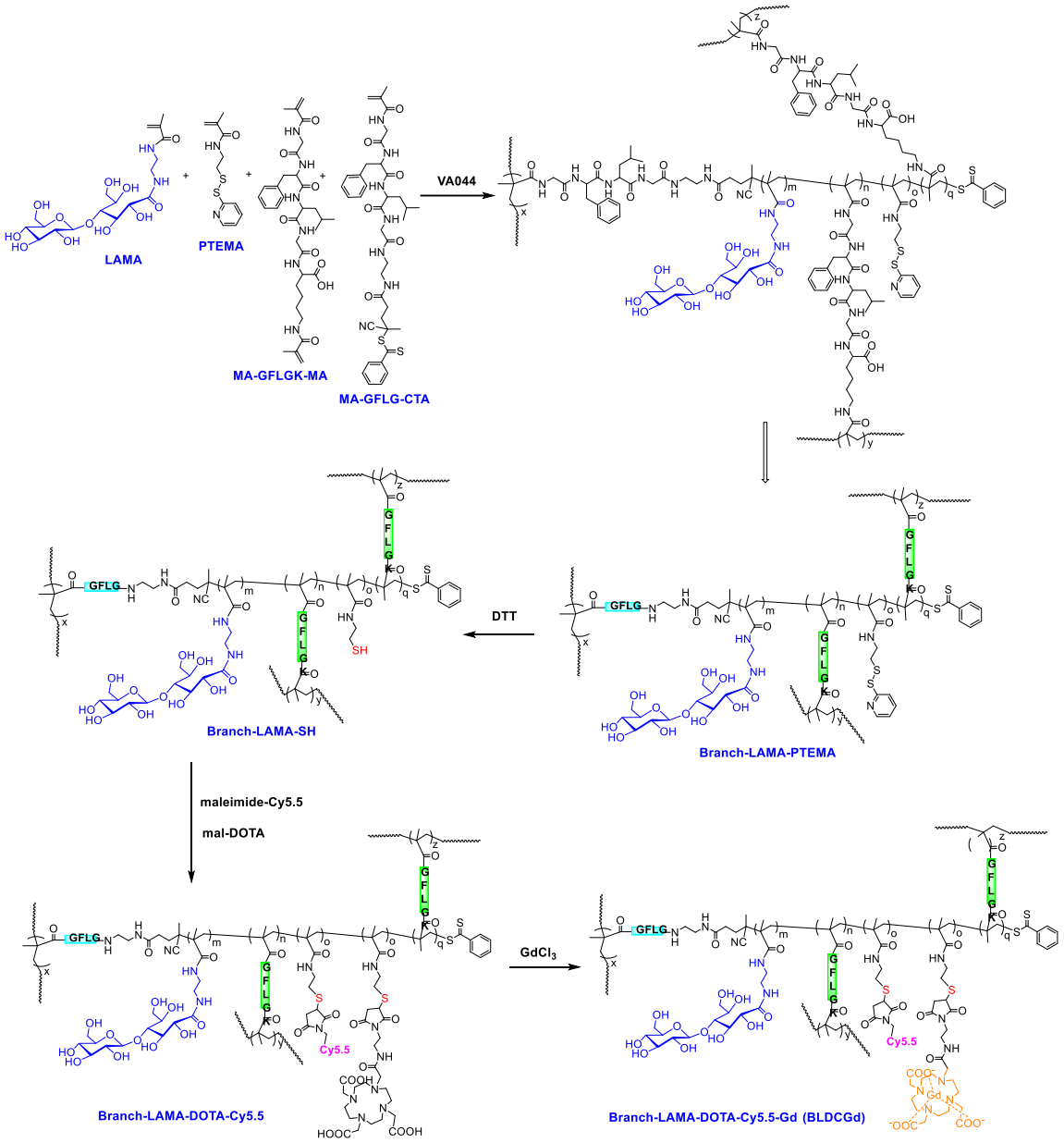

Fig. S1. Synthesis steps of Branched-LAMA-DOTA-Cy5.5-Gd (BLDCGd)

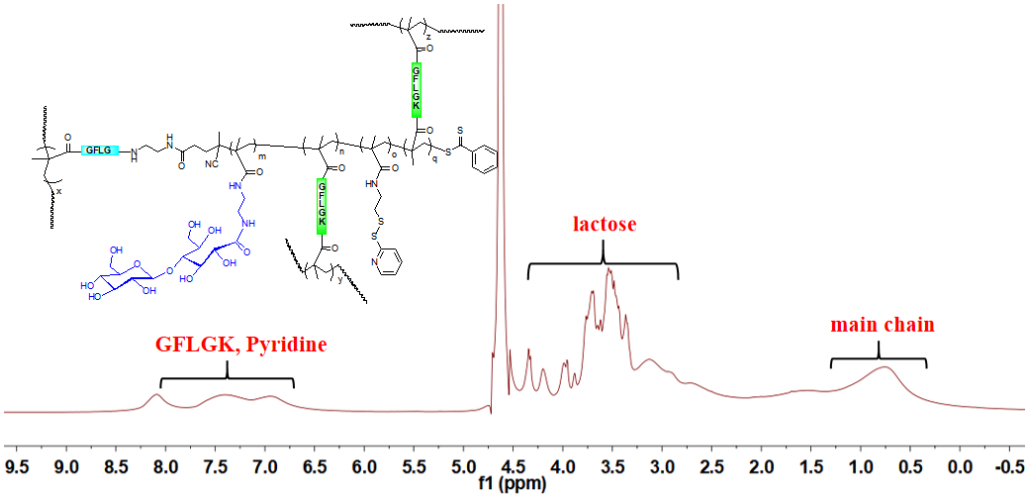

Fig. S2. ^{1}H NMR spectrum of Branched-LAMA-PTEMA in D_2O.

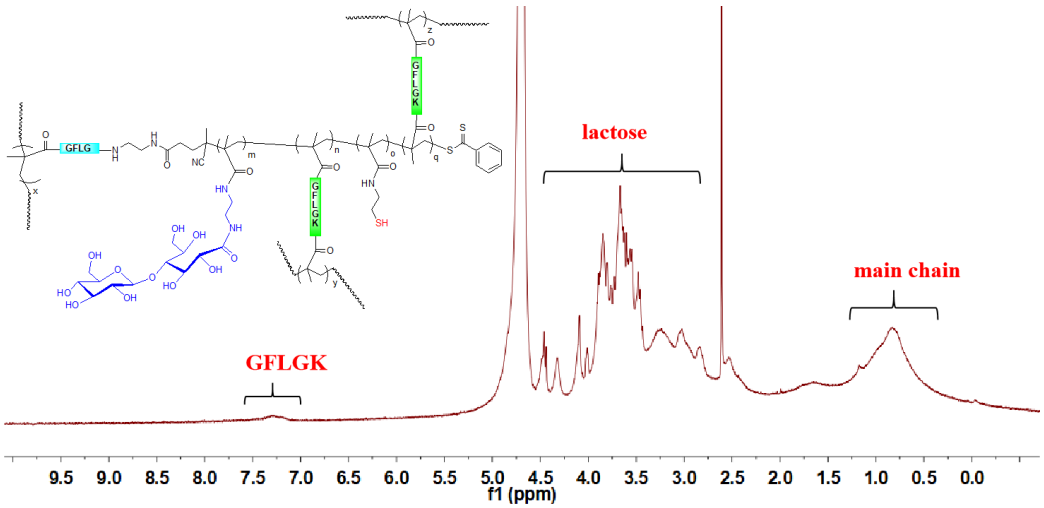

Fig. S3. ^{1}H NMR spectrum of Branched-LAMA-SH in D_2O/d6-DMSO (v/v:6/1).

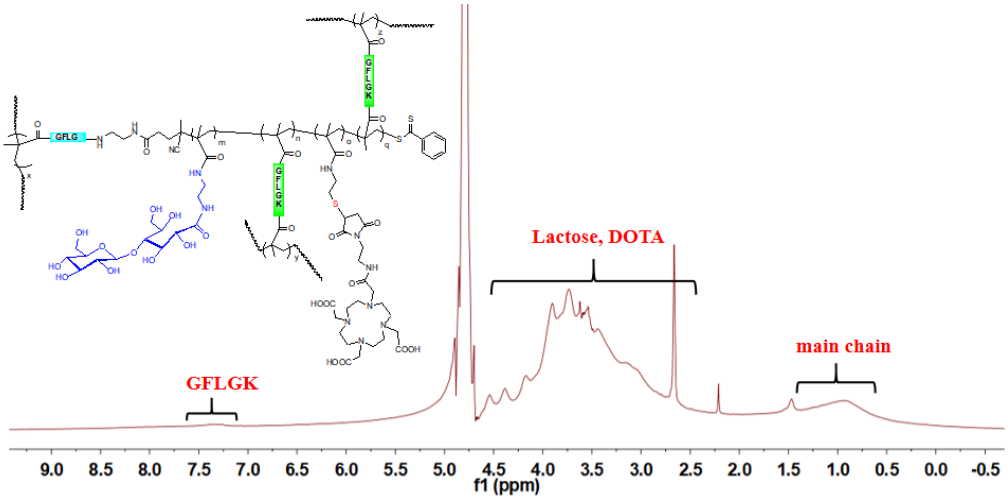

Fig. S4. ^{1}H NMR spectrum of Branched-LAMA-DOTA-Cy5.5 in D_2O/d6-DMSO (v/v:6/1).

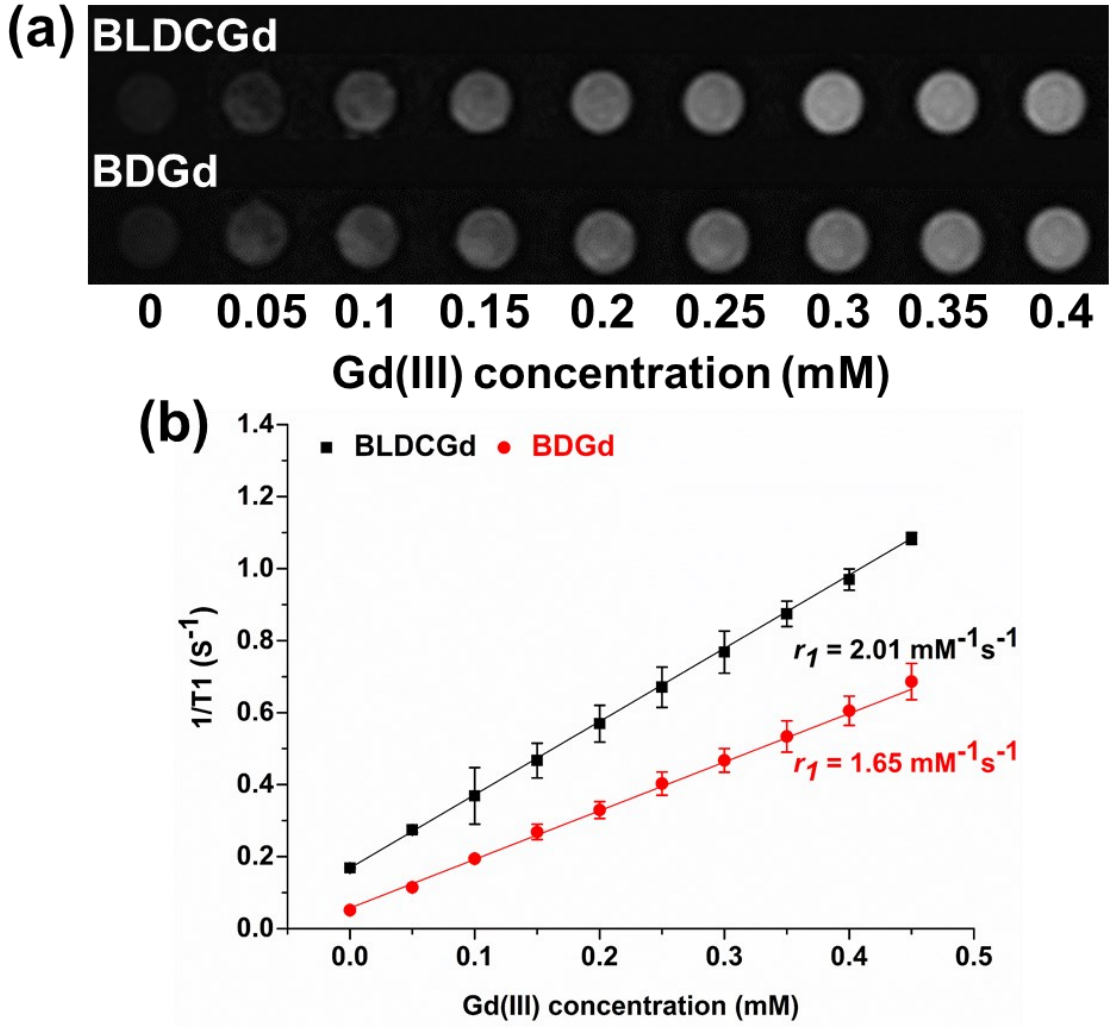

Fig. S5. (a) MR images and (b) relaxivities of BLDCGd and BDGd after incubation of them with papain for 24 h.

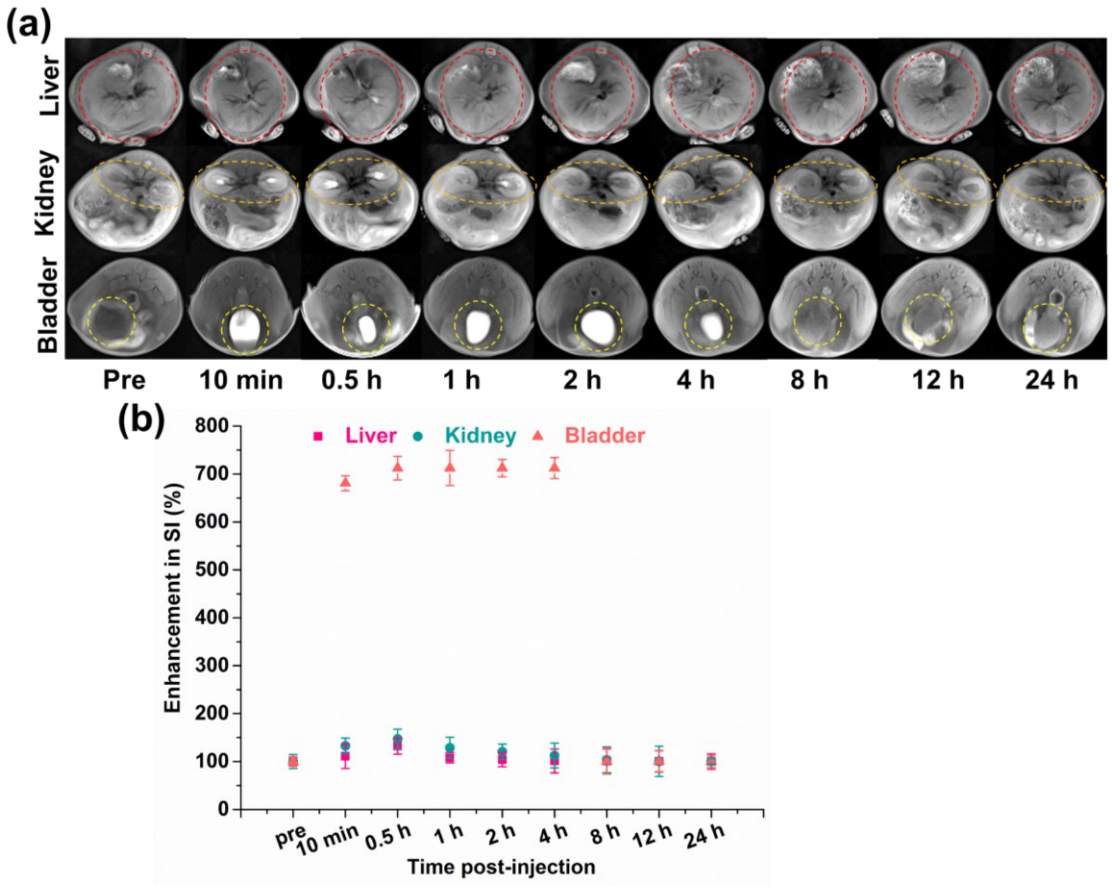

Fig. S6. MR images (a) and SI% (b) of the liver, kidney and bladder organs after injection of DTPA-Gd.

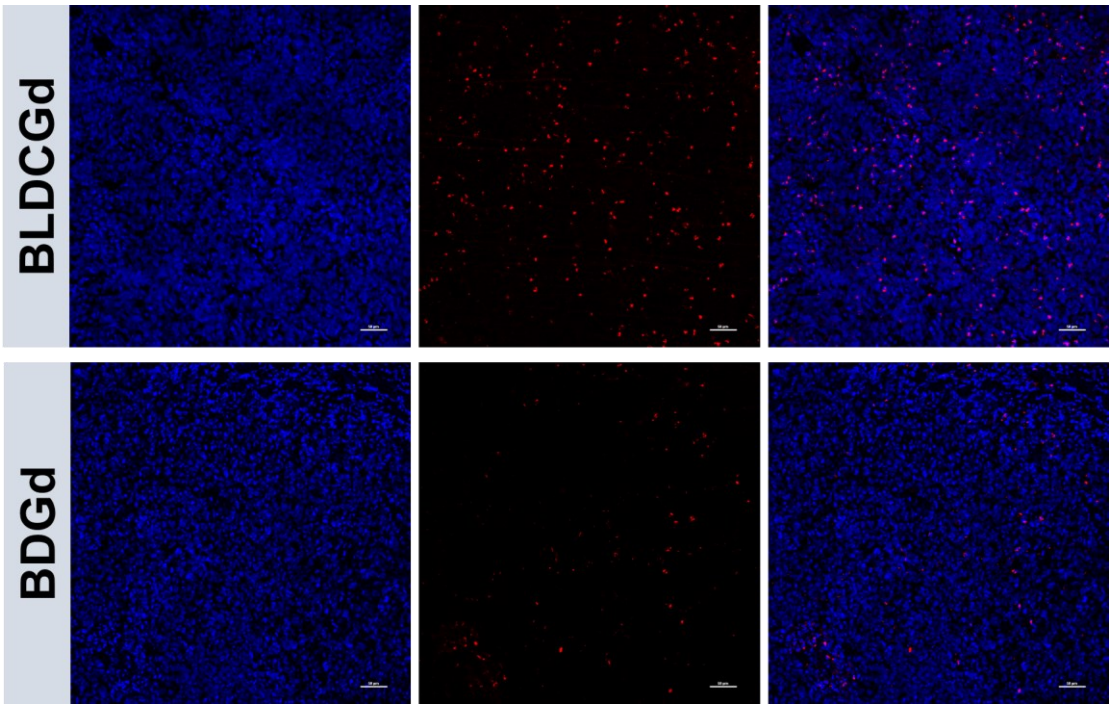

Fig. S7. Fluorescence images of liver cancer frozen sections at 2 h after injection of

BLDCGD and BDGd into mice. A few CA enrichment was observed in the tumor tissue of the BDGd-treated group, while pronounced CA enrichment in the tumor tissue of the BLDCGD-treated group. White scale bar: 50 μm.

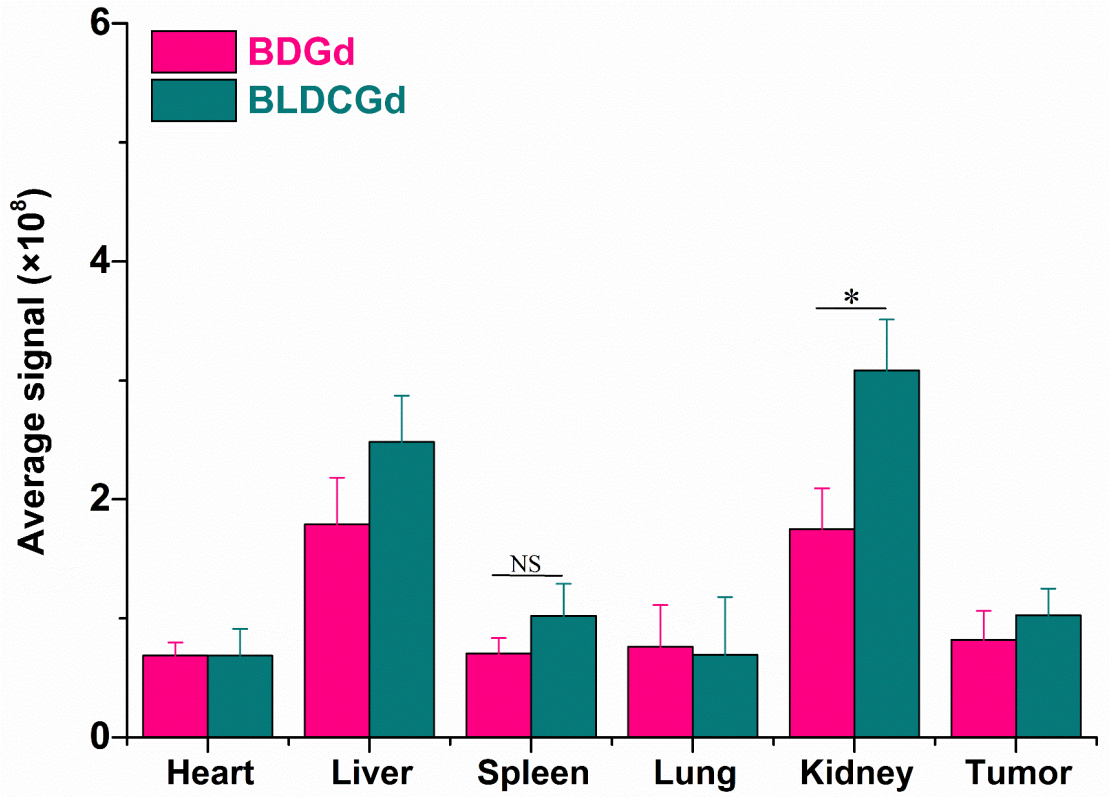

Fig. S8. The average signal intensity statistical analysis in major organs and tumor after injection of BDGd and BLDCGd at 24 h (*P＜0.05).

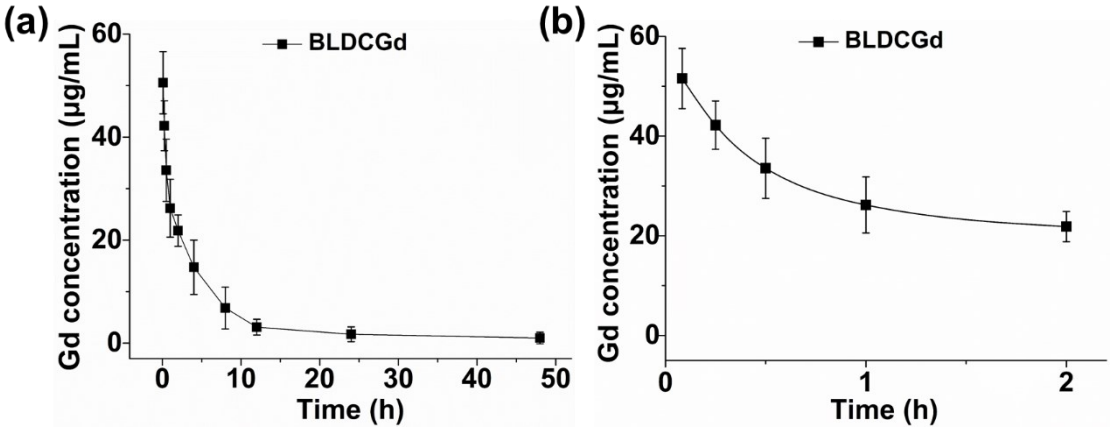

Fig. S9. (a) The concentration of Gd in the blood after injection of BLDCGd into normal mice for 48 h. (b) The concentration changes of Gd in the blood within 2 h.

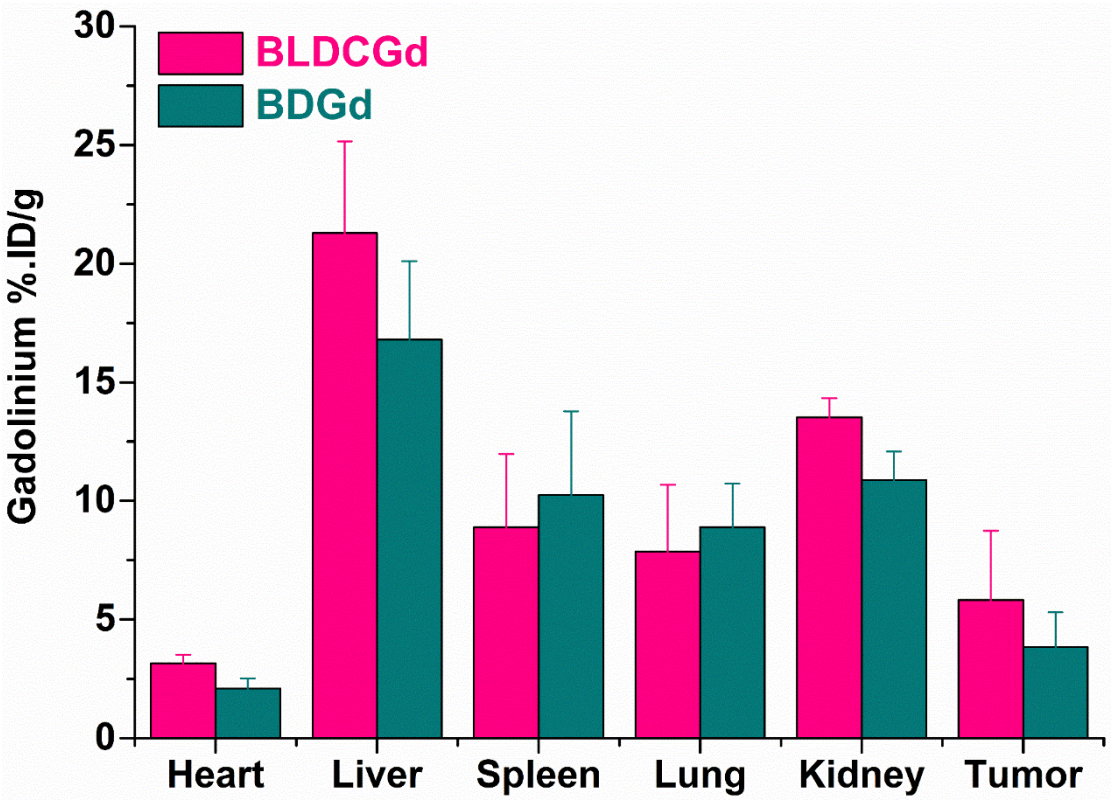

Fig. S10. Residual concentrations of Gd^{3+} in major organs and tumor tissues on day 1 after injection of the BLDCGd and BDGd into mice with liver cancer.

Table S1. Properties and characterizations of copolymers at each step.

polymers	Mw	PDI[a]	Size (nm)	PDI[b]	Zeta potential (mv)	content
Branch-LAMA-PTEMA	85 KDa	2.21	13±6	0.313	15.84±0.96	-
Branch-LAMA-SH	83 KDa	1.96	7±1	0.348	-0.04±0.95	-
Branch-LAMA-DOTA-Cy5.5	80 KDa	1.56	16±2	0.272	-9.13±2.83	-
Branch-LAMA-DOTA-Cy5.5Gd	81 KDa	1.67	59±12	0.209	7.28±1.34	1.5%Cy5.5 5.65%Gd

a the poly dispersion index of molecule weight; b the polydispersion index of particle size.

Reference

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