ASGP-R targeting magnetic resonance imaging contrast agent for liver cancer diagnosis

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Content

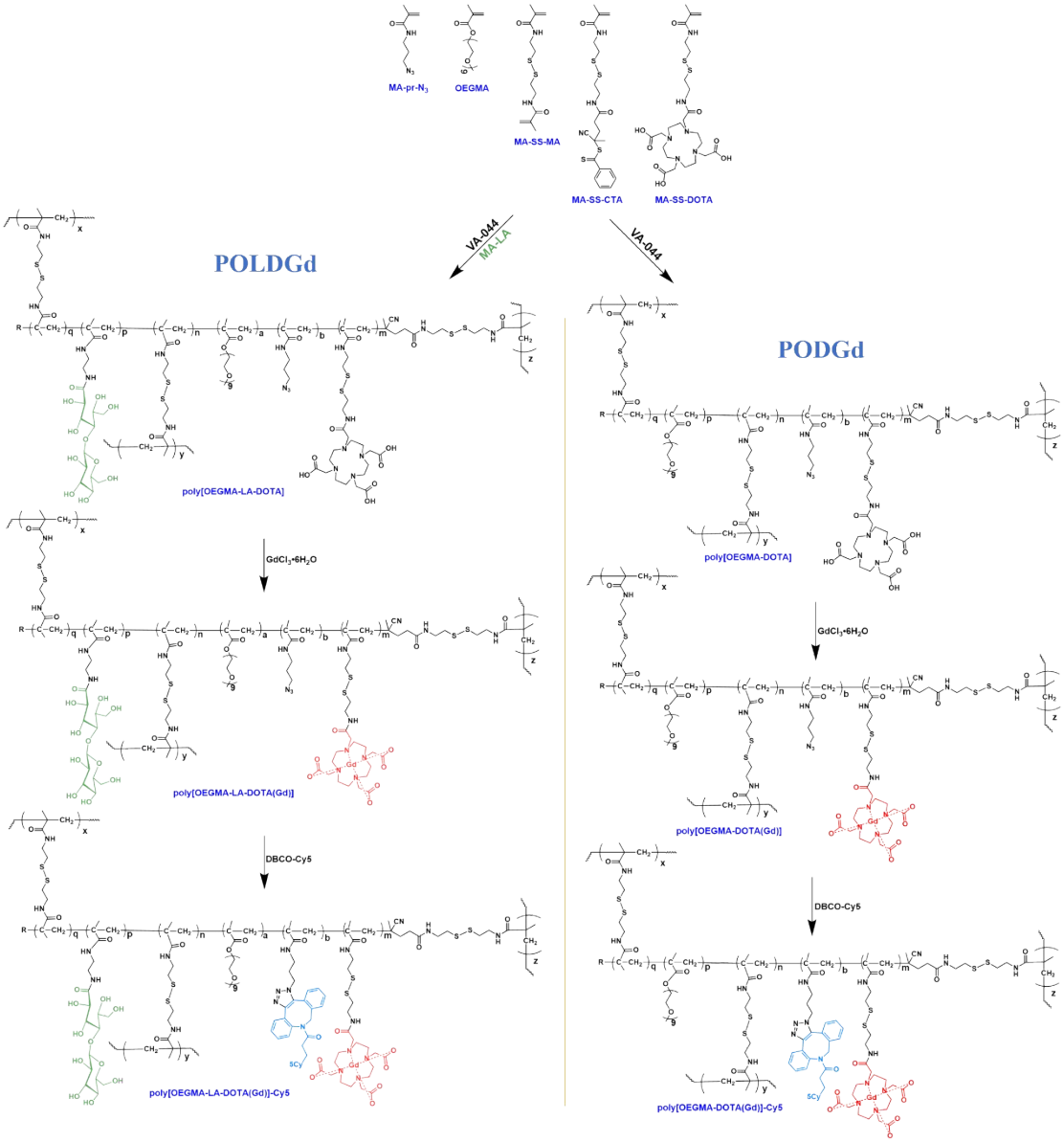
1. Experimental Section

1.1 Materials, reagents and method

All solvents used in this study were purchased from Chron Chemicals (Chengdu, China) unless otherwise noted and directly used without further purification. 2,2"-azobis[2-(2-imidazolin-2-yl)propane] dihydrochloride (VA-044) and gadolinium chloride hexahydrate ($GdCl_3 \cdot 6H_2O$) were acquired from Aladdin Rengent Inc. (Shanghai, China). DBCO-Cy5 was obtained from Confluore Biotechnology (Xi'an, China). Deuterium oxide (D_2O) was purchased from Energy Chemical (Shanghai, China). Oligo (ethylene glycol) monomethylether methacrylate (OEGMA) was obtained from Sigma-Adrich (Shanghai, China).

NMR spectra were recorded on a 400 MHz Bruker Advanced Spectrometer using D_2O as a solvent. Samples were mixed with KBr and extruded into sheets, and subjected to FT-IR spectrometry (Invenior, Bruker, USA). The average molecular weights (MWs) and polydispersity indexes (PDIs) of polymers were measured via Gel Permeation Chromatography (GPC; SHIMADZU, Japan). The hydrodynamic size and zeta potential of polymers were measured via a NanoBrook Omni (Brookhaven Instruments, USA). TEM images were used to characterize the morphology and size of nanoparticles, and they were acquired using JEM-2100Plus (Japan). Qualitative analysis of the elements in the samples was performed using EDS spectrometry. The monomers in this study were synthesized according to our previous articles[1, 2].

1.2 Synthesis of conjugates



Scheme S1. Synthesis routes of poly[OEGMA-LA-DOTA(Gd)]-Cy5 and poly[OEGMA-DOTA(Gd)]-Cy5.

1.2.1 Synthesis of poly[OEGMA-LA-DOTA(Gd)]-Cy5

Synthesis of poly[OEGMA-LA-DOTA]: A solution of MA-SS-CTA (7.67 mg), MA-SS-MA (41.30 mg) and MA-pr-N_3 (47.43 mg) in MeOH (1.00 mL) was added into a solution of MA-LA (294.03 mg), OEGMA (312.02 mg), MA-SS-DOTA (899.95 mg) and VA-044 (1.88 mg) in 6.00 mL of a mixed solvent (H_2O/MeOH = 1.5:1, v/v), and the resulting mixture was bubbled with argon under an ice bath for 30 min to strip oxygen. The mixture was stirred in the dark at 47 ˚C for 20 h and quenched with liquid nitrogen at the end of the reaction. The crude product was purified by dialysis against water (MWCO: 2 kDa) for 48 h in the dark. After filtration through a sterile syringe

filter, the filtrate was freeze-dried to give poly[OEGMA-LA-DOTA] (660 mg) as a pale pink solid. The structure of the product was characterized by ^{1}H NMR (**Figure S2**) and GPC (**Figure S3**).

Synthesis of poly[OEGMA-LA-DOTA(Gd)]: A solution of $GdCl_3 \cdot 6H_2O$ (500 mg) in RO H_2O (5 mL) was slowly dropped into a solution of poly[OEGMA-LA-DOTA] (500 mg) in RO H_2O (10 mL). The pH of the resulting mixture was adjusted to 5.20-5.40 using a dilute sodium hydroxide solution and the mixture was stirred overnight in the dark. The crude product was purified by dialysis against water (MWCO: 2 kDa) for 48 h in the dark. After filtration through a sterile syringe filter, the filtrate was freeze-dried to give poly[OEGMA-LA-DOTA(Gd)] (520 mg) as a pale pink solid. The poly[OEGMA-LA-DOTA(Gd)] product contained 7.58 % of Gd(III) measured by ICP-OES.

Synthesis of poly[OEGMA-LA-DOTA(Gd)]-Cy5: A solution of DBCO-Cy5 (2 mg) in DMSO (1mL) was added into a solution of poly[OEGMA-LA-DOTA(Gd)] (200 mg) in RO H_2O (10 mL), and the reaction mixture was stirred at room temperature overnight in the dark. The crude product was purified by dialysis against water (MWCO: 2000 Da) for 48 h in the dark. After filtration through a sterile syringe filter, the filtrate was freeze-dried to give poly[OEGMA-LA-DOTA(Gd)]-Cy5 (201 mg) as a bluish violet solid. The Cy5 content of poly[OEGMA-DOTA(Gd)]-Cy5 measured by UV-Vis spectrometry was 0.65 %.

1.2.2 Synthesis of poly[OEGMA-DOTA(Gd)]-Cy5

Synthesis of poly[OEGMA-DOTA]: A solution of MA-SS-CTA (4.68 mg), MA-SS-MA (23.45 mg) and MA-pr-N_3 (29.28 mg) in MeOH (1.00 mL) was added into a solution of OEGMA (308.73 mg), MA-SS-DOTA (503.90 mg) and VA-044 (1.37 mg) in 6.00 mL of a mixed solvent (H_2O/MeOH = 1.5:1, v/v), and the resulting mixture was bubbled with argon under an ice bath for 30 min to strip oxygen. The mixture was stirred in the dark at 47 ˚C for 20 h and quenched with liquid nitrogen at the end of the reaction. The crude product was purified by dialysis against water (MWCO: 2 kDa) for 48 h in the dark. After filtration through a sterile syringe filter, the filtrate was freeze-dried to give poly[OEGMA-DOTA] (1100 mg) as a pale pink solid. The structure of the product was characterized by ^{1}H NMR (**Figure S1**) and GPC (**Figure S3**).

Synthesis of poly[OEGMA-DOTA(Gd)]: A solution of $GdCl_3 \cdot 6H_2O$ (308 mg) in RO H_2O (5 mL) was slowly dropped into a solution of poly[OEGMA-DOTA] (305 mg) in RO H_2O (10 mL). The pH of the resulting mixture was adjusted to 5.20-5.40 using a dilute sodium hydroxide solution and

the mixture was stirred overnight in the dark. The crude product was purified by dialysis against water (MWCO: 2 kDa) for 48 h in the dark. After filtration through a sterile syringe filter, the filtrate was freeze-dried to give poly[OEGMA-DOTA(Gd)] (312 mg) as a pale pink solid. The poly[OEGMA-DOTA(Gd)] product contained 6.94 % of Gd(III) measured by ICP-OES.

Synthesis of poly[OEGMA-DOTA(Gd)]-Cy5: A solution of DBCO-Cy5 (2.50 mg) in DMSO (1mL) was added into a solution of the poly[OEGMA-DOTA(Gd)] (224 mg) in RO H_2O (10 mL), and the reaction mixture was stirred at room temperature overnight in the dark. The crude product was purified by dialysis against water (MWCO: 2000 Da) for 48 h in the dark. After filtration through a sterile syringe filter, the filtrate was freeze-dried to give poly[OEGMA-DOTA(Gd)]-Cy5 (225 mg) as a bluish violet solid. The Cy5 content of poly[OEGMA-DOTA(Gd)]-Cy5 measured by UV-Vis spectrometry was 0.75 %.

1.3 *In vivo* pharmacokinetics of gadolinium

Fifteen healthy BALB/C mice (8-10 weeks, 20 ± 2 g) were randomly divided into 3 groups (n = 5 per group). POLDGd, PODGd and DTPA-Gd at a dose of 0.08 mmol Gd^{3+}/kg were injected into the mice 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(III) concentration by ICP-MS.

1.4 *Flow cytometry analysis*

H22 and LO_2 cells were seeded in glass-bottom culture dishes overnight. POLDGd or PODGd at an equivalent Cy5 dose of 0.5 μg/mL was added for 1, 2 and 4 h-incubation. The cells were collected, washed with PBS, and detected via flow cytometry.

1.5 Biodistribution analysis

Twenty healthy female BALB/C mice (8-10 weeks, 20 ± 2 g) were randomly divided into 2 groups (n = 10 per group). POLDGd and PODGd at a dose of 0.08 mmol Gd^{3+}/kg were injected into these mice through their tail vein. On day 1 after injection, the mice were sacrificed and major organs (heart, liver, spleen, lung and kidney) were collected, lyophilized and weighed. These organs were digested with HNO_3 and H_2O_2 and the supernatants were analyzed to determine the residual concentration of gadolinium by ICP-MS.

1.6 CCK-8 Assay

H22 cells and HUVECs were seeded in 96-well plates at a concentration of 1×10^4 cells per well. POLDGd, PODGd and DTPA-Gd at different concentrations (25 μg/mL, 50 μg/mL, 100 μg/mL, 200 μg/mL and 400 μg/mL) were added to the modified eagle medium (MEM, Hyclone, Cytiva). Fresh media were used to replace spent 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 in an incubator, the absorbance at 450 nm was measured with a multifunctional enzyme labeler (Thermo Fisher SCIENTIFIC).

1.7 Complement activation and contact activation

Human platelet factor 4 (PF4) as a platelet activating factor (Cusabio Biotech, China), and human complement fragment 3a (C3a) and human complement fragment 5a (C5a) as indicators for complement activation (Cusabio Biotech, China) were measured via commercial enzyme-linked immunosorbent assays (ELISA). POLDGd and PODGd were prepared at a concentration of 1.0 mg/mL. 10 μL of the nanoparticle sample was added to 500 μL of human whole blood. After incubation for 1 h at 37°C, the whole blood was centrifuged (2500 g, 2-8 °C) for 10 min to obtain the plasma. Meanwhile, cellulose was used as a positive control group and the whole blood as a blank control group.

For the C3a assay, 5 μL of the obtained plasma was diluted 500 times with the C3a-sample diluent. 100 μL of the diluted plasma was added into an antibody-coated well provided from the C3a kit. For the C5a assay, 10 μL of the obtained plasma was diluted 10 times with the C5a-sample diluent. The diluted plasma was added into another antibody-coated well provided from the C5a kit. For the PF4 assay, 40 μL of the obtained plasma was diluted 10 times with the PF4-sample diluent. 200 μL of the diluted plasma was added into an antibody-coated well provided from the PF4 kit.

Finally, detections were conducted according to the instruction manuals. Five samples in each group were used, and the results were expressed as mean ± SD (n = 5).

1.8 Histopathological study

Fifteen healthy female BALB/C mice (8-10 weeks, 20 ± 2 g) were randomly divided into 3 groups (n = 5 per group). Two groups were injected with POLDGd and PODGd at a dose of 0.08 mmol Gd^{3+}/kg via tail vein and one group with saline as a control group. All mice were sacrificed after 1

day. The major organs (heart, liver, spleen, lung and kidney) and the blood samples were collected. The organs were fixed with a 4% paraformaldehyde solution for 48 h and embedded in paraffin. HE staining and blood biochemical index analysis were then performed.

2. Results and Discussion

2.1 Figures

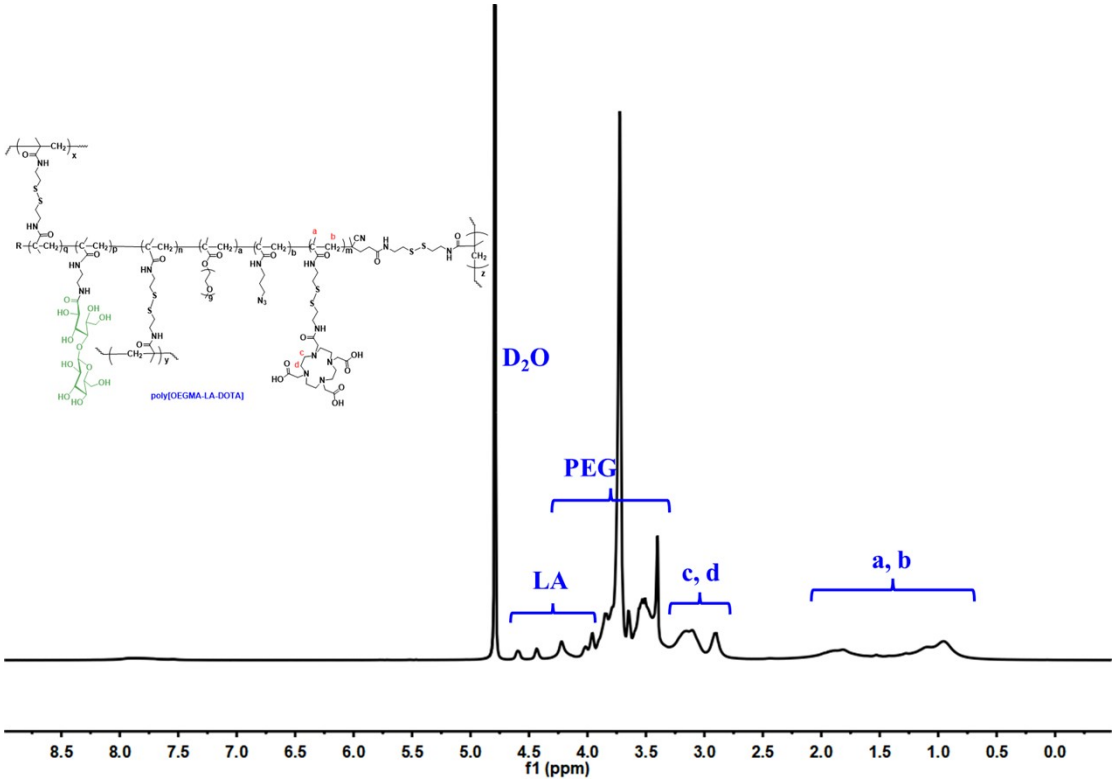


Figure S1. ^{1}H NMR spectrum of poly[OEGMA-LA-DOTA] (recorded in D_2O). The distinct peaks appeared at 4.6–3.9 ppm, indicating that lactobionic acid was polymerized into the main chain of poly[OEGMA-LA-DOTA].

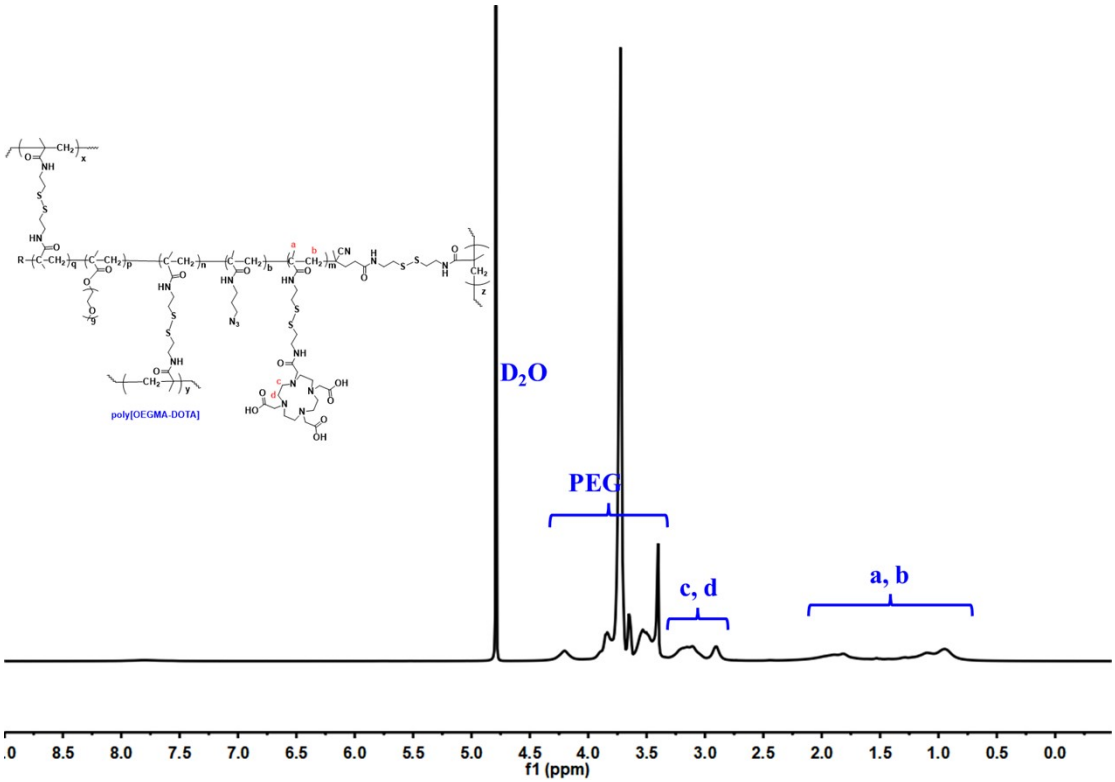


Figure S2. ^{1}H NMR spectrum of poly[OEGMA-DOTA] (recorded in D_2O).

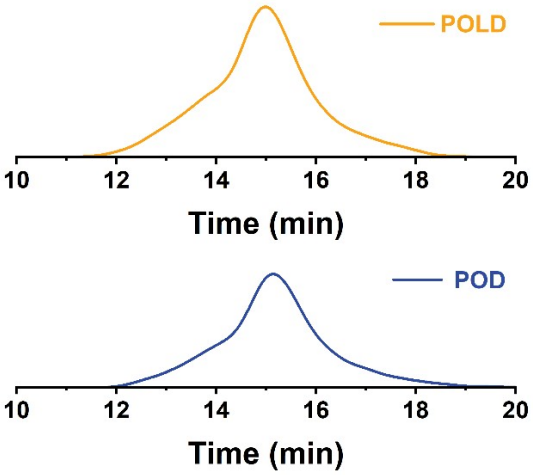


Figure S3. GPC results of the prepared polymers POLD and POD. The mobile phase was a 0.2 M lithium chloride solution in a mixed solvent (H_2O: DMF=35:65, v/v) at a flow rate of 0.5 mL/min (45 °C).

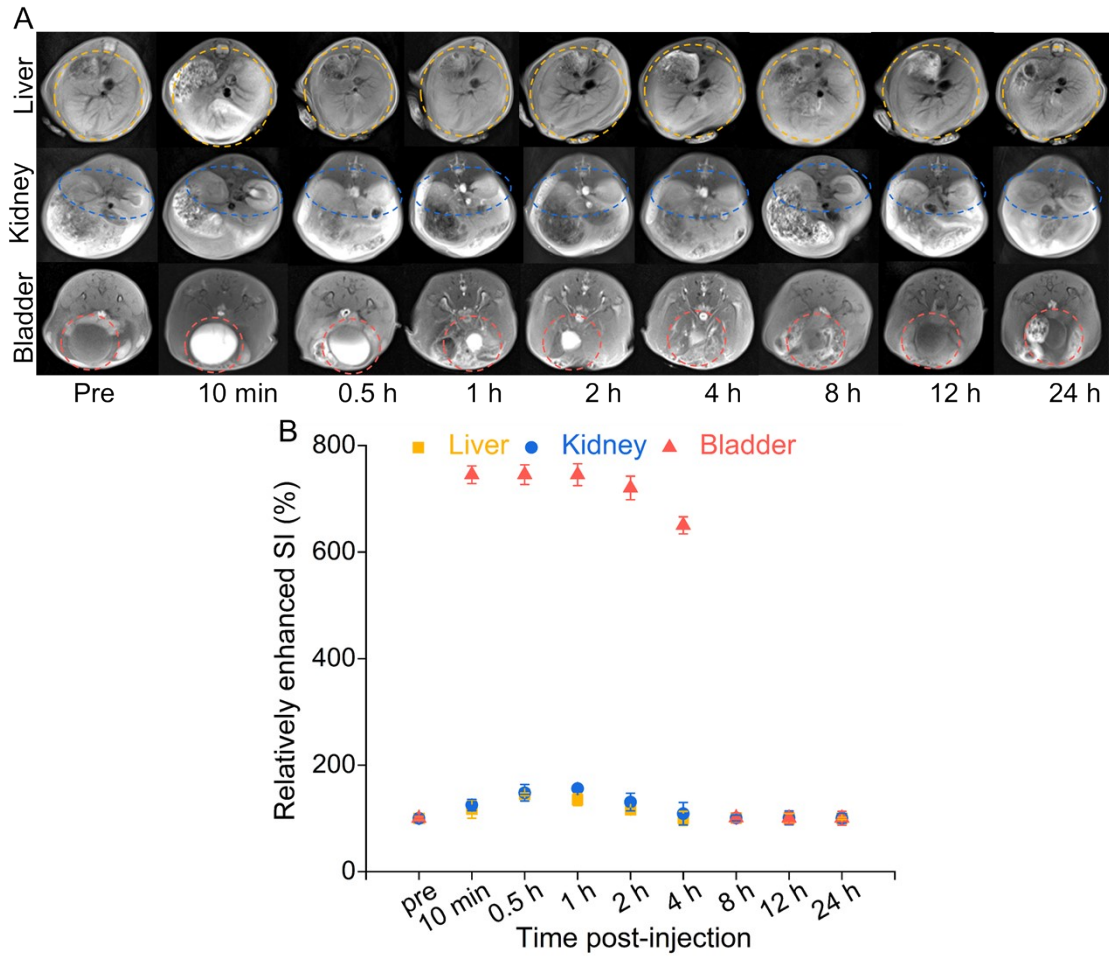


Figure S4. (A) MR images of main excretory organs and (B) relatively enhanced SI% in the MR images after injection of DTPA-Gd.

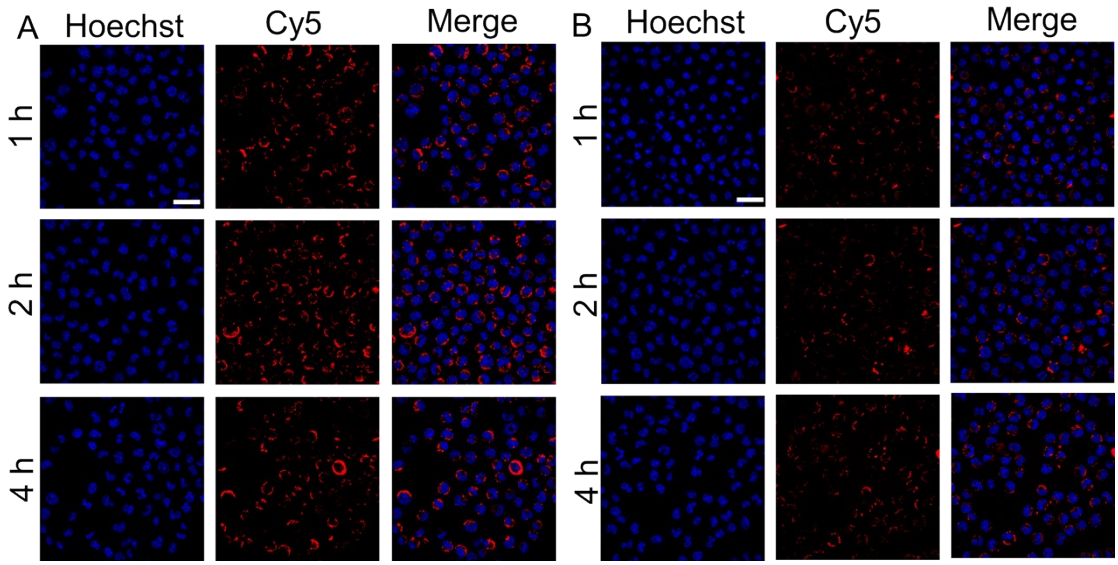


Figure S5. Fluorescence images of H22 cells incubated with POLDGd (A) and PODGd (B) for 1, 2 and 4 h. Scale bar: 25 μm.

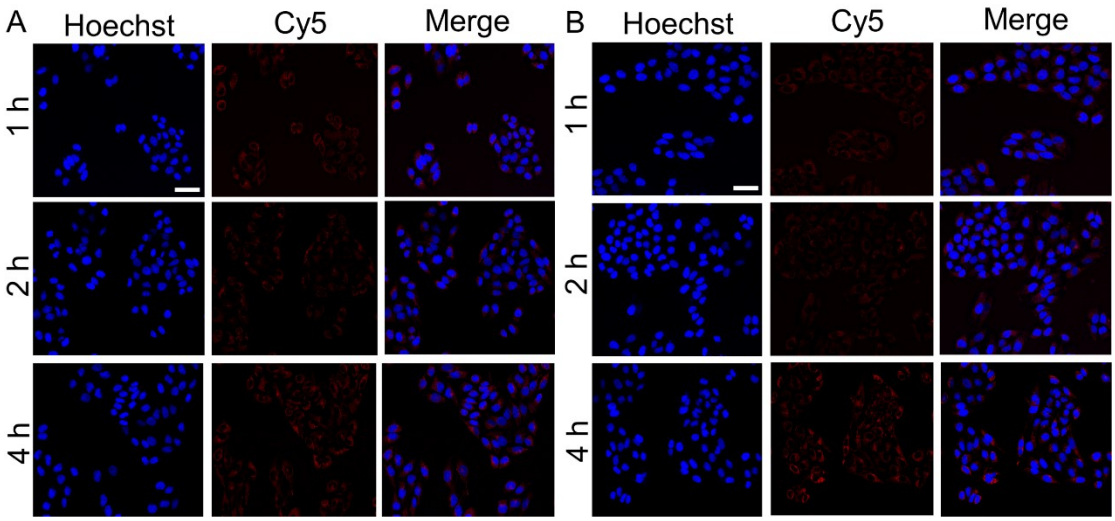


Figure S6. Fluorescence images of LO_2 cells incubated with POLDGd (A) and PODGd (B) were added to each well at a Cy5 dose of 0.5 μg/mL. Scale bar: 25 μm.

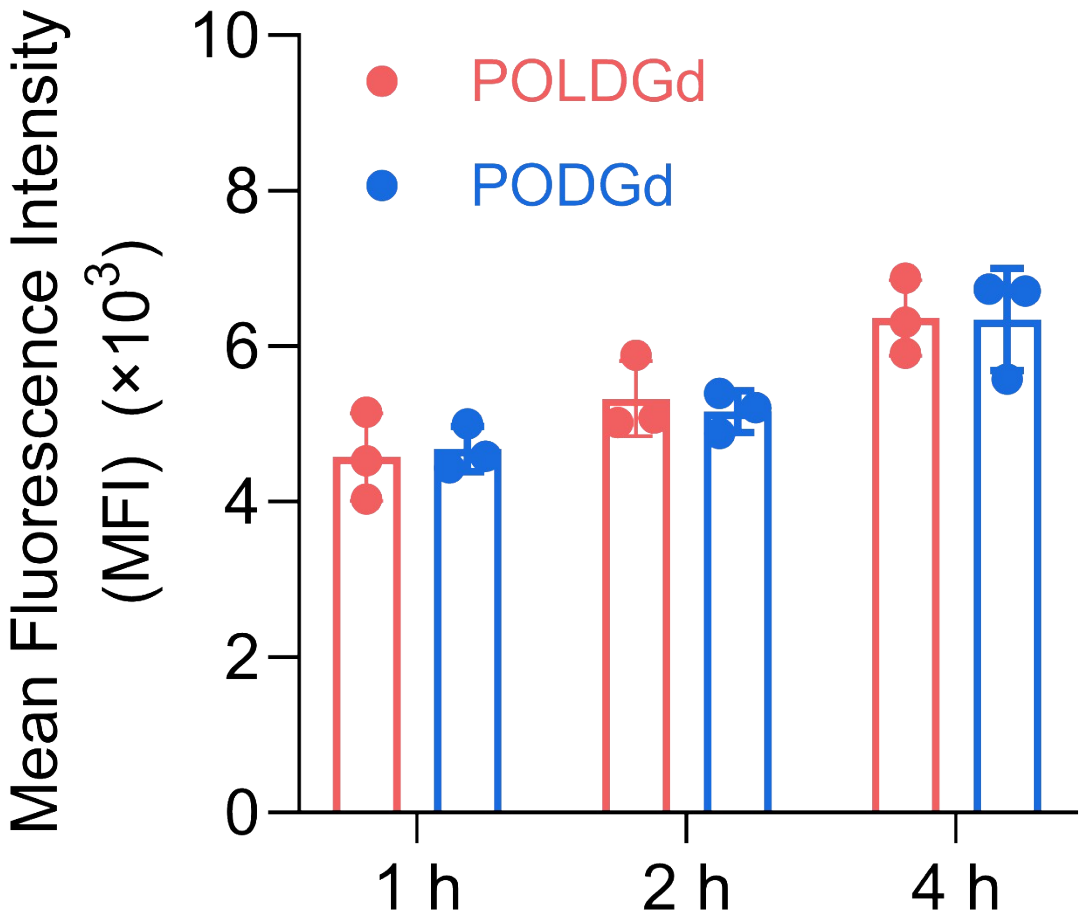


Figure S7. Flow cytometry analysis LO_2 cells after incubation with POLDGd and PODGd for 1, 2 and 4 h.

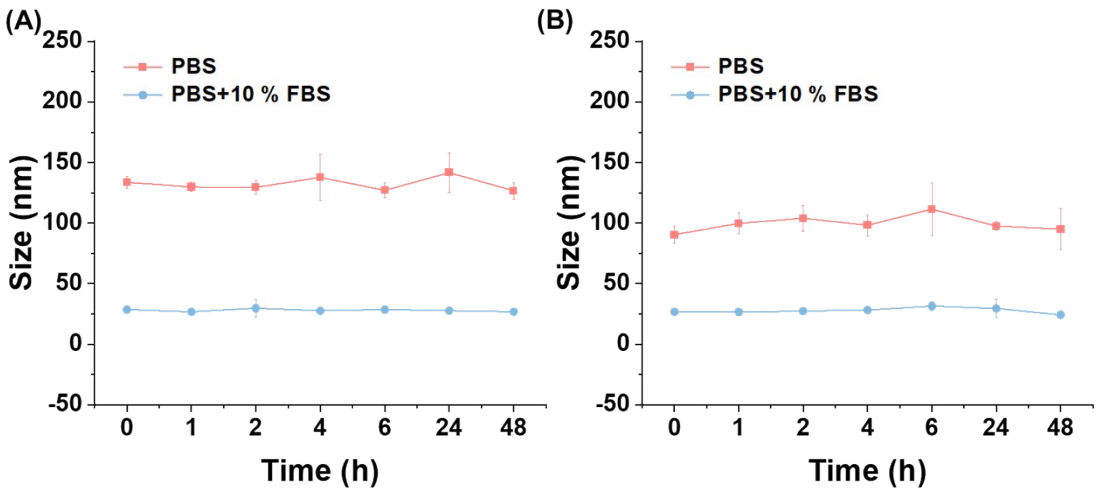


Figure S8. Stability of PODGd and POLDGd in various solutions including PBS and PBS + 10% FBS.

2.2 Tables

Table S1. GPC results of poly[OEGMA-LA-DOTA] and poly[OEGMA-DOTA].

Polymers	Mn (kDa)	Mw (kDa)	PDI
poly[OEGMA-LA-DOTA]	33.82	45.24	1.34
poly[OEGMA-DOTA]	38.73	48.16	1.24

Table S2. Physiochemical properties of synthetic polymers.

Polymers	Size (nm)	Zeta Potential (mV)	Gd(III) Loading Efficiency (wt%)
Poly[OEGMA-LA-DOTA(Gd)]	103.67±5.76	-2.39±1.06	7.58 %
Poly[OEGMA-DOTA(Gd)]	135.28±5.38	-3.93±1.26	6.94 %

3. References

1. X. Wang, J. Chen, Z. Li, Y. Li, Y. Zhang, Q. Gong and K. Luo, *Journal of Controlled Release*, 2024, **373**, 905-916.
2. X. Zhang, X. Wang, Z. Li, J. Du, X. Xiao, D. Pan, H. Zhang, X. Tian, Q. Gong and Z. Gu, *Nanoscale*, 2023, **15**, 809-819.