Electronic Supplementary Information

Phosphorous core-shell tecto dendrimers for enhanced tumor imaging: the rigidity of backbone matters

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Experimental section

Materials

Generation 3 (G3) poly(amidoamine) (PAMAM) dendrimers were purchased from Dendritech (Midland, MI). Generation 2.5 phosphorus dendrimer (P-G2.5) was from Professor Jean-Pierre Majoral (Laboratoire de Chimie de Coordination du CNRS, Toulouse, France). 1-(3-Dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDC·HCl), N-hydroxysuccinimide (NHS), tetraazacyclododecane tetraacetic acid (DOTA)-NHS, and Cyanine5.5 (Cy5.5)-NHS were from Sigma-Aldrich (St. Louis, MO). 1-Adamantaneacetic acid (Ad-COOH), β-cyclodextrin (CD), 1,3-propane sulfonate (1,3-PS) and N, N-carbonyldiimidazole (CDI) were from J&K Scientific Ltd. (Shanghai, China). Dimethyl sulfoxide (DMSO) was from Shanghai Lingfeng Chemical Reagent Co., Ltd. (Shanghai, China). Dulbecco's Modified Eagle Medium (DMEM), fetal bovine serum (FBS), 0.25% trypsin-ethylene diamine tetraacetic acid (EDTA), penicillin and streptomycin were from Gibco (Carlsbad, CA). Cell Counting Kit-8 (CCK-8) and BCA Protein Assay Kit were from Beyotime Biotech Co., Ltd. (Shanghai, China). 4'6-Diamidino-2-phenylindole (DAPI) was acquired from BestBio Biotechnology Co., Ltd. (Shanghai, China). Regenerated cellulose dialysis membranes were from Shanghai Yuanye Biotechnology Co., Ltd. (Shanghai, China). Water used in all experiments was purified using a PURIST UV Ultrapure Water System (RephiLe Bioscience, Ltd., Shanghai, China) with a resistivity higher than 18.2 MΩ·cm.

Preparation of P-G2.5/G3-DOTA-Cy5.5-PS and G3/G3-DOTA-Cy5.5-PS core-shell tecto dendrimers (CSTDs)

The P-G2.5/G3 CSTDs and G3-CD/Ad-G3 CSTDs (G3/G3 CSTDs) were synthesized according to the procedures described in our previous reports.[1-3] To synthesize P-G2.5/G3-DOTA CSTDs, a DMSO solution (5 mL) containing DOTA-NHS (3.13 mg) was dropwise added to a DMSO solution (5 mL) of P-G2.5/G3 CSTDs (30 mg) under stirring for 24 h. Then, a DMSO solution (5 mL) of Cy5.5 (2.94 mg) was dropwise added to the above mixture and stirred for 24 h at room temperature. After that, the reaction mixture was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with

a molecular weight cut-off (MWCO) of 3500 and lyophilized to obtain the P-G2.5/G3-DOTA-Cy5.5 CSTDs. Afterwards, a water solution (5 mL) containing P-G2.5/G3-DOTA-Cy5.5 (25 mg) was mixed with an aqueous solution (5 mL) of 1,3-PS (1.4 mg) under stirring for 24 h. The reaction mixture was dialyzed and lyophilized to obtain the final product of P-G2.5/G3-DOTA-Cy5.5-PS CSTDs. The synthesis process of G3/G3-DOTA-Cy5.5-PS CSTDs was the same as above. To be brief, a DMSO solution (5 mL) containing DOTA-NHS (2.69 mg) was dropwise added to a DMSO solution (5 mL) of G3/G3 CSTDs (30 mg) under stirring for 24 h to obtain the G3/G3-DOTA CSTDs. Then, a DMSO solution (5 mL) of Cy5.5 (2.53 mg) was dropwise added to the above mixture while stirring at room temperature for 24 h. After that, the reaction mixture was dialyzed against water (9 times, 2 L) for 3 days using a dialysis membrane with a molecular weight cut-off (MWCO) of 3500, and lyophilized to obtain the G3/G3-DOTA-Cy5.5 CSTDs. Afterwards, a water solution containing G3/G3-DOTA-Cy5.5 (25 mg, 5 mL) was mixed with 1,3-PS (1.24 mg, in 5 mL water) under stirring for 24 h, and the reaction mixture was dialyzed and lyophilized to obtain the final product of G3/G3-DOTA-Cy5.5-PS CSTDs.

For comparison and characterization, P-G2.5/G3-DOTA, P-G2.5/G3-DOTA-PS, P-G2.5/G3-DOTA-Cy5.5, G3/G3-DOTA, G3/G3-DOTA-PS, G3/G3-DOTA-Cy5.5, and G3/G3-DOTA-Cy5.5-PS were also synthesized, purified through dialysis, and lyophilized to obtain the respective solid products, which were stored at -20 °C for further use.

Characterization techniques

^{1}H NMR was performed on a Bruker AV400 NMR spectrometer (Karlsruhe, Germany) using D_2O as the solvent. The P-G2.5/G3-DOTA, P-G2.5/G3-DOTA-PS, G3/G3-DOTA, or G3/G3-DOTA-PS with a mass of 10 mg was dissolved in 600 μL D_2O before characterization. Atomic force microscopy (AFM) was performed using a Molecular Force Probe 3D (MFP-3D) AFM (Asylum Research, Santa Barbara, CA). Each sample was dissolved in water at a concentration of 1 mg/mL before AFM measurements. The P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS was dropped onto a clean silicon wafer, settled naturally for 2 h and dried with nitrogen to prepare the respective AFM sample. UV-vis spectra were acquired using a Lambda 25 UV-vis spectrophotometer (Perkin Elmer,

Boston, MA) and all samples were dispersed in water (0.125, 0.25, and 0.5 mg/mL, respectively) before assessments. Fluorescence spectroscopy was carried out using a QuantMaster-40 fluorescence spectrometer (Protein Technologies Inc., Tucson, AZ) at the excitation wavelength of 675 nm. Each sample was dispersed in water at a corresponding Cy5.5 concentration of 5 μM before evaluation. Dynamic light scattering (DLS) and zeta potential measurements were performed by a Malvern Zetasizer Nano ZS system (Worcestershire, UK) equipped with a standard 633-nm laser. Each sample was dissolved in water at a concentration of 2 mg/mL before measurements. To examine the stability of the prepared CSTDs, the P-G2.5/G3-DOTA-Cy5.5-PS (2 mg/mL) or G3/G3-DOTA-Cy5.5-PS (2 mg/mL) CSTDs were dispersed in water, phosphate buffered saline (PBS) or cell culture medium (DMEM with 10% FBS), and their hydrodynamic size was measured by DLS every two days for one week. The radiochemical purity of ^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS and ^{99m}Tc-G3/G3-DOTA-Cy5.5-PS was tested by instant thin layer chromatography (ITLC) with the assistance of silica gel-coated fiber glass sheets (Macherey-Nagel, GmbH&Co. KG, Düren, Germany). Saline was used as a mobile phase, and the sheets were measured using a thin layer chromatogram scanner (Bioscan Inc., Tucson, AZ).

Protein Resistance Assay

The antifouling property of P-G2.5/G3-DOTA-Cy5.5, G3/G3-DOTA-Cy5.5, P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS was confirmed by protein resistance assay *via* UV-vis spectroscopy. Briefly, bovine serum albumin (BSA, 2 mg in 1 mL PBS) solution was mixed with P-G2.5/G3-DOTA-Cy5.5, G3/G3-DOTA-Cy5.5, P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS at different concentrations (0.125, 0.5, or 1.5 mg/mL, respectively) and the mixture solution was incubated at 37 °C for 4 h. Next, each mixture solution was centrifuged at 7500 rpm for 15 min to obtain the non-adsorbed BSA in the supernatant. The BSA content in the supernatant was measured using the BCA Protein Assay Kit according to the kit instructions to determine the antifouling property of different complexes.

***In vitro* cytotoxicity assay**

CCK-8 assay was performed to analyze the cytotoxicity of P-G2.5/G3-DOTA-Cy5.5-PS and G3/G3-DOTA-Cy5.5-PS using normal cells (L929 cells, a mouse fibroblast cell line) and cancer cells (B16 cells, a murine melanoma cell line). L929 cells were seeded in a 96-well plate at a density of 1×10^4 cells per well with 100 μL of complete RPMI-1640 medium for each well and incubated overnight. B16 cells were seeded in a 96-well plate at a density of 1×10^4 cells per well with 100 μL of complete DMEM medium for each well and incubated overnight. Afterwards, the medium in each well was replaced with fresh medium containing P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS at different concentrations (0, 100, 500, 1000, 2000, 3000, and 5000 nM, respectively) and cells were cultured for 24 h. Subsequently, the medium in each well was taken out and the cells were washed with PBS for three times, followed by incubation with 100 μL serum-free medium containing 10% CCK-8 for additional 3 h in the cell incubator. Finally, the absorbance of each well was recorded by a Thermo Scientific Multiskan MK3 ELISA reader (Waltham, MA) at 450 nm. For each sample, 6 parallel wells were tested to give a mean value and standard deviation.

Cellular uptake assays

In brief, B16 cells were seeded in a 12-well plate at a density of 1×10^5 cells per well with 1 mL of medium and cultured overnight. Afterwards, the medium was replaced with 1 mL of medium containing P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS at different concentrations (0, 0.5, 1, 2, and 5 μM, respectively) and cells were incubated for additional 4 h. Then, the cells were washed with PBS, trypsinized, and collected for flow cytometry analysis using a FACS Calibur flow cytometer (Becton Dickinson, Franklin Lakes, NJ).

Confocal laser scanning microscopy (CLSM, Carl Zeiss LSM 700, Jena, Germany) was used to observe the intracellular uptake of P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS *in vitro*. In brief, B16 cells were seeded in confocal dishes at a density of 1×10^5 cells per dish with 1.0 mL medium and incubated overnight. The next day, the medium in each dish was substituted with fresh medium containing P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS at a Cy5.5 concentration of 5 μM and the cells were incubated for 4 h. Thereafter, the culture medium was

removed, and the cells were washed with PBS for three times, fixed with glutaraldehyde (2.5%) for 15 min, and counter stained with DAPI for 15 min at 37 °C for CLSM observation.

Penetration of multicellular tumor spheroids (MCTSs)

MCTSs composed of B16 cells were constructed according to the literature with some modifications.[4] Briefly, 500 μL of agarose solution (1%, w/v, in sterile saline) was dropped into the micro-mold to form 3D Petri Dish gels. Then, the solidified 3D Petri Dish gels were separated from the micro-mold, placed to each well of a 12-well plate, and equilibrated for more than 15 min with 2.5 mL DMEM. Then, the culture medium outside and inside the cell seeding chamber of the 3D Petri Dish was removed. After that, the cell suspension (190 μL) containing 5×10^5 B16 cells was carefully seeded into the cell seeding chamber of each 3D Petri Dish. After 10 min, the additional fresh culture medium (2.5 mL) was slowly added to each well of the 12-well plate to allow the cells to aggregate and grow. After about 7 days of culture, the MCTSs with a diameter of 300 μm were built up successfully.

To study the penetration of P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS complexes, the MCTS of B16 cells were incubated with P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS complexes at a Cy5.5 concentration of 5 μM for 6 h. Then, the MCTSs were washed with PBS and observed by CLSM with Z-stack scanning from the top to bottom with 5 μm per section. The Cy5.5 fluorescence intensity in each B16 MCTS sample was analyzed by Zeiss CLSM software.

Hemolysis assay

All animal experiments were performed following the protocols approved by the Animal Care and Use Committee of Donghua University (approval # DHUEC-STCSM-2021-03) and also in accordance with the policy of National Ministry of Health of China. Briefly, 1.5 mL of blood collected from the inner canthus vein plexus of mice was diluted with 3.5 mL of normal saline (NS), and then the pure red blood cells (RBCs) were obtained *via* repeated centrifugation/redispersion steps (2000 rpm, 10 min, 3 times). The RBCs were then diluted with 5 mL of NS. Afterwards, 500 μL of the obtained RBC suspension was mixed with 500 μL water (positive control), NS (negative control), and

NS solutions of P-G2.5/G3-DOTA-Cy5.5-PS at different concentrations (1, 2.5, 5, or 10 μM), respectively. After 2 h incubation at 37 °C, all the samples were centrifuged at 10000 rpm for 5 min. Then, the photos of the samples were taken and the absorbance of the obtained supernatant at 540 nm was measured *via* UV-vis spectrometry. The hemolysis rate was calculated according to the following formula: hemolysis rate (%) = $[(A_{(sample,\ 540\ nm)} - A_{(negative,\ 540\ nm)}) / (A_{(positive,\ 540\ nm)} - A_{(negative,\ 540\ nm)})] \times 100\%$.

***In vivo* fluorescence imaging of tumors**

Male C57BL/6 mice (15-20 g, 4-6 weeks old, Shanghai Slac Laboratory Animal Center, Shanghai, China) were used to establish the subcutaneous melanoma tumor model. Each mouse was subcutaneously injected with 2×10^6 B16 cells into the right hind leg. When the tumor volume reached about 100 mm^3, each tumor-bearing mouse was intravenously injected with 100 μL PBS containing P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS complexes at a corresponding Cy5.5 concentration of 5 μM to evaluate tumor imaging ability *in vivo*. The fluorescence images of the mice were recorded at 0, 0.5, 1, 2.5, 3.5, or 12 h postinjection using a Tanon ABL X6 *in vivo* imaging system (Shanghai, China). The tumors were extracted from the mice at different time points post intravenous injection to measure the fluorescence intensity. The mice were sacrificed at 12 h post intravenous injection of P-G2.5/G3-DOTA-Cy5.5-PS or G3/G3-DOTA-Cy5.5-PS and the tumors and main organs (heart, liver, spleen, lungs, and kidneys) of mice were extracted to analyze the *ex-vivo* fluorescence signal intensity.

***In vivo* single-photon emission computed tomography (SPECT) imaging and distribution**

The B16 tumor-bearing mice with a tumor volume of 100 mm^3 were randomly divided into two groups and intravenously administrated with ^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS or ^{99m}Tc-G3/G3-DOTA-Cy5.5-PS (^{99m}Tc = 1 mCi, 200 μL in saline). Then, the SPECT images of the mice were recorded at 30, 60, 90, 150, or 210 min postinjection using an Infinia GE SPECT imaging system. The mice were sacrificed at different time points postinjection, and the tumors and main organs of mice were extracted

to analyze the *ex-vivo* SPECT signal intensity.

Biosafety examinations

To evaluate the biosafety of the prepared ^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS and ^{99m}Tc-G3/G3-DOTA-Cy5.5-PS CSTDs, at 7 days post intravenous injection of the respective material (5 mg/kg, ^{99m}Tc = 1 mCi, 100 μL in saline), the major organs (heart, liver, spleen, lungs, and kidneys) of mice were collected, fixed, embedded in paraffin, and sectioned for hematoxylin & eosin staining to observe the histological changes.

Statistical analysis

All experimental data were represented as the mean ± standard deviation through at least three parallel experiments. One-way analysis of variance statistical method was used to analyze the experimental results through IBM SPSS Statistic 26 software (IBM, Armonk, NY). A p value of 0.05 was selected as a significance level, and the data were indicated with (*) for $p < 0.05$, (**) for $p < 0.01$, and (***) for $p < 0.001$, respectively.

Table S1. Hydrodynamic size and polydispersity index (PDI) of various complexes.

Sample	Hydrodynamic size (nm)	Polydispersity index (PDI)	Zeta potential (mV)
G3-CD/Ad-G3	207.9 ± 14.8	0.64 ± 0.08	15.6 ± 3.6
G3/G3-DOTA-Cy5.5-PS	298.9 ± 14.7	0.39 ± 0.05	5.1 ± 0.4
P-G2.5/G3	144.5 ± 44.5	0.30 ± 0.01	21.4 ± 2.1
P-G2.5/G3-DOTA-Cy5.5-PS	176.8 ± 0.5	0.38 ± 0.03	13.0 ± 1.9

Table S2. The radiochemical purity of ^{99m}Tc-G3/G3-DOTA-Cy5.5-PS and ^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS in PBS after stored for different time periods (n = 3).

Sample	^{99m}Tc-G3/G3-DOTA-Cy5.5-PS	^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS
0 h	99.6 ± 0.30%	99.7 ± 0.10%
1 h	99.1 ± 0.40%	99.1 ± 0.44%
3 h	98.7 ± 0.44%	98.2 ± 0.95%
6 h	98.8 ± 0.26%	96.9 ± 0.26%

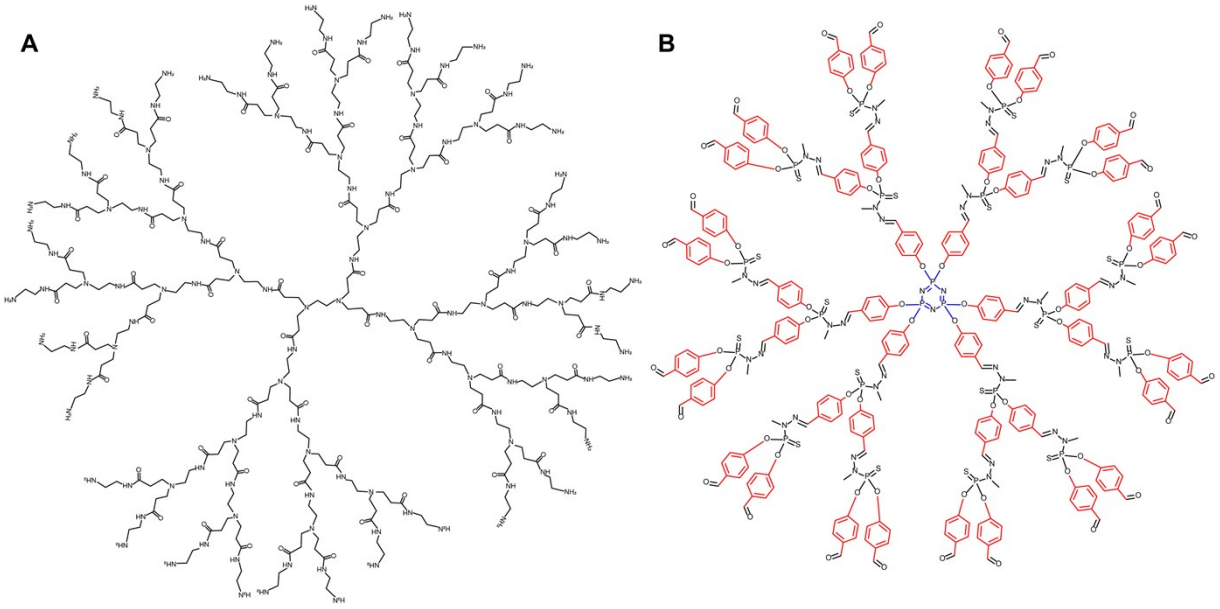

Figure S1. 2D chemical structures of (A) generation 3 PAMAM dendrimer and (B) generation 2.5 phosphorus dendrimer.

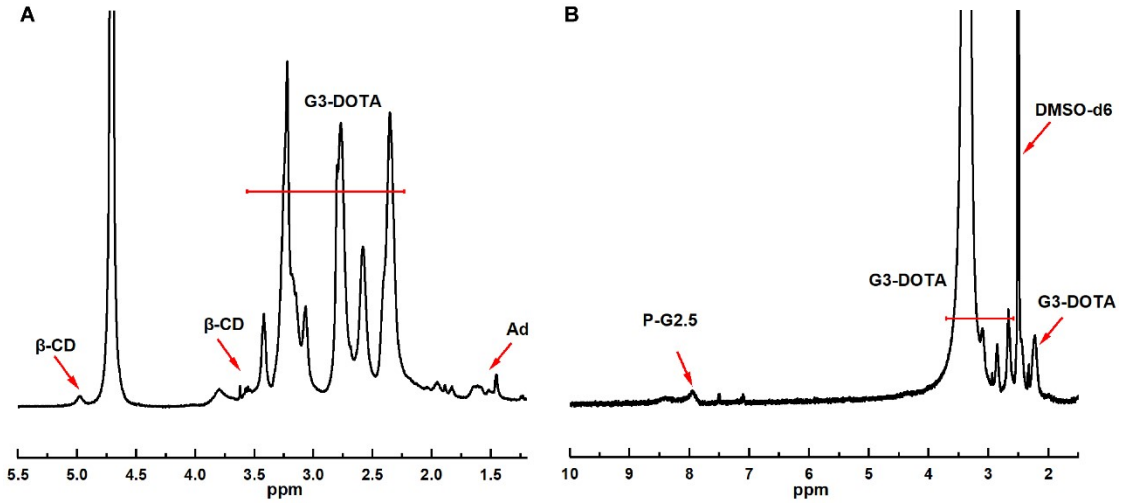

Figure S2. ^{1}H NMR spectra of (A) G3/G3-DOTA and (B) P-G2.5/G3-DOTA.

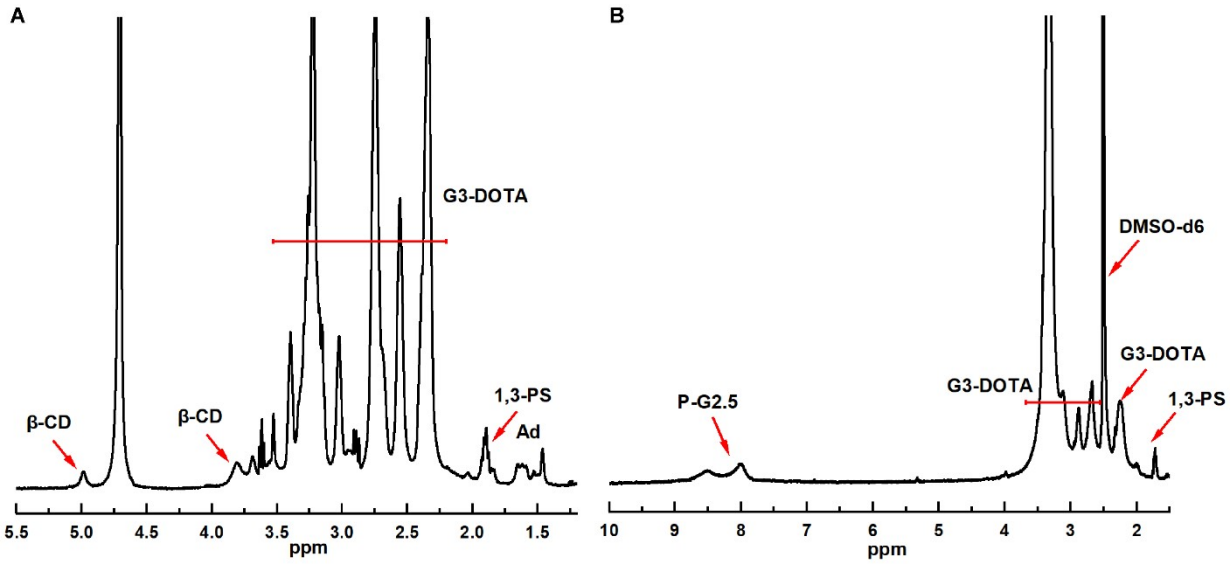

Figure S3. ^{1}H NMR spectra of (A) G3/G3-DOTA-PS and (B) P-G2.5/G3-DOTA-PS.

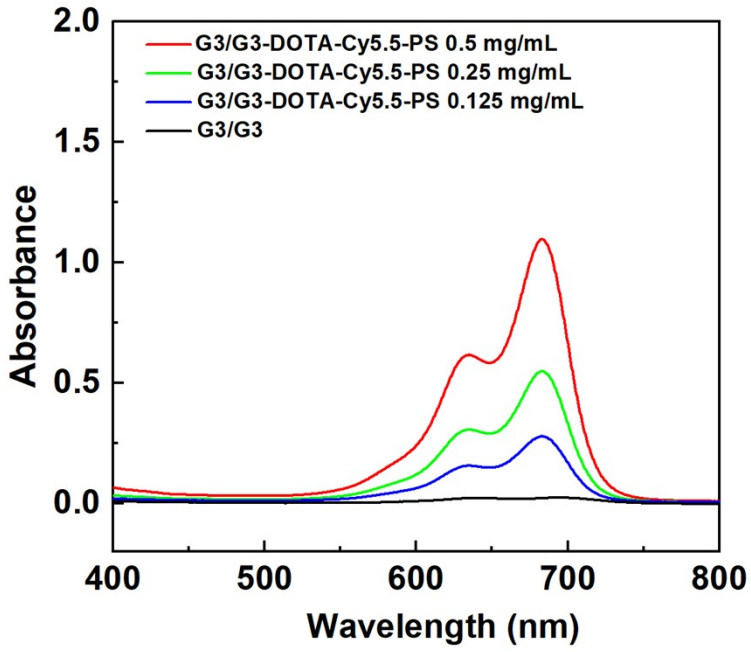

Figure S4. UV-vis spectra of G3/G3 CSTDs and G3/G3-DOTA-Cy5.5-PS complex at different concentrations (0.125, 0.25, and 5 mg/mL, respectively).

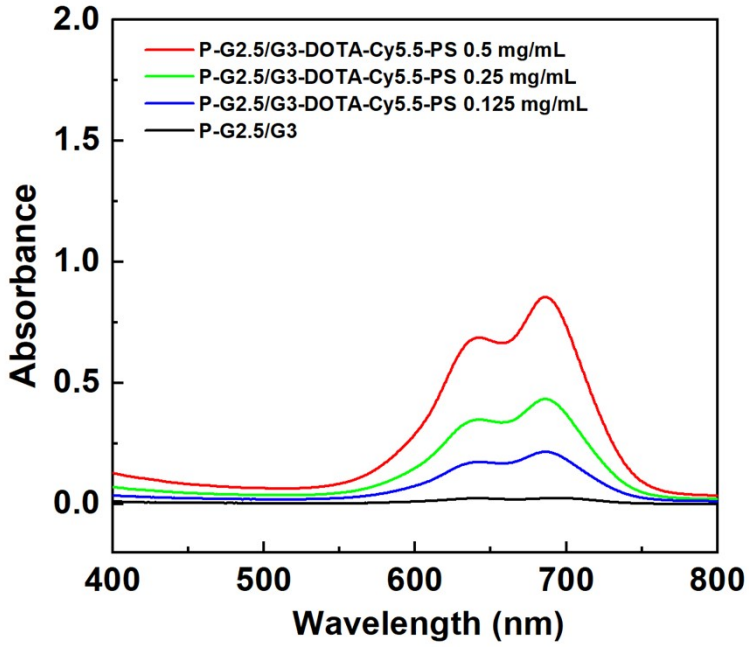

Figure S5. UV-vis spectra of P-G2.5/G3 CSTDs and P-G2.5/G3-DOTA-Cy5.5-PS CSTDs at different concentrations (0.125, 0.25, and 5 mg/mL, respectively).

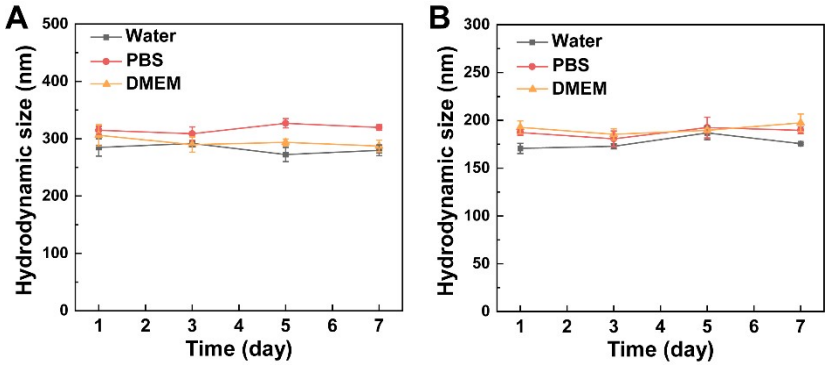

Figure S6. Hydrodynamic size variation of (A) G3/G3-DOTA-Cy5.5-PS and (B) P-G2.5/G3-DOTA-Cy5.5-PS within a week when dispersed in water, PBS, and DMEM, respectively.

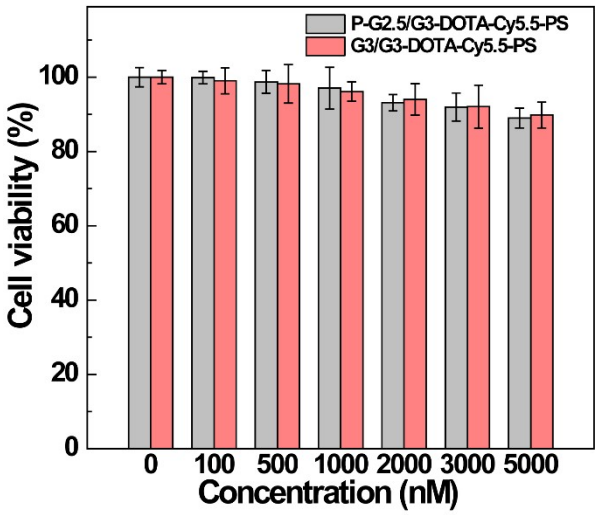

Figure S7. Viability of L929 cells treated with the P-G2.5/G3-DOTA-Cy5.5-PS and G3/G3-DOTA-PS-Cy5.5 under different concentrations for 24 h (n = 6).

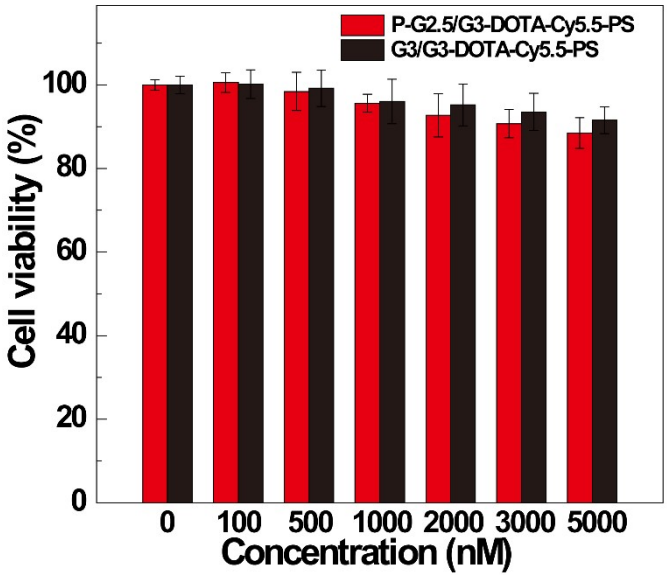

Figure S8. Viability of B16 cells treated with the P-G2.5/G3-DOTA-Cy5.5-PS and G3/G3-DOTA-PS-Cy5.5 under different concentrations for 24 h (n = 6).

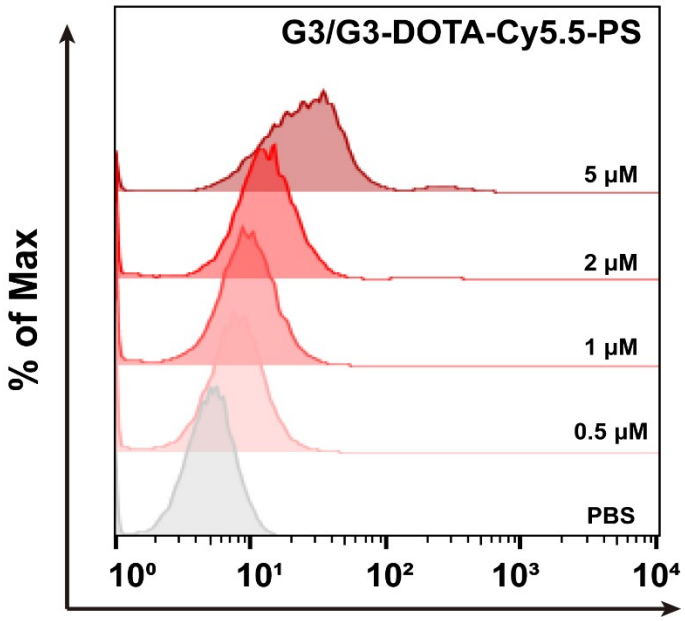

Figure S9. Fluorescence intensity histograms of Cy5.5 in B16 cells after incubation with G3/G3-DOTA-Cy5.5-PS at different Cy5.5 concentrations (0.5, 1, 2, and 5 μM, respectively).

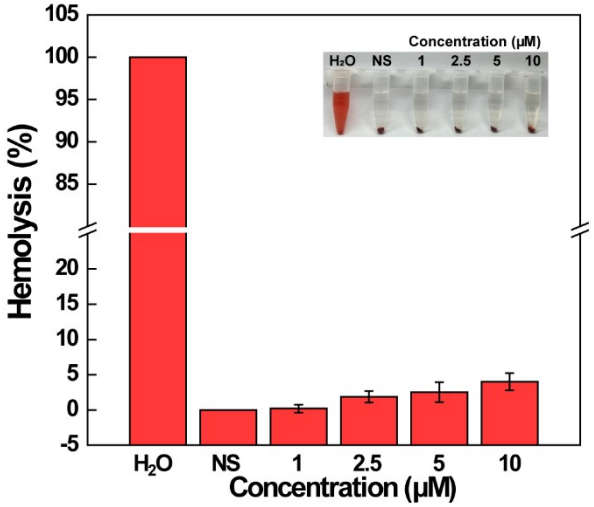

Figure S10. Hemolysis percentages of the mouse RBCs treated with the P-G2.5/G3-DOTA-Cy5.5-PS solution at different concentrations (mean ± SD, n = 3). Inset shows the corresponding digital photos of the supernatants after 2 h co-incubation, followed by centrifugation.

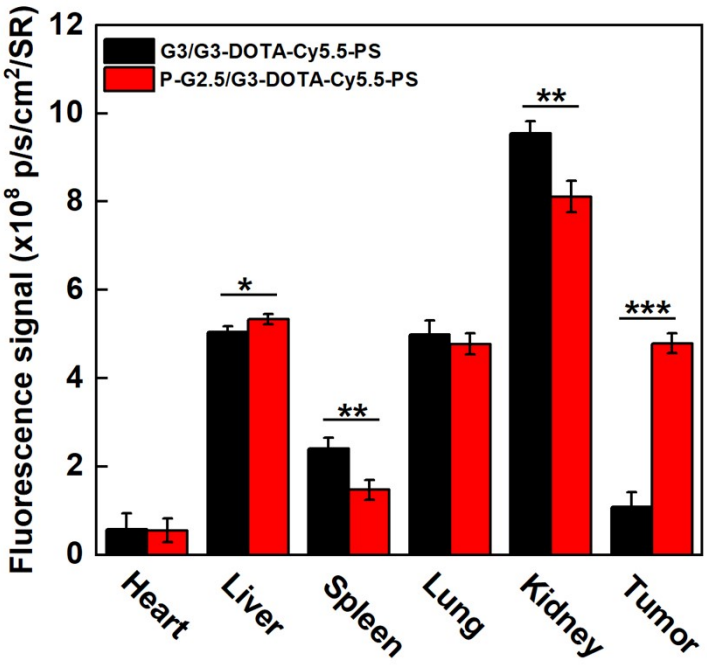

Figure S11. The fluorescence intensity of major organs (heart, liver, spleen, lung, and kidney) and tumors from the mice intravenously injected with G3/G3-DOTA-Cy5.5-PS or P-G2.5/G3-DOTA-Cy5.5-PS after 12 h. *$p < 0.05$, **$p < 0.01$ and ***$p < 0.001$, respectively (n = 3).

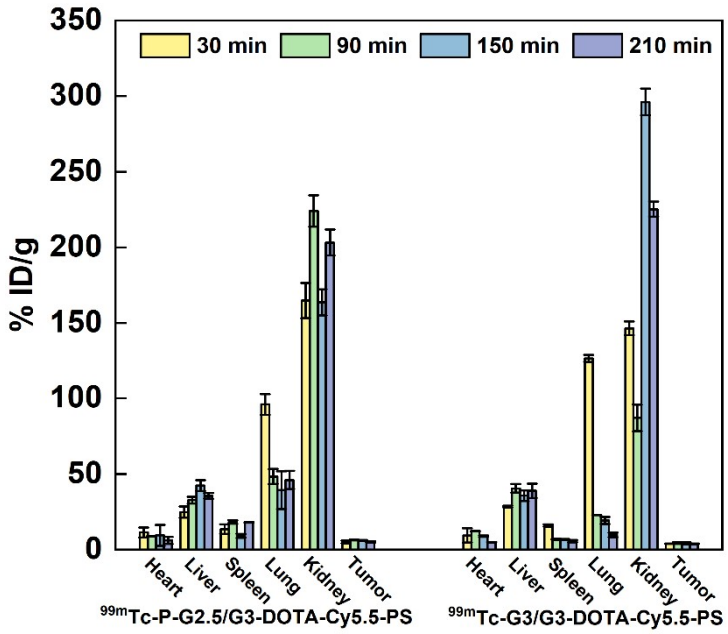

Figure S12. The radioactive contents in main organs (heart, liver, spleen, lung, and kidney) and tumors after intravenous injection of ^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS and ^{99m}Tc-G3/G3-DOTA-Cy5.5-PS for different time points (n = 3).

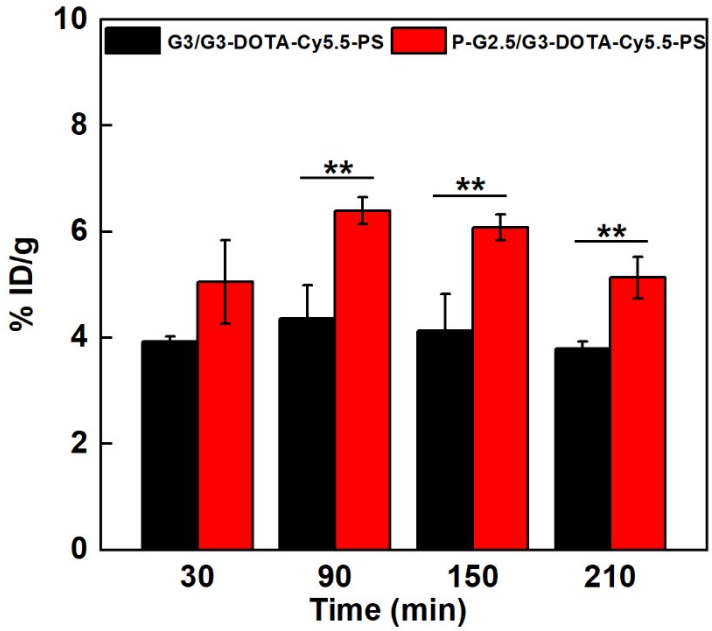

Figure S13. The radioactive contents of tumors after intravenous injection of ^{99m}Tc-P-G2.5/G3-DOTA-Cy5.5-PS or ^{99m}Tc-G3/G3-DOTA-Cy5.5-PS for different time periods (** $p < 0.01$, $n = 3$).

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