Electronic Supplementary Information

Radionuclide ^{131}I-labeled multifunctional dendrimers for targeted SPECT imaging and radiotherapy of tumors†

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Part of Experimental Details:

Synthesis of G5.NHAc-HPAO-PEG-FA dendrimers. FA with 1.5 molar equivalents of NH_2-PEG-COOH (11.37 mg, 0.0257 mmol) dissolved in 5 mL DMSO was mixed with a DMSO solution (2 mL) containing EDC (4.94 mg, 0.0257 mmol), and the reaction mixture was stirred for 3 h to activate the γ-carboxylic acid group of FA. Then the activated FA was dropwise added to a DMSO solution (5 mL) containing NH_2-PEG-COOH (85.86 mg, 0.0172 mmol) under vigorous magnetic stirring at room temperature. The reaction was stopped after 24 h. Then, the reaction mixture was extensively dialyzed against phosphate buffered saline (PBS, 3 times, 4 L) and water (3 times, 4 L) through a 1 000 MWCO membrane for 3 days to remove the excess of reactants, followed by lyophilization to get the product FA-PEG-COOH.

G5.NH_2 (10.0 mg) dissolved in DMSO (5 mL) was reacted with 12 molar equivalents of HPAO (1.21 mg, 5 mL in DMSO) under vigorous magnetic stirring. The reaction was stopped after 24 h to get the raw product of G5.NH_2-HPAO, which was subsequently reacted with the EDC-activated FA-PEG-COOH. In brief, FA-PEG-COOH (100.95 mg) dissolved in 5.0 mL DMSO with 50 molar equivalents of G5.NH_2 was reacted with EDC (73.7 mg, in 5.0 mL DMSO) under vigorous magnetic stirring for 3 h. The EDC-activated FA-PEG-COOH was then added dropwise into the DMSO solution of the raw product of G5.NH_2-HPAO under magnetic stirring. The reaction was continued for 3 days to obtain the raw product of G5.NH_2-HPAO-PEG-FA conjugates, into which triethylamine (35.68 μL) was added and the solution was thoroughly mixed for 30 min. Excess acetic anhydride (20.19 μL, 5 molar equivalents of dendrimer terminal amines) was then added dropwise into the above mixture solution under stirring for 24 h. The final reaction mixture was purified and lyophilized to get the G5.NHAc-HPAO-PEG-FA dendrimers according to the procedure similar to the purification of FA-PEG-COOH, except that a dialysis membrane with MWCO of 14 000 was used. For comparison, G5.NHAc-FI-HPAO-*m*PEG dendrimers without FA were also synthesized using *m*PEG-COOH and characterized.

***In vitro* cytotoxicity assay and cell morphology observation.** MTT assay was used to evaluate the cytotoxicity of the G5.NHAc-HPAO-PEG-FA dendrimers *in vitro*. Briefly, 1×10^4 C6-HFAR cells per well were seeded into a 96-well plate. After overnight incubation to bring the cells to confluence, the medium was replaced with 200 μL fresh medium containing G5.NHAc-HPAO-PEG-FA dendrimers with different concentrations. After 24 h incubation at 37 ^oC, an MTT solution (20 μL in PBS, 5 mg/mL) was added to each well and the cells were incubated for another 4 h at 37 ^oC. After that, the medium in each well was carefully removed, and DMSO (200 μL) was added to dissolve the formed formazan crystals. The assays were carried out according to the manufacturer's instructions at 570 nm using a Thermo Scientific Multiskan MK3 ELISA reader (Thermo Scientific, Waltham, MA). For each sample, mean and standard deviation for the triplicate wells were analyzed.

After treatment with the G5.NHAc-HPAO-PEG-FA dendrimers with different concentrations for 24 h, the morphology of cells was observed using a Leica DM IL LED inverted phase contrast microscope. The magnification was set at 200 × for all samples.

Confocal microscopy. Confocal microscopy (Carl Zeiss LSM 700, Jena, Germany) was used to observe the specific uptake of the G5.NHAc-FI-HPAO-PEG-FA dendrimers by C6-HFAR cells according to our previous reports [1, 2]. Briefly, cover slips with a diameter of 14 mm were pretreated with 5% HCl, 30% HNO_3, and 75% alcohol and then fixed in a 24-well tissue culture plate. 5×10^4 C6 cells were seeded into each well with 1 mL fresh medium and cultured at 37 °C and 5% CO_2 for 12 h to allow the cells to attach onto the cover slips. Then, the medium was discarded, and replaced with 1 mL fresh medium containing PBS (control), G5.NHAc-FI-HPAO-*m*PEG dendrimers (1000 nM), and G5.NHAc-FI-HPAO-PEG-FA dendrimers (1000 nM), respectively. After 2 h incubation at 37 °C and 5% CO_2, the cells were rinsed with PBS for 3 times, fixed with glutaraldehyde (2.5%) for 15 min at 4 °C, and counterstained with Hoechst 33342 (1 μg/mL) for 20 min at 37 °C using a standard procedure. The FI fluorescence was excited with a 488 nm argon blue laser, and the emission was collected through a 505-525 nm barrier filter. The optical section thickness was set at 5 mm. Samples were scanned using a 63× oil-immersion objective lens.

Targeted tumor radiotherapy *in vivo*. The tumor model was established according to the procedures described above. When the tumor nodules attained a volume of 0.5-1.2 cm^3, a solution of the ^{131}I-G5.NHAc-HPAO-*m*PEG or ^{131}I-G5.NHAc-HPAO-PEG-FA dendrimers ([^{131}I] = 37 MBq/mL, 200 μL) was intravenously injected into each nude mouse *via* the tail vein every third day. Mice injected with saline (200 μL), Na^{131}I (200 μL), G5.NHAc-HPAO-*m*PEG dendrimers (200 μL) with the same concentration of the ^{131}I-G5.NHAc-HPAO-*m*PEG dendrimers, and G5.NHAc-HPAO-PEG-FA dendrimers (200 μL) with the same concentration of the ^{131}I-G5.NHAc-HPAO-PEG-FA dendrimers every third day were also tested for comparison. The tumor sizes were measured by calipers every third day and the tumor volume was calculated according to a formula of (tumor length×(tumor width)^2)/2. The relative tumor volume (denoted as V/V_0, where V_0 and V are the tumor volume without treatment (i.e., day 0) and treated at different time points, respectively) and body weight of each mouse were recorded at pre-determined time points.

H&E staining. Mice treated with saline, Na^{131}I, G5.NHAc-HPAO-*m*PEG, G5.NHAc-HPAO-PEG-FA, ^{131}I-G5.NHAc-HPAO-*m*PEG, and ^{131}I-G5.NHAc-HPAO-PEG-FA were euthanized after 21 days' treatment, then the tumors and the major organs including the heart, liver, spleen, lung, and kidney were harvested. The tumors and organs were fixed with 10% neutral buffered formalin, embedded in paraffin, sectioned into slices with a thickness of 4 μm, stained with hematoxylin and eosin (H&E) using a standard procedure, and observed using a Leica DM IL LED inverted phase contrast microscope.

TUNEL assay. Apoptosis was assessed on tumor tissues using a terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) method through the use of *in situ* apoptotic detection kit (Roche, Basel, Switzerland) according to the manufacturer's guidelines. Briefly, the tumors harvested after 21 days' treatment were fixed in paraformaldehyde, dehydrated, paraffin-embedded, and sectioned. The section was then deparaffinized, rehydrated, washed, stained using a TUNEL Kit, and examined with a Leica DM IL LED inverted phase contrast microscope. The number and percentage of TUNEL-positive cells were counted and determined from five random selected fields

per mouse.

References

[1] Li J, He Y, Sun W, Luo Y, Cai H, Pan Y, et al. Hyaluronic acid-modified hydrothermally synthesized iron oxide nanoparticles for targeted tumor MR imaging. Biomaterials. 2014;35 3666-77.
[2] Li J, Zheng L, Cai H, Sun W, Shen M, Zhang G, et al. Polyethyleneimine-mediated synthesis of folic acid-targeted iron oxide nanoparticles for *in vivo* tumor MR imaging. Biomaterials. 2013;34:8382-92.

Table S1. Radiochemical purity of the ^{131}I-G5.NHAc-HPAO-PEG-FA dendrimers at different time periods.

Time (h)	0	1	3	27
Radiochemical purity (%)	100	98.41	96.9	92.84

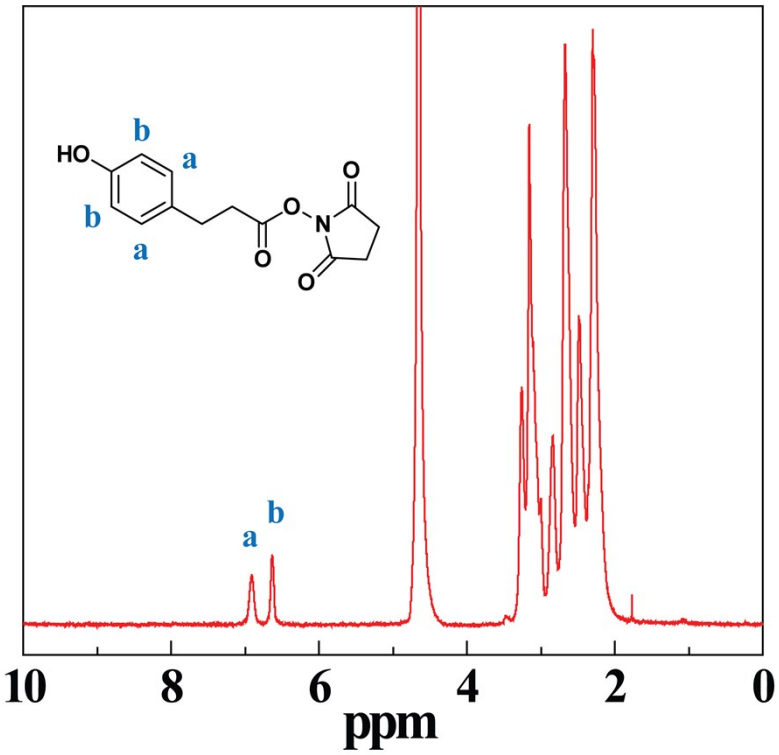

Figure S1. ^{1}H NMR spectrum of G5.NH_2-HPAO dendrimer dissolved in D_2O. Inset shows the molecular structure of HPAO.

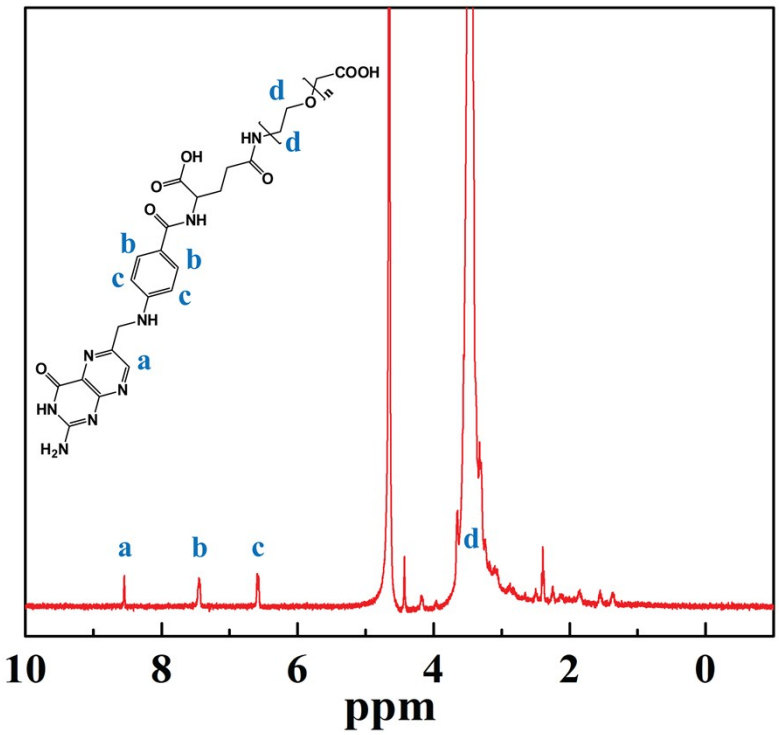

Figure S2. ^{1}H NMR spectrum of FA-PEG-COOH dissolved in D_2O. Inset shows the molecular structure of FA-PEG-COOH.

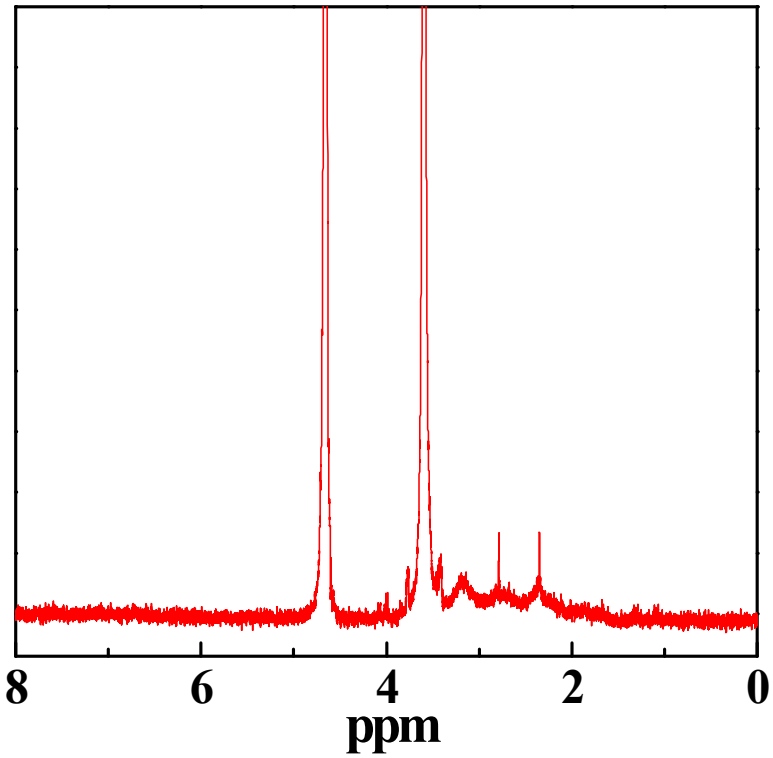

Figure S3. ^{1}H NMR spectrum of G5.NHAc-HPAO-PEG-FA dendrimer dissolved in D_2O.

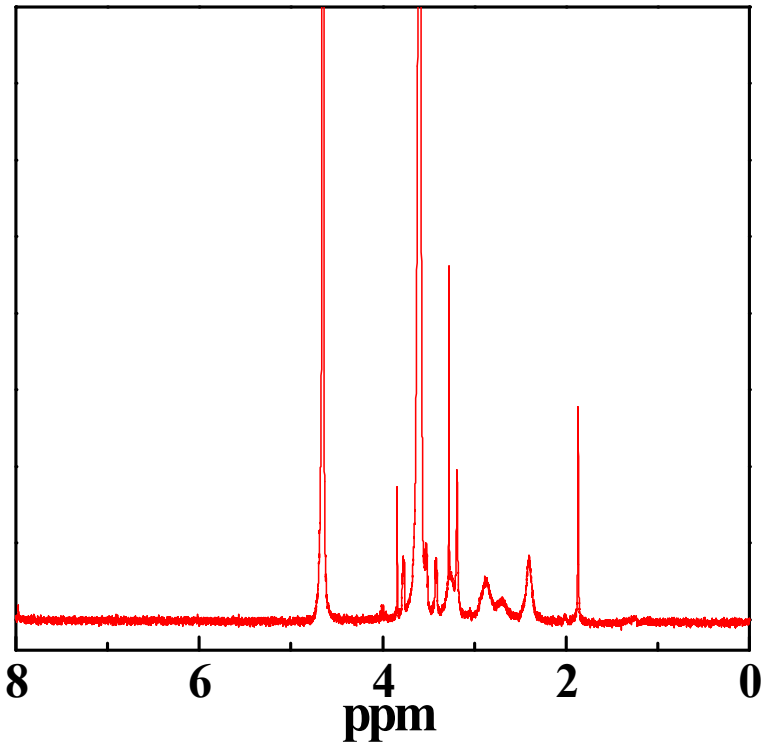

Figure S4. ^{1}H NMR spectrum of G5.NHAc-HPAO-*m*PEG dendrimer dissolved in D_2O.

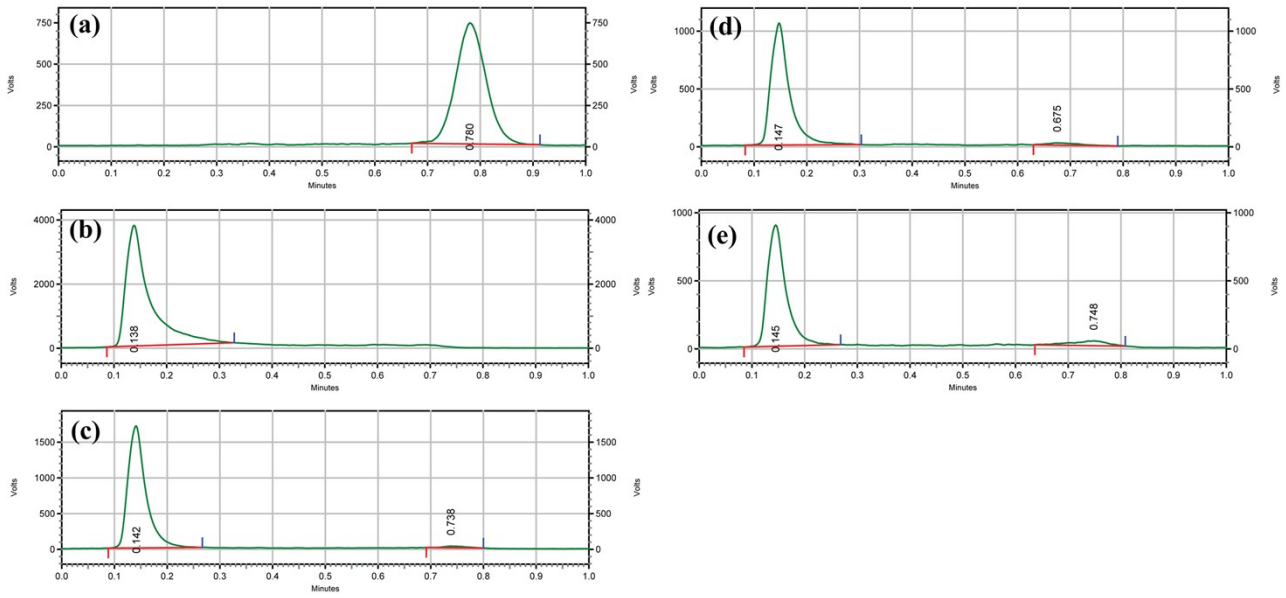

Figure S5. Radiochemical purity of Na^{131}I (a) and the formed ^{131}I-G5.NHAc-HPAO-PEG-FA dendrimers at (b) 0 h, (c) 1 h, (d) 3 h, and (e) 27 h.

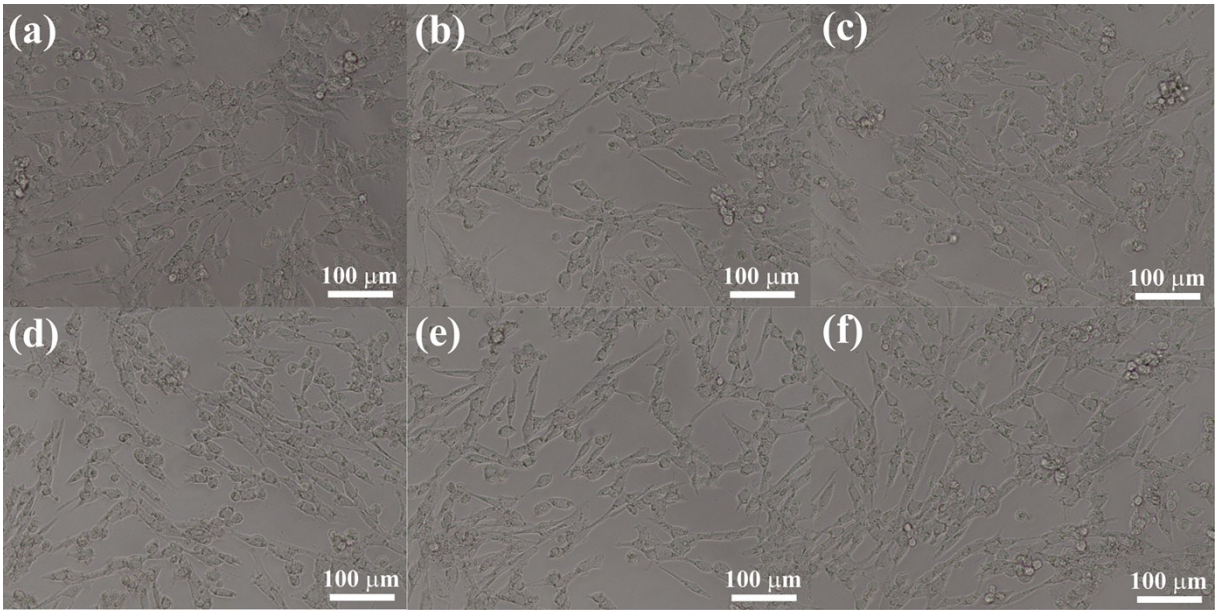

Figure S6. The morphology of C6 cells treated with PBS (a) and G5.NHAc-HPAO-PEG-FA at concentrations of 0.1 μM (b), 1 μM (c), 5 μM (d), 10 μM (e), 20 μM (f), respectively for 24 h.

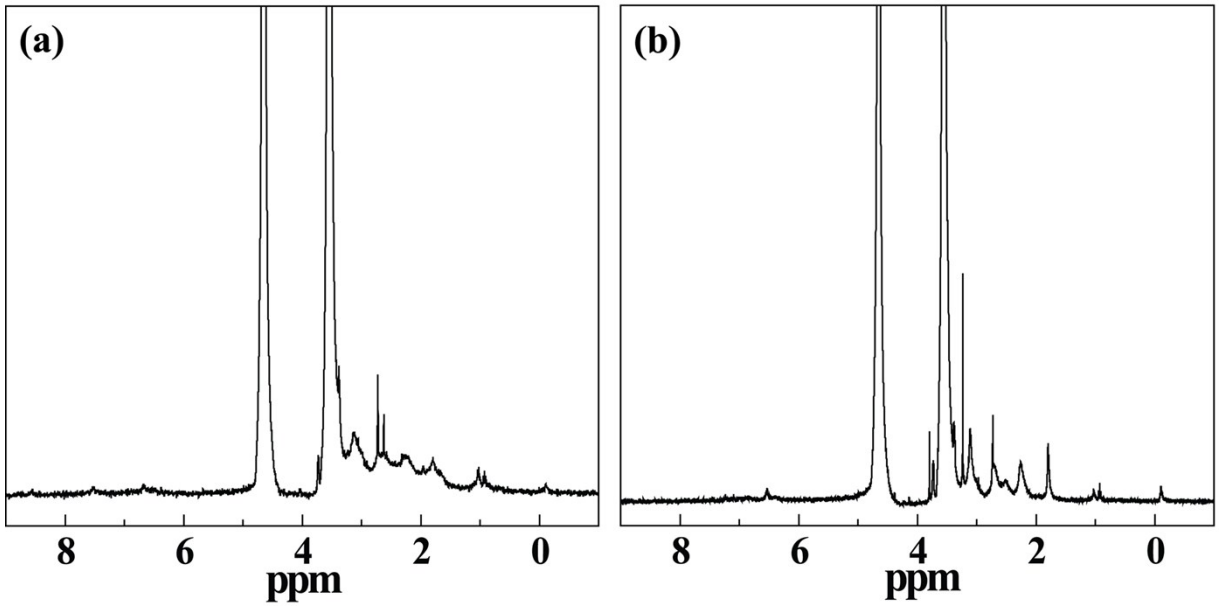

Figure S7. ^{1}H NMR spectra of the G5.NHAc-FI-HPAO-PEG-FA (a) and G5.NHAc-FI-HPAO-*m*PEG (b) dendrimers dissolved in D_2O.

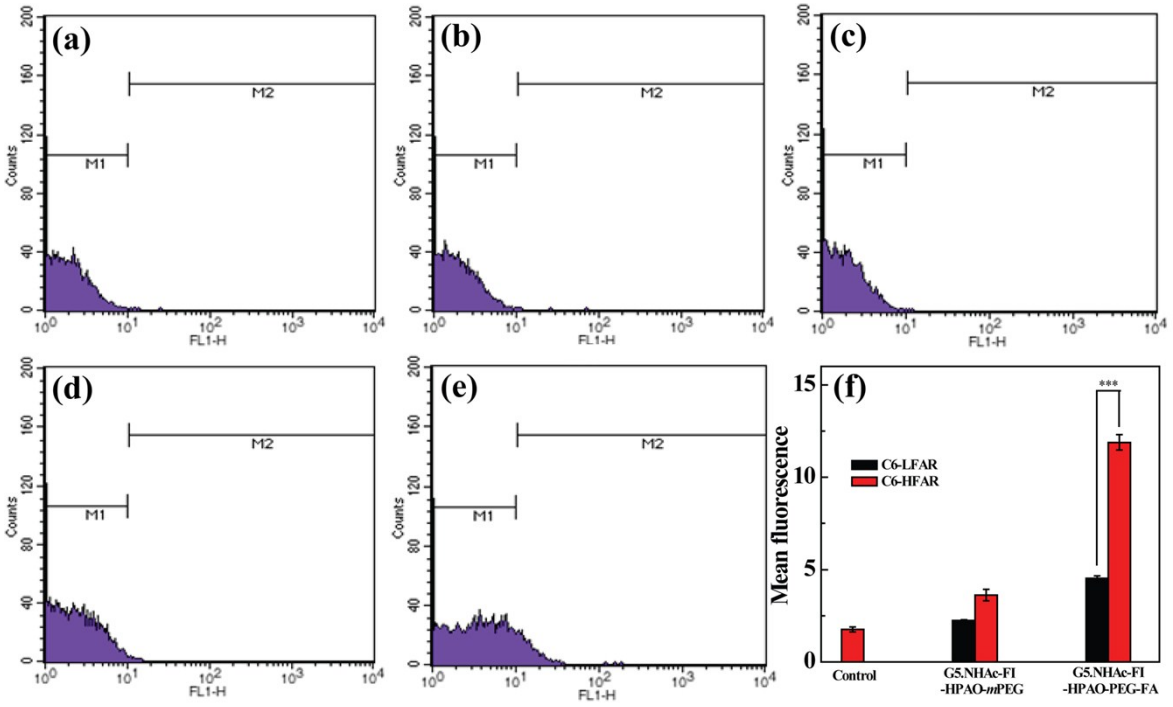

Figure S8. Flow cytometric analysis of C6-HFAR cells treated with PBS (a), C6-LFAR (b) and C6-HFAR (c) cells treated with the G5.NHAc-FI-HPAO-*m*PEG dendrimers, C6-LFAR (d) and C6-HFAR (e) cells treated with the G5.NHAc-FI-HPAO-PEG-FA dendrimers (1000 nM) for 2 h, respectively. (f) shows the comparison of the binding of different dendrimers with different cells.

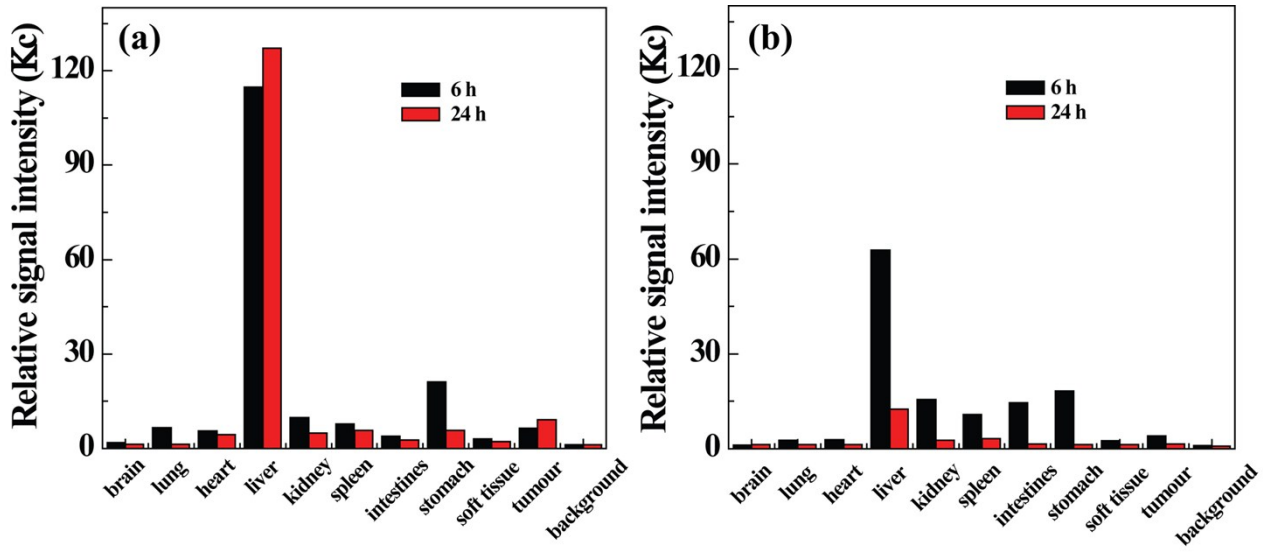

Figure S9. Relative SPECT signal intensity of different organs at different time points post intravenous injection of ^{131}I-G5.NHAc-HPAO-PEG-FA (a) and ^{131}I-G5.NHAc-HPAO-*m*PEG (b).

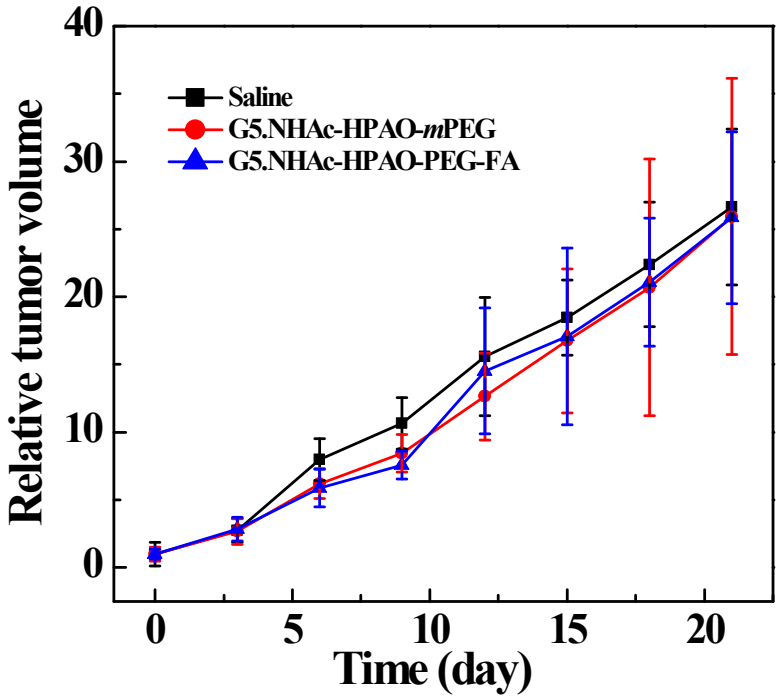

Figure S10. The growth of C6 xenografted tumors after various treatments. The relative tumor volumes were normalized according to their initial tumor volumes (Mean ± SD, n = 5).

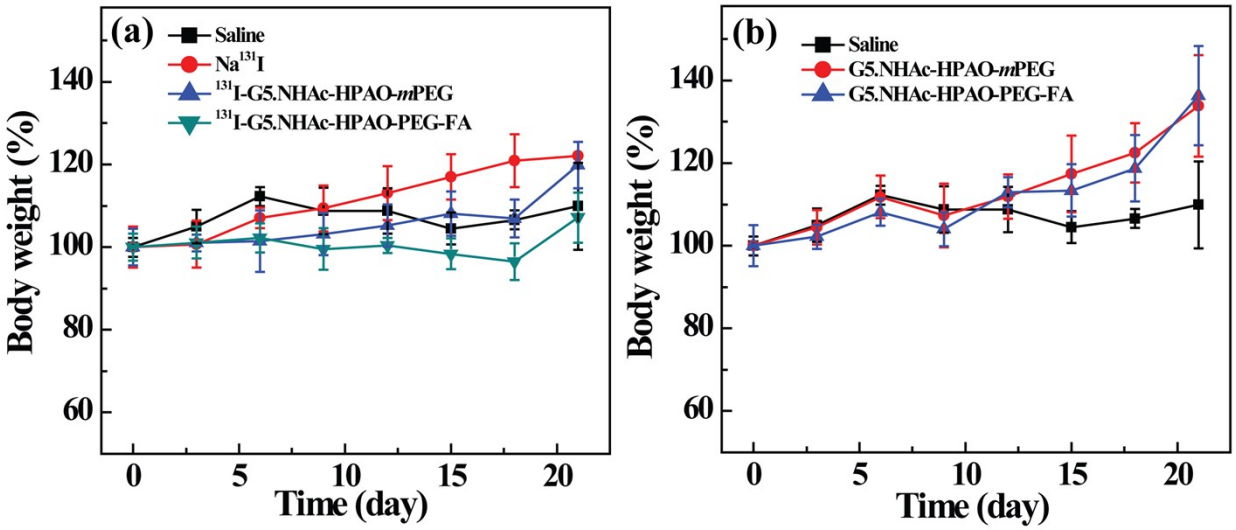

Figure S11. The body weight of C6 tumor-bearing mice after various treatments with ^{131}I (a) and without ^{131}I (b). The relative body weight were normalized according to their initial weights (Mean ± SD, n = 5).