Supporting Information

Salicylic Acid Conjugated Dendrimers Are a Tunable, High Performance CEST MRI Nanoplatform

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1. Materials and methods

1.1. General

The G5-SA-D-Am, G5-SA-D-Diol and G5-SA-D-Ac dendrimers were synthesized using a commercially available generation 5 poly(amidoamine) ethylenediamine (EDA) core - dendrimer terminated with 126 primary amines (for short G5-Am), 1,4,7,10-tetraazacyclododecane-1,4,7,10-tetraacetic acid mono (N- hydroxysuccinimide ester) (D) and 5-aminomethylsalicylic acid methyl ester (SA), glycidol and acetic anhydride.

1.2. Chemicals

All chemicals were purchased from Sigma-Aldrich or Fisher Scientific unless otherwise specified. Diisopropylethylamine (DIPEA), N-(3-Dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) were purchased from Chem Impex International Inc. 5-aminomethylsalicylic acid methyl ester (SAME) was acquired from Astatech Inc. All reagents and solvents were used as received without further purification.

1.3. Synthesis of 5-N-succinamylmethylsalicylic acid methyl ester

5-N-succinamylmethylsalicylic acid methyl ester (SAME) was synthetized several times using the same procedure. 0.15 g ($6.89x10^{-4}$ mole) of 5-aminomethylsalicylic acid methyl ester was dissolved in 10 mL of DMF, followed by addition of 1.2 mole equivalent of 4-dimethylaminopyridine (DMAP, 0.101 g) and succinic anhydride (0.083 g). After 16 hrs of stirring the reaction mixture at room temperature DMF was removed on a rotary evaporator. Obtained residue was dissolved in 50% water-methanol solution and purified on a reversed phase-high performance liquid chromatography (RP-HPLC) system (Varian ProStar) with an Agilent Technology 1260 Infinity photodiode array detector using a semipreparative C-18 Luna column (5 mm, 10 × 250 mm Phenomenex) and a gradient elution starting with 98% H_2O (0.1% TFA) and 2% MeOH (0.1% TFA) reaching 100% of MeOH in 60 min at a flow rate of 4 mL/min. The desired SAME was collected between 28 and 31 min (Figure s1), evaporated on a rotary evaporator and dried for several hours under high vacuum, which resulted in white powder. Typical reaction yield was ~91%.

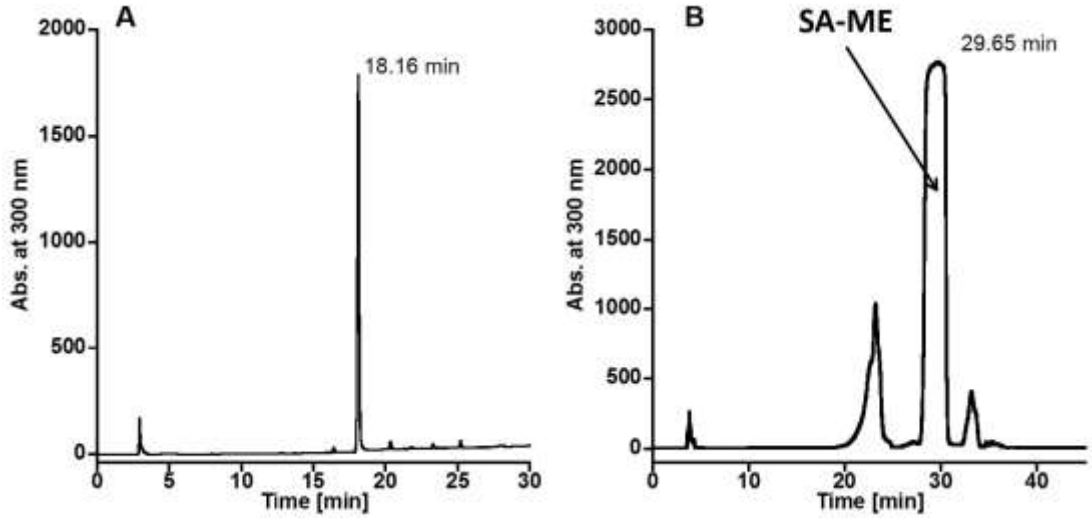

Figure s1. RP-HPLC chromatograms of (**A**) 5-aminomethylsalicylic acid methyl ester (starting material) and (**B**) the reaction mixture for synthesis of SAME. Absence of the peak at 18.16 min in the chromatogram of the reaction mixture indicates formation of the product with elution time at 29.65 min, which was identified by ^{1}H NMR .

1.4. Electrospray Ionization Mass Spectrometry (ESI-MS)

Spectra were recorded on a Bruker Daltonics Esquire 3000 Plus spectrometer using diluted solutions in H_2O-MeOH 50% (v/v) and 0.1% of formic acid.

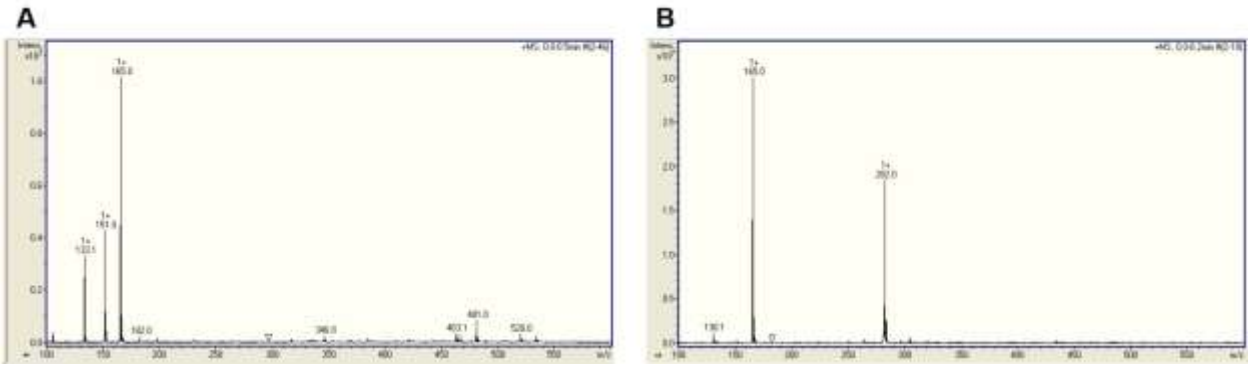

Figure s2. ESI-MS spectra of (**A**) 5-aminomethylsalicylic acid methyl ester, theoretical chemical formula: $C_9H_{11}NO_3$, exact mass: 181.07, molecular weight: 181.19, observed m/z: 182 - $(M + 1)^{+1}$ and fragments formed during ionization 165 $(M + 1)^{+1}$ (5-methylsalicylic acid methyl ester) 151 $(M + 1)^{+1}$ (salicylic acid methyl ester) and 133.1 $(M + 1)^{+1}$ benzoic acid methyl ester and (**B**) *5*-N-succinamylmethylsalicylic acid methyl ester. Theoretical chemical formula: $C_{13}H_{15}NO_6$, exact mass: 281.09, molecular weight: 281.26, observed m/z: 282 - $(M + 1)^{+1}$ and 165 $(M + 1)^{+1}$ fragment formed during ionization - (5-methylsalicylic acid methyl ester).

1.5. Potentiometric Titration

Dendrimer was dissolved in 0.1M NaCl at a concentration of 0.6 mg/mL and the pH was adjusted to ~3.0 with a small amount of concentrated HCl. The titrations were performed at room temperature, using NaOH (0.1 M) used as a titrant and a Mettler Toledo G20 automated titration equipped with an InLab electrode controlled by Mettler Toledo LabX software.

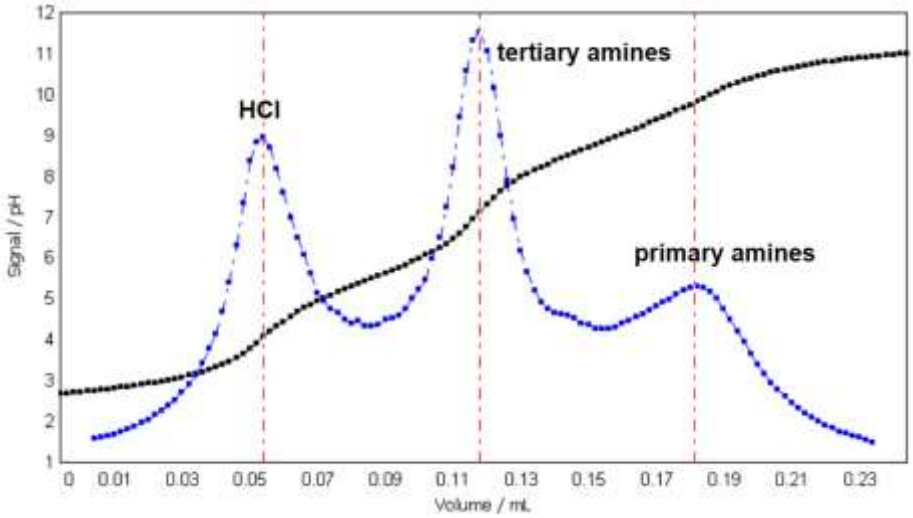

Figure s3. Potentiometric titration curve used to calculate average number of terminal primary amine (126) in the generation 5 PAMAM dendrimer G5-Am, which was utilized as a starting material for synthesis of the nanoparticles.

1.6. Nuclear magnetic resonance (NMR)

All NMR spectra were recorded using a Bruker Avance III 500 MHz NMR spectrometer using DMSO or D_2O as solvents.

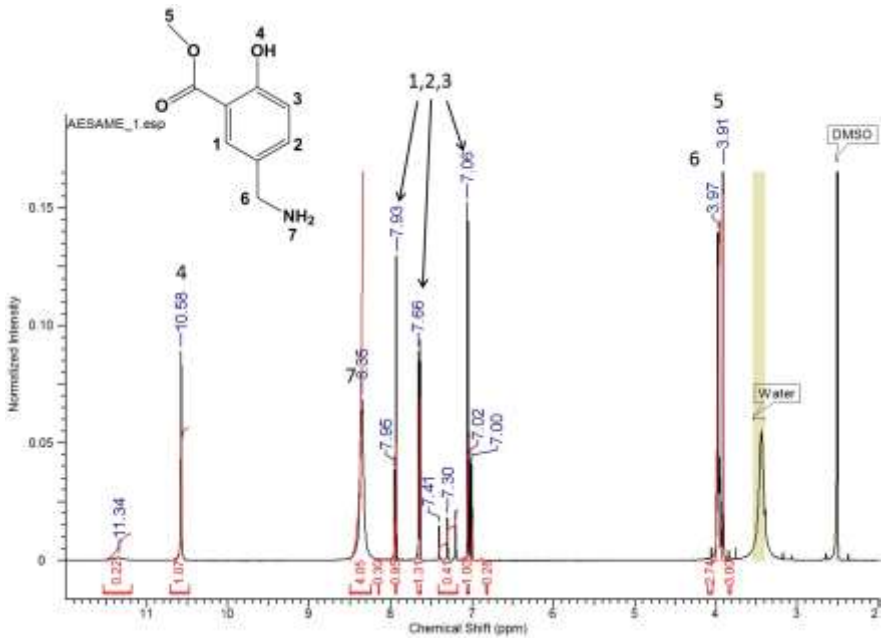

Figure s4A. ^{1}H NMR spectrum of 5-aminomethylsalicylic acid methyl ester recorded using DMSO as a solvent. Values of the integrals correlate with the structure of the purchased compound.

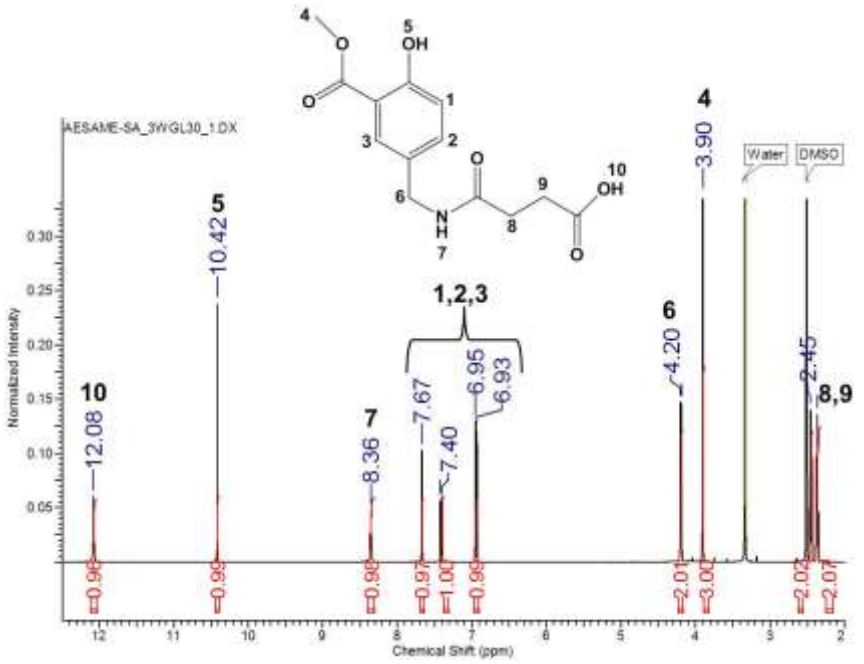

Figure s4B. ^{1}H NMR spectrum of 5-N-succinamylmethylsalicylic acid methyl ester recorded using DMSO as a solvent. Presence of addition peaks at 2.37, 2.45 and 12.08 ppm related to the succinamic acid moiety clearly indicate modification of 5-aminomethylsalicylic acid methyl ester. Spectrum demonstrates high purity of SAME through the exclusive presence of signals related to the analyte.

1.6. Synthesis of G5-SA-D-Ac, G5-SA-D-Diol and G5-SA-D-Ac

Preparation of G5-SA-D-Ac, G5-SA-D-Diol and G5-SA-D-Ac involved multiple step synthesis as presented in Figure 1A. (step **1**) 0.15 g of SAME was dissolved in DMF followed by addition of 2 mole equivalent of N-(3-dimethylaminopropyl)-N′-ethylcarbodiimide hydrochloride (EDC) and 1-hydroxybenzotriazole (HOBt) to activate the carboxyl group. After 30 min DMSO containing 0.02 mole equivalents of G5-Am (MW=28826, terminated with 126 NH_2 groups, dendrimer:SAME molar ratio 1:50) was added. Resulting reaction mixture was stirred at room temperature for 16 h and solvent was removed on a rotary evaporator. The residue was re-suspended in 1xPBS buffer (pH=7.4) and transferred to 15 mL Amicon centrifugal filters with 10k Da MWCO. After centrifugation for 30 minutes at 4100 rpm, 1xPBS buffer was added to re-dissolve the material. This process was repeated twice with PBS buffer and 6 times of DI water. Purified product was dissolved in DI H_2O and lyophilized to give G5-SAME-Am. (step **2**) Subsequently G5-SAME-Am was reacted with 4 mole equivalent DOTA-NHS in DMSO in the presence of DIPEA for 3 h, which was followed by evaporation of the solvent and purification as described above, providing G5-SAME-D-Am. In the next steps (step **4 and 5**) G5-SAME-D-Am was allowed to react with 10 mole equivalent (on the basis of remaining terminal NH_2 groups) of either glycidol or acetic anhydride in methanol for 24 h to provide G5-SAME-D-Diol and G5-SA-D-Ac, respectively. Finally (step **3,5,7**) carboxyl groups of salicylic acid moieties were deprotected (removal of methyl ester - ME) by dissolving obtained conjugates in 2 M NaOH 50% methanolic solution and stirring for 6 h at room temperature. Resulting solutions were diluted with DI H_2O and extensively dialyzed against DI water using regenerated cellulose membrane with 10k Da MWCO. The retentates were filtered, excess of H_2O evaporated and lyophilized.

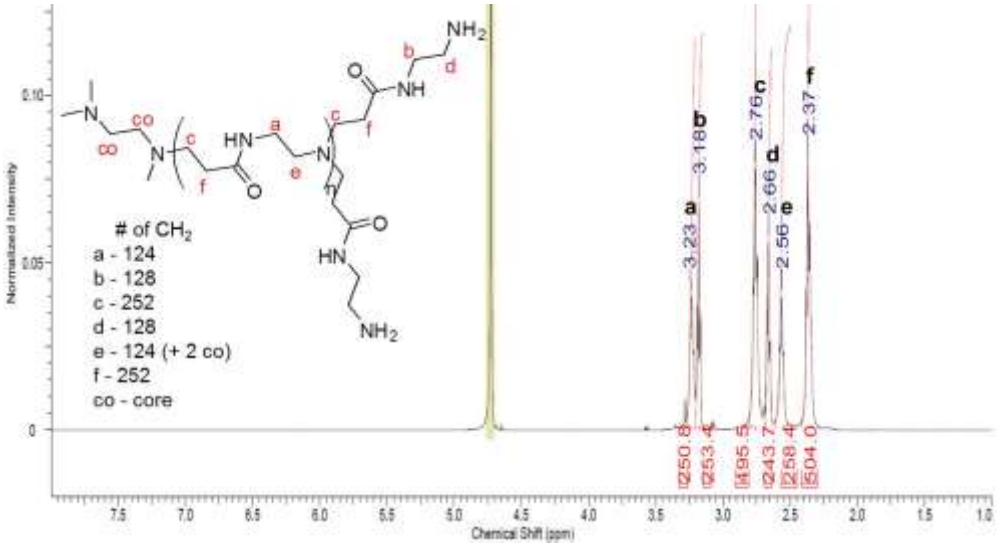

Figure s5A. ^{1}H NMR spectrum of G5-PAMAM ethylenediamine (EDA) core - dendrimer terminated with 126 primary amines (**G5-Am**) recorded using D_2O as a solvent. Insert - partial structure of dendrimer (n=4) with theoretical number of CH_2 groups. Assignment of the peaks was performed based on a previous report [1] and values of integrals are in good agreement with the number of protons in each group of CH_2 moieties.

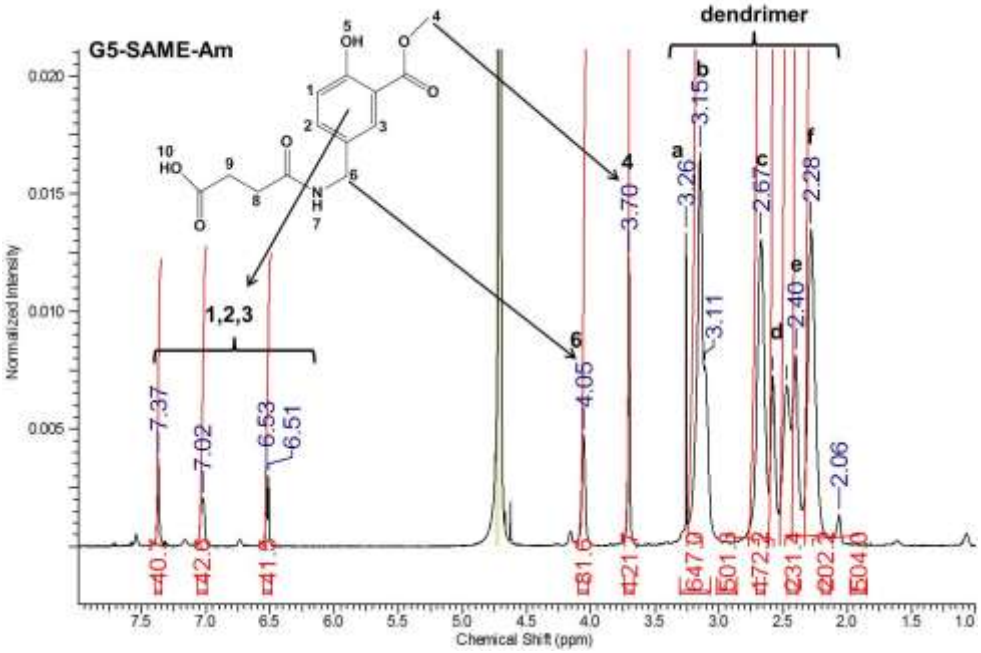

Figure s5B. ^{1}H NMR spectrum of G5 PAMAM dendrimer conjugated with ~40 molecules of 5-N-succinamylmethylsalicylic acid methyl ester (step **1**, **G5-SAME-Am**) recorded using D_2O as a solvent. Only signals related to protons 8 and 9 of SAME overlap with a peak associated with group of protons **e** of dendrimer. Values of integrals related the signals of group **f** of dendrimer (504 protons) and attached SAME (peaks 1, 2, 3, 4 and 6), indicate ~1:40 G5-Am:SAME molar ratio, which was further confirmed by the MALID-TOF and UV-Vis analysis (Figure s5 and s7).

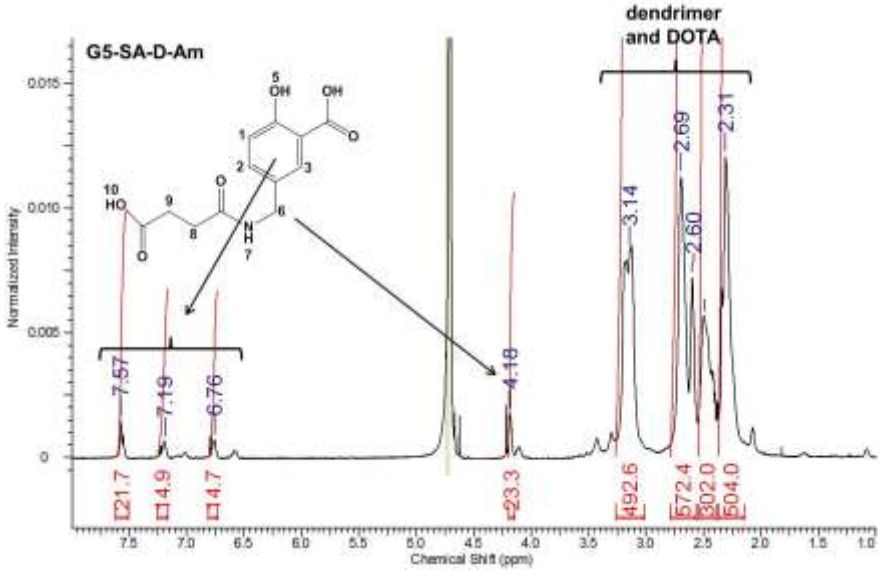

Figure s5C. ^{1}H NMR spectrum of G5 PAMAM dendrimer conjugated with ~40 molecules of 5-N-succinamylmethylsalicylic acid,~4 molecules of DOTA and ~82 unmodified primary amines (step **3**, **G5-SA-D-Am**) recorded using D_2O as a solvent. Conjugation of DOTA provided an

additional 64 and 32 protons with signals at ~2.46 and ~3.3 ppm, respectively. The signals of these protons overlap with peaks associated with the dendrimer, leading to a decrease of the signals related to 5-N-succinamylmethylsalicylic acid. For this reason, ^{1}H NMR could not be used for calculation of the number of DOTA molecules attached to the dendrimer, which was calculated instead based MALDI-TOF and UV-Vis analysis. Absence of the peak at 3.7 ppm related to protons of methyl ester indicates successful deprotection of SAME.

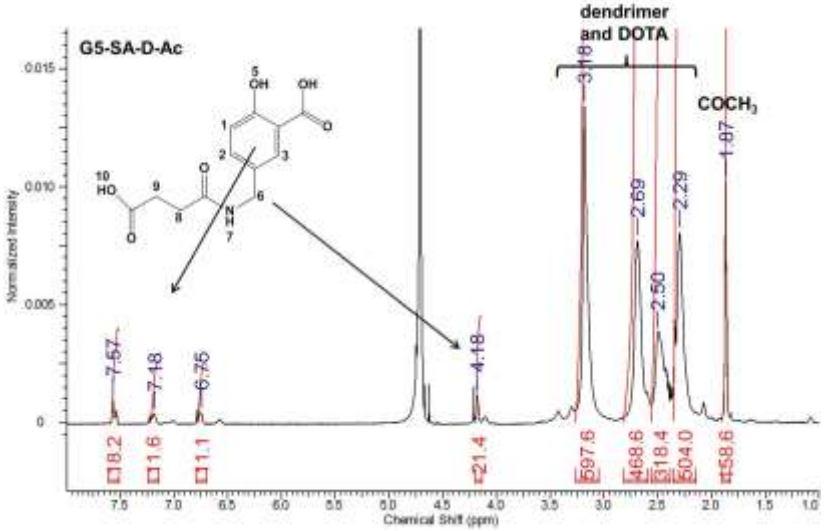

Figure s5D. ^{1}H NMR spectrum of generation 5 PAMAM dendrimer conjugated with ~40 molecules of 5-N-succinamylmethylsalicylic acid,~4 molecules of DOTA, ~56 acetamide groups and 26 unmodified primary amines (step **5**, **G5-SA-D-Ac**) recorded using D_2O as a solvent. Acylation of G5-SAME-D-Am resulted in the presence of intense peak at 1.87 ppm in the spectrum of G5-SA-D-Ac and number of the acetamide groups (Ac) was calculated based on increase of the molecular weight detected by MALDI-TOF.

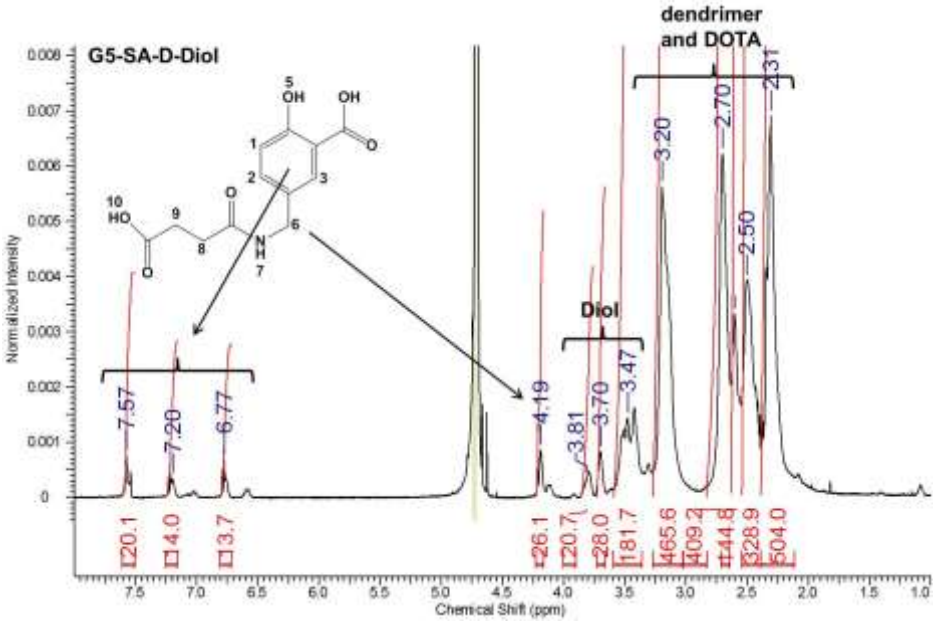

Figure s5E. ^{1}H NMR spectrum of generation 5 PAMAM dendrimer conjugated with ~40 molecules of 5-N-succinamylmethylsalicylic acid,~4 molecules of DOTA, ~ 40 groups of 1,2-propanediol (Diol) and 42 unmodified primary amines (step **7**, **G5-SA-D-Diol**) recorded using D_2O as a solvent. The presence of additional signals between 3.4 and 3.84 ppm indicates successful glycidolation of G5-SAME-D-Am and number of 1,2-propanediol moieties in the nanoparticles was calculated based on MALDI-TOF analysis.

1.7. Matrix-Assisted Laser Desorption Ionization-Time-of-Flight (MALDI-TOF)

Spectra of dendritic nanomaterials were recorded on a Voyager DE-STR spectrophotometer, using 2,5- dihydroxybenzoic acid (DHB) as a matrix. First 10 µL of matrix at concentration of 10 mg/mL was mixed with 10 µL of dendrimer or conjugate at concentration of 4 mg/mL. Then 1 µL of resulting mixture was placed on the target plate (in triplicate) and evaporated. Matrix and dendrimer were dissolved in 50% MeOH and 0.1% TFA aqueous solution. Number of shots and laser power was adjusted according to spectrum quality.

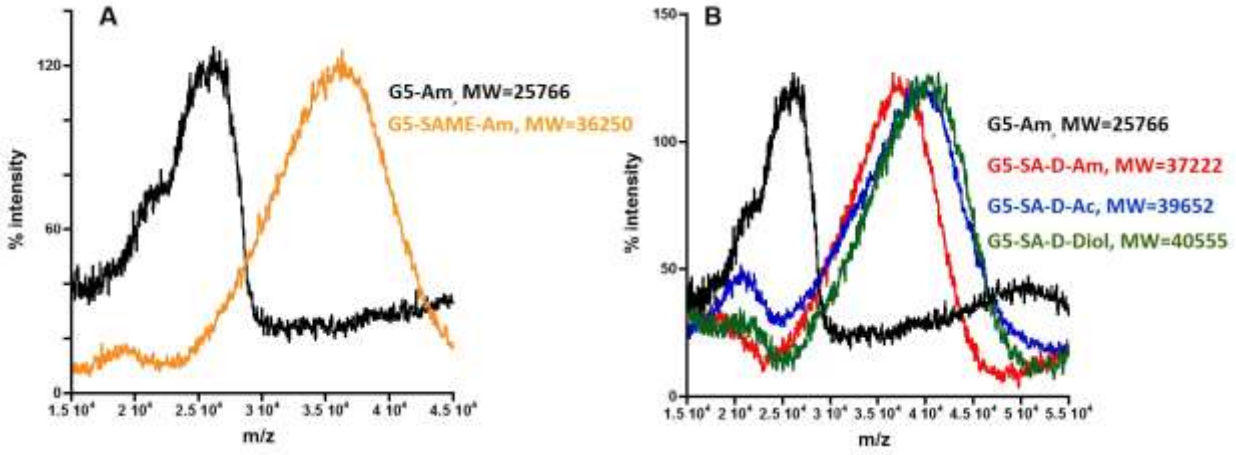

Figure s6. MALDI-TOF of (**A**) starting G5 PAMAM dendrimer (G5-Am) and G5-SAME-Am (nanoparticle obtained in the first synthesis step), increase of the molecular weight indicates attachment of ~42 SAME molecules to the dendrimer, which in good agreement with ^{1}H NMR and UV-Vis analysis; (**B**) G5-Am, G5-SA-D-Am, G5-SA-D-Diol and G5-SA-D-Ac. Significant increase of molecular weight of final products compared to starting material indicates covalent attachment of functional groups to the dendrimer molecule. Similar spectra were recorded for all intermediate products and were used to calculate number of salicylic acid and DOTA molecules attached to dendrimer as well as degree of acylation and hydroxylation (data not shown).

1.8. Dynamic light scattering and zeta potential

Dynamic light scattering and zeta potential analyses were performed using a Malvern Zetasizer Nano ZEN3600. Results are presented as a mean of three sequential measurements, which confirmed satisfying reproducibility. All the analyzed conjugates were prepared at a

concentration of 2 mg/ml in PBS (c= 0.01M, pH 7.4). DLS measurements were performed at a 90° scattering angle at 25°C.

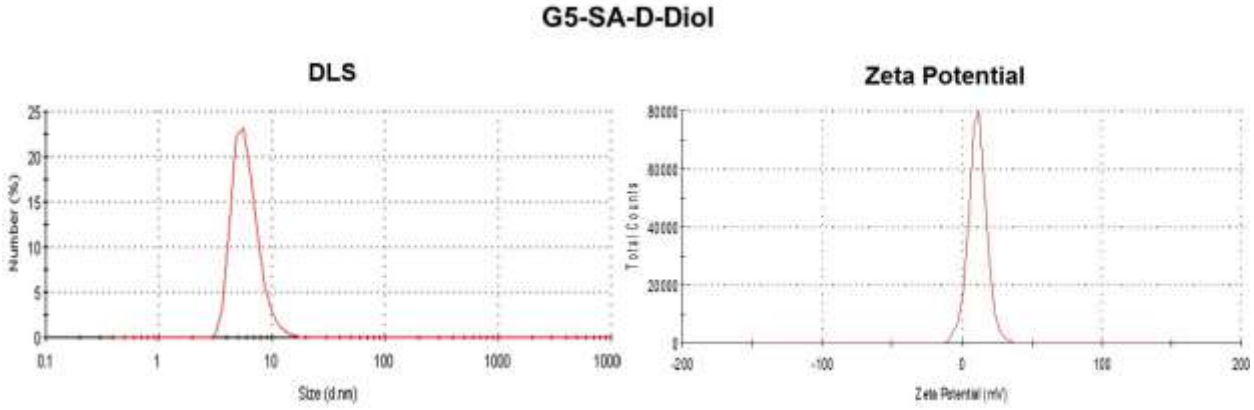

Figure s7. Representative size and zeta potential distributions obtained for G5-SA-D-Diol. Similar size distributions with appropriate change in zeta potential distribution were observed for all other nanoparticles.

1.9. UV-Vis spectroscopy

UV-Vis spectra were collected on a NanoDrop 2000 spectrophotometer. The concentration of SAME, DOTA-NHS, G5-Am, G5-SA-D-Am were adjusted to the intensity of the observed peaks to stay within measurable range. Samples of dendrimer, conjugates and DOTA-NHS were prepared in PBS (c= 0.1M, pH 7.4) and were titrated with an aqueous solution of $CuCl_2$ at a concentration to achieve appropriate molar ratios.

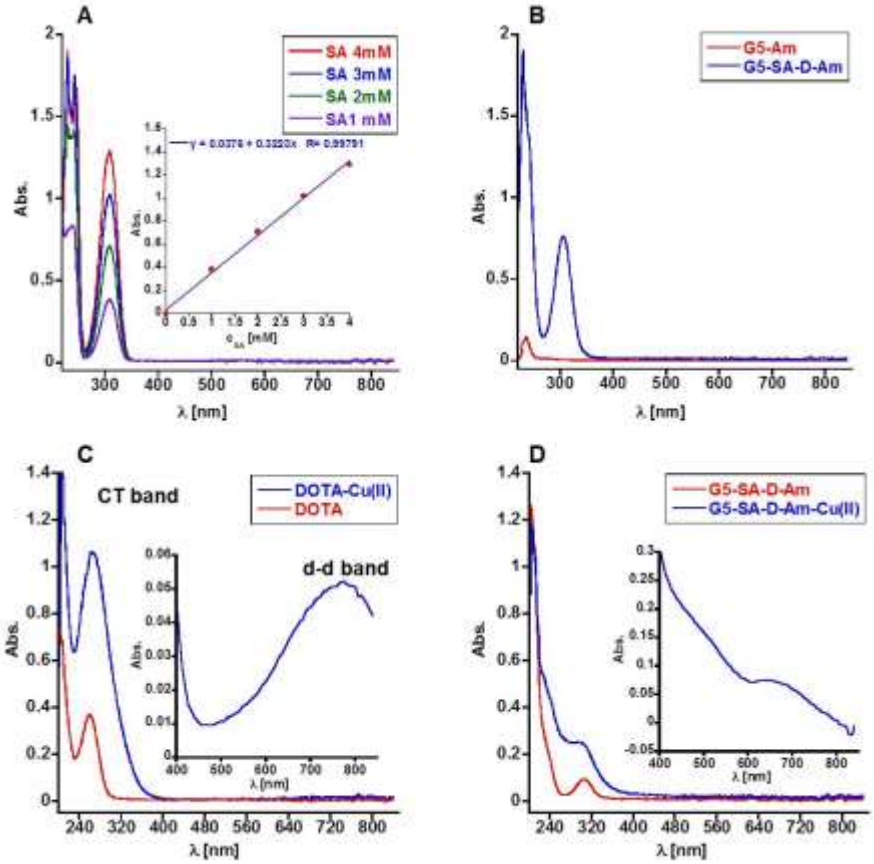

Figure s8. Representative UV-Vis spectra acquired for: (**A**) SAME recorded for concentrations ranging for 1 to 4 mM. Insert shows calibration obtained using maximum of absorbance at 309 nm, which was used to calculate number of SA groups conjugated to the dendrimer; (**B**) G5 PAMAM dendrimer at a concentration of 1 mM, showing only a low intensity peak at 235 nm and G5-SA-D-Am (synthetic step **3**) recorded at concentration of 0.055 mM revealing a strong signal at 309 nm, that is related to SA. Intensity of this peak confirms conjugation of 42 SA to the dendrimer; (**C**) DOTA w/o Cu(II), showing strong charge transfer (CT) in the UV range and d-d transitions (insert) in the visible range upon chelation of copper ions. For CT and d-d measurement, concentration of DOTA used was 1 mM and 10 mM, respectively with 1 mole equivalent of Cu(II) added; (**D**) G5-SA-D-Am w and w/o 4 eq. of Cu(II), with the presence of d-d band and increase in intensity in the UV range related to the CT transition indicating the presence of DOTA on the conjugate. Precipitation and no increase in signal was observed upon further addition of Cu(II). PAMAM dendrimer is a relatively weak Cu(II) chelator and in PBS buffer upon addition of copper ions to dendrimer without DOTA (G5-Am) precipitation of the copper phosphate was observed.

1.10. In vitro MRI measurements

MR data of dendrimer conjugate solutions (concentration of 360 μM dendrimer conjugate in 1X PBS; 42 salicylic acid residues per dendrimer give the concentration ~15 mM, pH 7.2-7.5) were acquired at 37 ^{o}C on an 11.7 T Bruker Avance system (Bruker Biosciences, Billerica, MA, USA) using a 20 mm birdcage transmit/receive coil. MR data was also acquired on solutions of

normalized human serum (Seronorm, Sero AS, Billingstad, Norway) titrated to pH 7.3 with and without 360 μM dendrimer conjugate. The Quantitation of Exchange using Saturation Power (QUESP) dataset was acquired using saturation pulses with B_1= 1.2, 2.4, 3.6, 4.7, 5.9, 7.2 and 10.8 μT, with the saturation time (T_{sat})= 4 s followed by a RARE readout (RARE=8, TR/TE=6000ms/19.09ms). Saturation offsets were incremented from -12 to +12 ppm with a 0.2 ppm step size for the MTR_{asym} spectra.

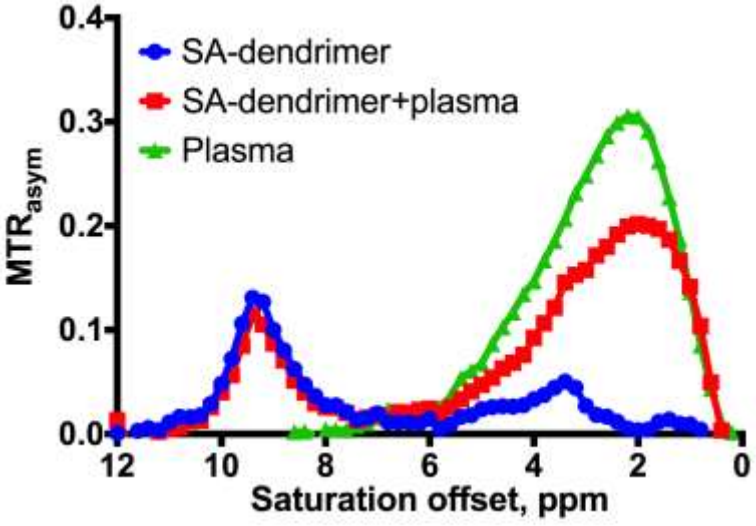

Figure s9. Comparison of CEST contrast for dendrimer agent in PBS vs. dendrimer in normalized human serum vs free normalized human serum (Seronorm™). ω_1 = 3.6 μT.

1.11. Calculation of exchange rate constants

The dendrimer data was fit to numerical solutions to a 4 pool set of Bloch equations which includes phenolic protons (pool 1, Δω = 9.4 ppm), dendrimer amine protons (pool 2, Δω = 3.6 ppm; pool 3, Δω = 2.2 ppm) and water (pool w, Δω = 0 ppm). The Bloch equations were solved numerically using home written Matlab code to fit the data as described in detail previously to obtain the three exchange rates: k_{1w}, k_{2w}, k_{3w} where k_{1w} = rate of exchange for proton 1 (phenolic proton) with water [2]. The longitudinal (R_1) and transverse (R_2) relaxation parameters were fixed to R_{1w} = 0.3 s^{-1}, R_{2w} = 0.9 s^{-1}, $R_{11} = R_{12} = R_{13}$ = 0.71 s^{-1}, and $R_{21} = R_{22} = R_{23}$ = 39 s^{-1}. Error limits for the phenolic OH rates were also determined as described previously [2].

Table s1. Best fit exchange rates obtained from fitting QUESP data

G5	Δω = 9.4 ppm k_{1w} [s^{-1}]	Δω = 3.6 ppm k_{2w} [s^{-1}]	Δω = 2.2 ppm k_{3w} [s^{-1}]
1	N/A	N/A	N/A

2	4500	1000	100
3	3600	470	150
4	950	210	210

1.12. Implantation of U87 glioblastoma cells into mouse brain

U87 glioblastoma cells (ATCC, Manassas, VA) were maintained in monolayer culture (37°C, 5% CO_2, 95% O_2) in minimum essential medium (MEM) with Eagle's salts supplemented with 10% fetal bovine serum, penicillin, and streptomycin (Gibco, Grand Island, NY). Cells were subcultured and used for implantation when they were in an exponential phase of growth. The suspension was diluted with PBS to a final concentration of 20000 cells per µL. A total of 3 SCID mice (20-25 g) obtained from the National Cancer Institute (Frederick, MD), were anesthetized with ketamine (80 mg kg^{-1}) and xylazine (13 mg kg^{-1}) administered intraperitoneally They were placed in a stereotactic apparatus, and after the skull was exposed, and a 0.7 mm burr hole was drilled over the right hemisphere 2.0 mm lateral to the midline and 1.0 mm anterior to the bregma. The needle of a 10µL Hamilton syringe was inserted to a depth of 2.5 mm beneath the dura through the center of the skull hole and 10^5 U87 cells in 5 µL PBS were injected intracerebrally during a 10 min. The incision was closed with 4-0 silk sutures (Ethicon, Somerville, NJ).

1.13. In vivo MRI measurements

Mice bearing U87 cell derived glioblastoma xenografts were anesthetized prior to intracranial infusion of 5 µl of either G5-SA-D-Ac at a concentration of 500 µM or 1xPBS over 10 min. The animals were then positioned in an 11.7 T horizontal bore Bruker Biospec scanner (Bruker Biosciences, Billerica, MA) and were under isoflurane anesthesia for the entire image collection period. Single slice images were acquired from 0.5-1.5 hrs after intracranial injection. To produce the CEST images, two sets of saturation images were collected, a WASSR set for B_0 mapping and a CEST data set for characterizing contrast. For the WASSR images, the saturation parameters were t_{sat} = 500 ms, B_1 = 0.5 µT, TR = 1.5 sec with saturation offset incremented from −1 to +1 ppm with respect to water in 0.1 ppm steps and acquisition time 1 min 18 s, while for the CEST images, t_{sat} = 2.2 sec, B_1 = 3.4 µT, TR = 5.5 sec, with offset sampling from -7.8 to -10.8ppm and +7.8 to +10.8ppm with a 0.3 ppm step and acquisition time 6min 19s 500ms. The acquisition parameters were: TE=4.8 ms, number of averages = 2, matrix size = 64x48, spatial resolution = 0.24x0.23 mm^2, field of view = 15.5x14.5 mm, slice thickness = 1 mm, RARE factor=16. The CEST contrast was calculated using the asymmetry in the magnetization transfer ratio (MTR_{asym})[3]:

$$MTR_{asym} = \frac{S_w(-\Delta w) - S_w(+\Delta w)}{S_{0w}}$$

For all in vivo images, the average MTR_{asym} from 8.7 to 9.9 ppm was calculated. The signal intensity data (S_w) was processed using Matlab 2015 and Prism 6 software. For the region of interest within the tumor, the relative MTR_{asym} was calculated as described previously [3].

In order to determine the tumor coverage shown in Fig. 3, tumor borders were defined according to T_{2w} images, ROIs were drawn over this region and histograms of the average MTR_{asym} from 8.7 to 9.9 ppm prepared. % coverage was then calculated by # of pixels above

– 0.035 / total # of tumor pixels.

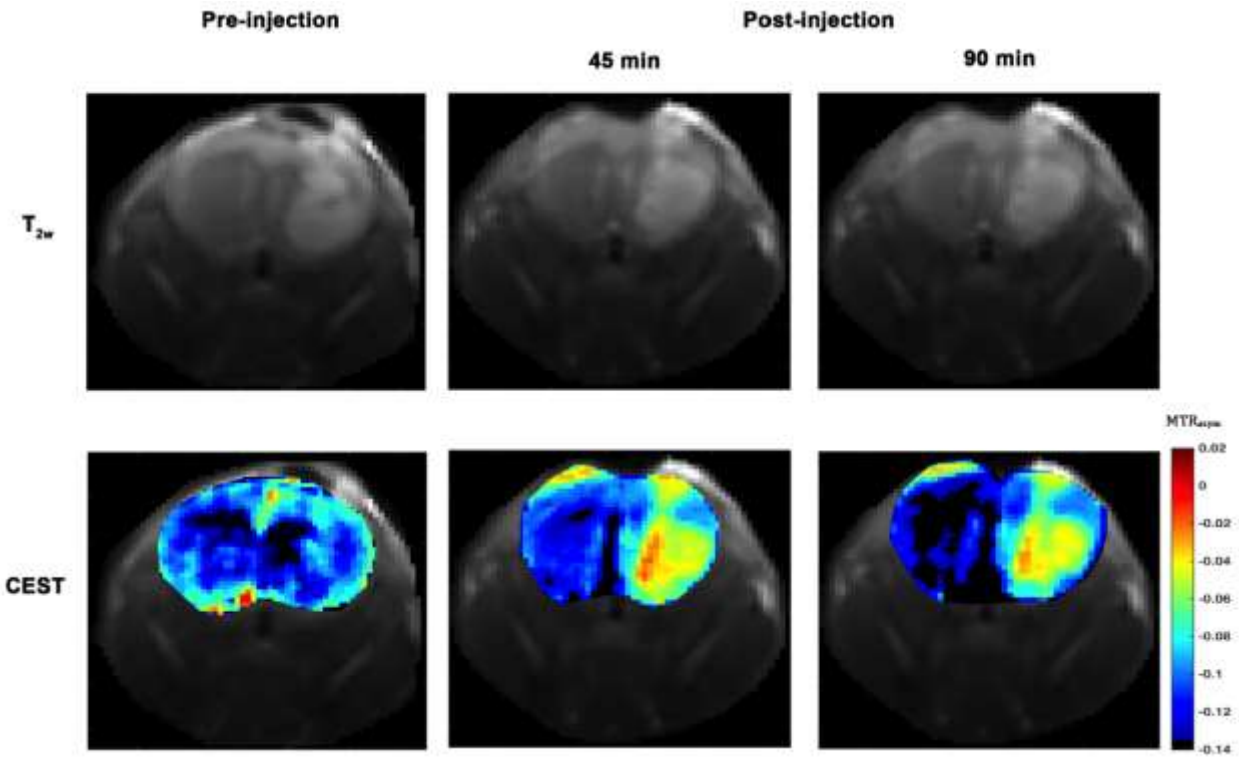

Figure s10. In vivo characterization of G5-SA-D-Ac conjugate prior and up to 1.5 hours after intratumoral infusion into U87 glioblastoma xenograft mouse model using CEST MRI, upper panels T_{2w} images, lower panels average MTR_{asym} from 8.7 to 9.9 ppm.

References

1. Shi, X. Y.; Banyai, I.; Islam, M. T.; Lesniak, W.; Davis, D. Z.; Baker, J. R.; Balogh, L. P. *Poly* 2005, 46, 3022-3034.
2. Yang, X.; Yadav, N. N.; Song, X.; Ray Banerjee, S.; Edelman, H.; Minn, I.; van Zijl, P. C.; Pomper, M. G.; McMahon, M. T. *Chem Eur J* 2014, 20, 15824-32.

3. Chan, K. W.; Yu, T.; Qiao, Y.; Liu, Q.; Yang, M.; Patel, H.; Liu, G.; Kinzler, K. W.; Vogelstein, B.; Bulte, J. W.; van Zijl, P. C.; Hanes, J.; Zhou, S.; McMahon, M. T. *J Cont Rel.* 2014, 180, 51-9.