Supporting Information For:

Use of a Genetically Engineered Protein for the Design of a Multivalent MRI Contrast Agent

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Materials and Instruments

Unless otherwise noted, all starting materials were obtained from commercial sources and used without further purification. Analytical TLC was performed on Merck KgaA silica gel 60 F254 TLC plates. Silica for flash chromatography was ICN Silitech 32-63 D 60 Å. ^{1}H NMR spectra were recorded on Varian 500, 400 or 300 MHz NMR spectrometers. ^{13}C NMR spectra were on a Varian 500, 400 or 300 MHz NMR spectrometer. MALDI-TOF mass spectrometry was performed on a PE Voyager DE-Pro MALDI-TOF-MS instrument. Relaxivity measurements were performed in triplicate using a Bruker mq60 NMR Analyzer (Bruker Canada, Milton, Ont., Canada). Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine Gd(III) concentration and was performed on a Thermo Jarrell Ash Atomscan Model 25 Sequential ICP Spectrometer.

Synthesis of the gene for the protein polymer

The cloning of the gene for the protein polymer was accomplished through first polymerase chain reaction (PCR) amplifying the single-stranded oligonucleotide sequence of three repeats of the monomer unit, shown in Figure S1. This construct was then digested with the enzyme Eam1104 I to create adhesive ends on the gene. It was allowed to self ligate and form a multimer and then ligated into a pUC18 cloning plasmid. To create a controlled doubling of a multimer, the insert in the pUC18 plasmid was amplified through PCR with primers that eliminate one of the Sap I sites that flank the insert in the pUC18 plasmid. Two different batches of the PCR amplified insert were separately digested by Eam1104 I and Sap I. The phosphate group of the Eam1104 I

digested batch was removed through digestion with calf intestinal phosphatase and the two batches were subsequently ligated together. After replacing the phosphate group by using T4 polynucleotide kinase and another Eam1104 I digestion, the gene was ligated into the pUC18 plasmid and transformed into novablue cloning cells. This process was repeated with the previous gene product from the cloning plasmid to obtain the desired length of the protein polymer. All enzymes are from New England Biolabs except for Taq polymerase, which is from Promega.

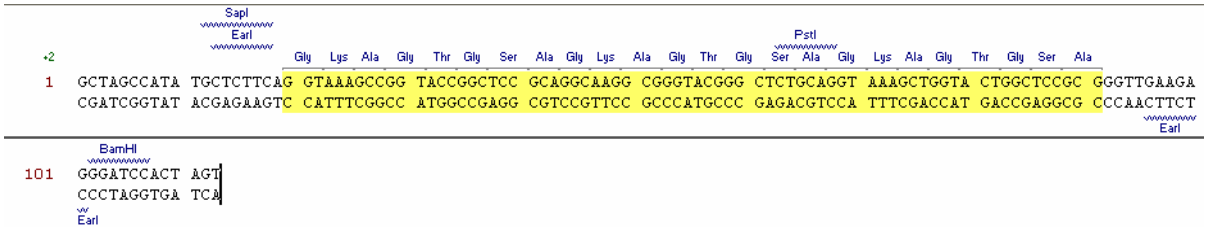


Figure S1: Oligonucleotide sequence used for three repeats of the monomer unit. The Sap I and Ear I (Eam 1104 I) sites are identified.

Synthesis and characterization of protein polymers

The gene for $(GKAGTGSA)_{30}$ was Eam1104 I digested out of the pUC18

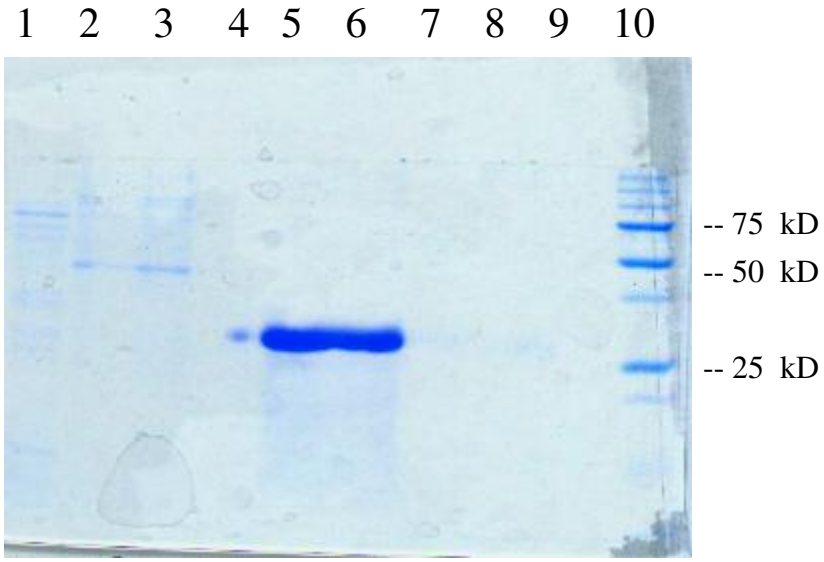


Figure S2: PAGE of protein polymer nickel affinity purification. Lane 1: flow through. Lane 2: guanidine hydrochloride buffer wash. Lane 3: 10 mM imidazole wash. Lane 4: 20 mM imidazole wash. Lane 5: elution 1 with 250 mM imidazole. Lane 6: elution 2. Lane 7: elution 3. Lane 8: elution 4. Lane 9: elution 5. Lane 10: molecular weight marker.

plasmid, ligated into the pET19b plasmid and then transformed into chemically competent *E. coli* strain BLR(DE3) cells. Cultures were grown in 1L of Difco Terrific Broth (Fisher Scientific) supplemented with ampicillin (Sigma, 200 μg/mL) and tetracycline (ICN Biomedicals, 12.5 μg/mL). After reaching an OD_{600} of 0.6 to 0.8, the cultures were induced with 1 mM of isopropyl thiogalactoside (IPTG) (US Biologicals) and harvested after 3-4 hours with centrifugation (20 min, 4000 rpm, 4 °C). The supernatant was decanted and the cell pellet was frozen overnight in a –20 °C freezer, thawed, and then resuspended in a 6 M guanidine hydrochloride, 20 mM sodium phosphate, 500 mM NaCl, pH 7.8 buffer. After three freeze (-80 °C)/thaw cycles, the cell suspension was sonicated and centrifuged for 30 minutes at 8000 rpm, 4 °C. The protein is expressed as a fusion protein with a 10X histidine tag for purification by nickel affinity chromatography. The supernatant was purified with Chelating Sepaharose Fast Flow nickel resin (GE Healthcare) under denaturing conditions with 250 mM imidazole competitive elution (Invitrogen procedure). The elutions, which were determined via sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) (Figure S2), were dialyzed batchwise against deionized water for three days. After lyophilization, the dried solid was resuspended in deionized water and filtered with a 0.45 μm polytetrafluoroethylene (PTFE) syringe filter. The filtered solution was then lyophilized.

The protein was analyzed with matrix assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) (Perseptive Biosystems Voyager Pro DE) using a 2-(4-Hydroxyphenylazo)benzoic acid (HABA) matrix (Figure S3).

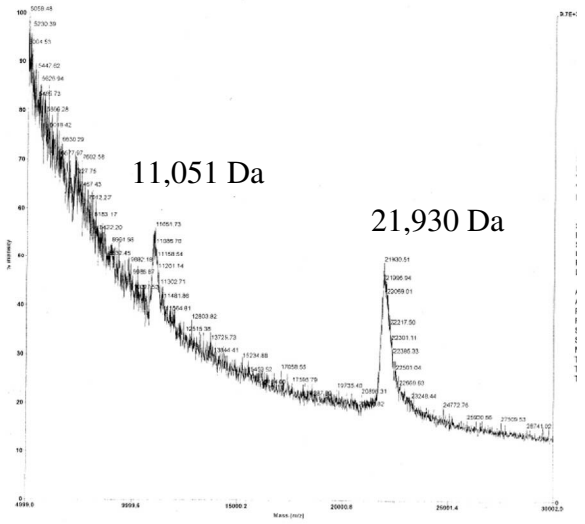


Figure S3: MALDI of nickel affinity chromatography purified protein polymer. The mass detected for z=1, 21,930 Da is a little higher than the calculated mass of 21,825 Da.

Circular dichroism (CD) was performed at 25 ºC in water using a Jasco J-715 CD Spectrophotometer. The CD spectrum shown in Figure S4 indicates that the protein is random coil.

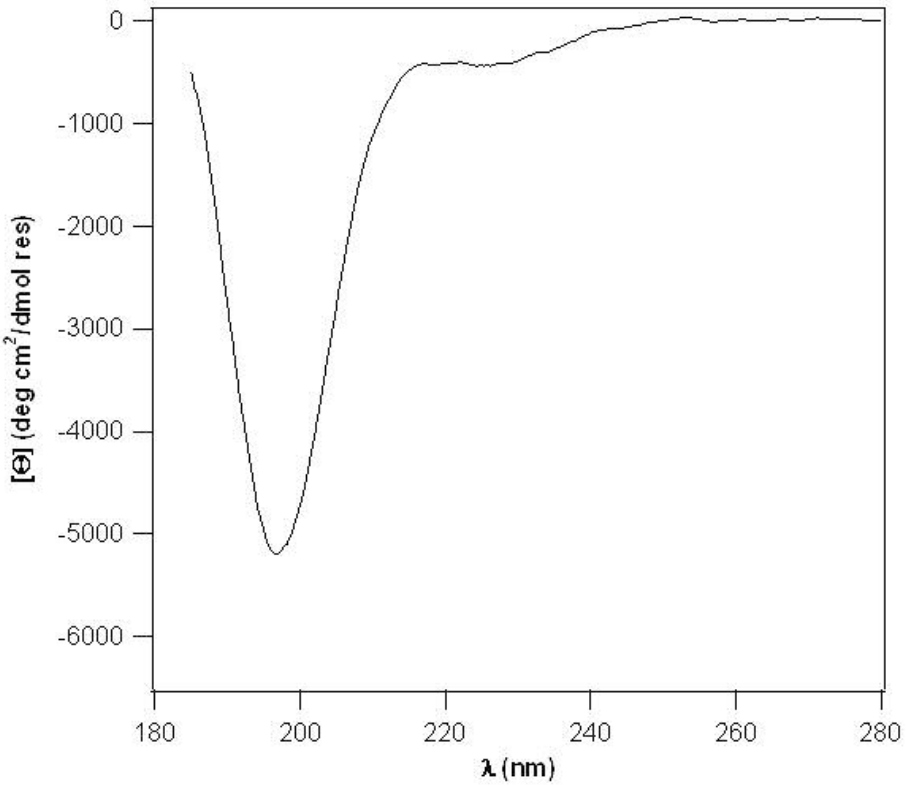
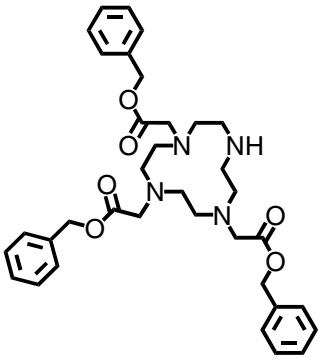
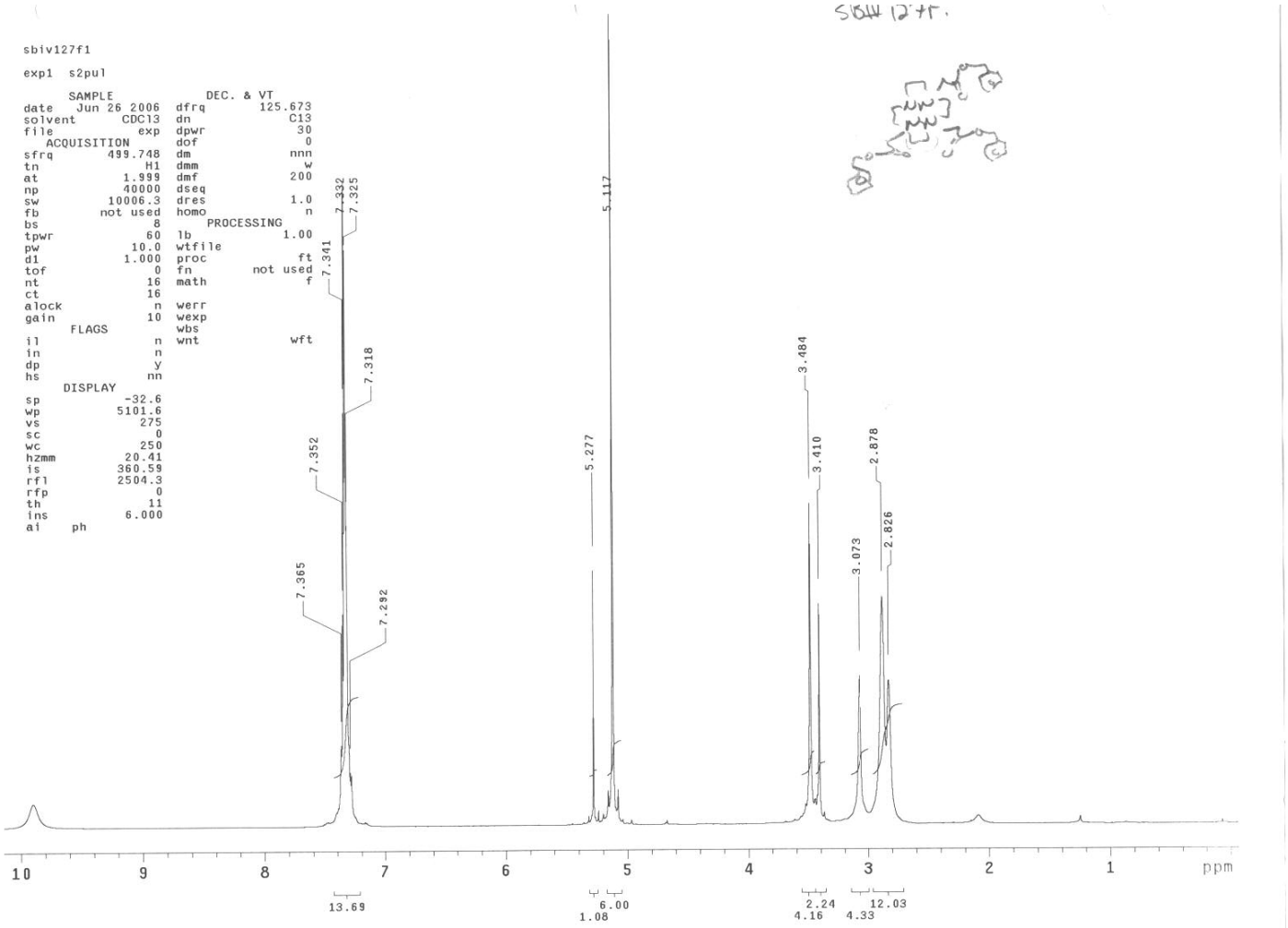


Figure S4: Circular Dichroism spectrum of $(GKAGTGSA)_{30}$ in water at 25 ºC.

Synthesis of the DOTA-based Gd(III) chelator



Synthesis of **2**: In a dry round bottom flask was combined cyclen (2.89 g, 17 mmol), sodium bicarbonate (4.71 g, 56 mmol) and dry acetonitrile (200 mL). The solution was stirred under N_2, for 5 minutes and a solution of dry acetonitrile (30 mL) and benzyl bromoacetate (11.6 g, 51 mmol) was added dropwise. The reaction proceeded overnight then the solid was filtered, and the solvent was removed by rotary evaporation. Flash chromatography was performed on silica gel using water saturated with KNO_3/H_2O/ACN in a ratio of 1/9/90 to give a white hygroscopic solid (10 g, 68% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.34 (bs, 15H), 5.12 (s, 6H), 3.48 (s, 4H), 3.42 (s, 2H), 3.08 (s, 4H), 2.88-2.83 (bm, 12H): ^{13}C (125 MHz, $CDCl_3$) δ 171.09, 170.28, 135.44, 128.79, 128.72, 128.66, 128.55, 66.71, 57.28, 51.55, 49.42, 47.38. ESI MS (methanol) Calcd. 616.33, found 639.63 M+Na.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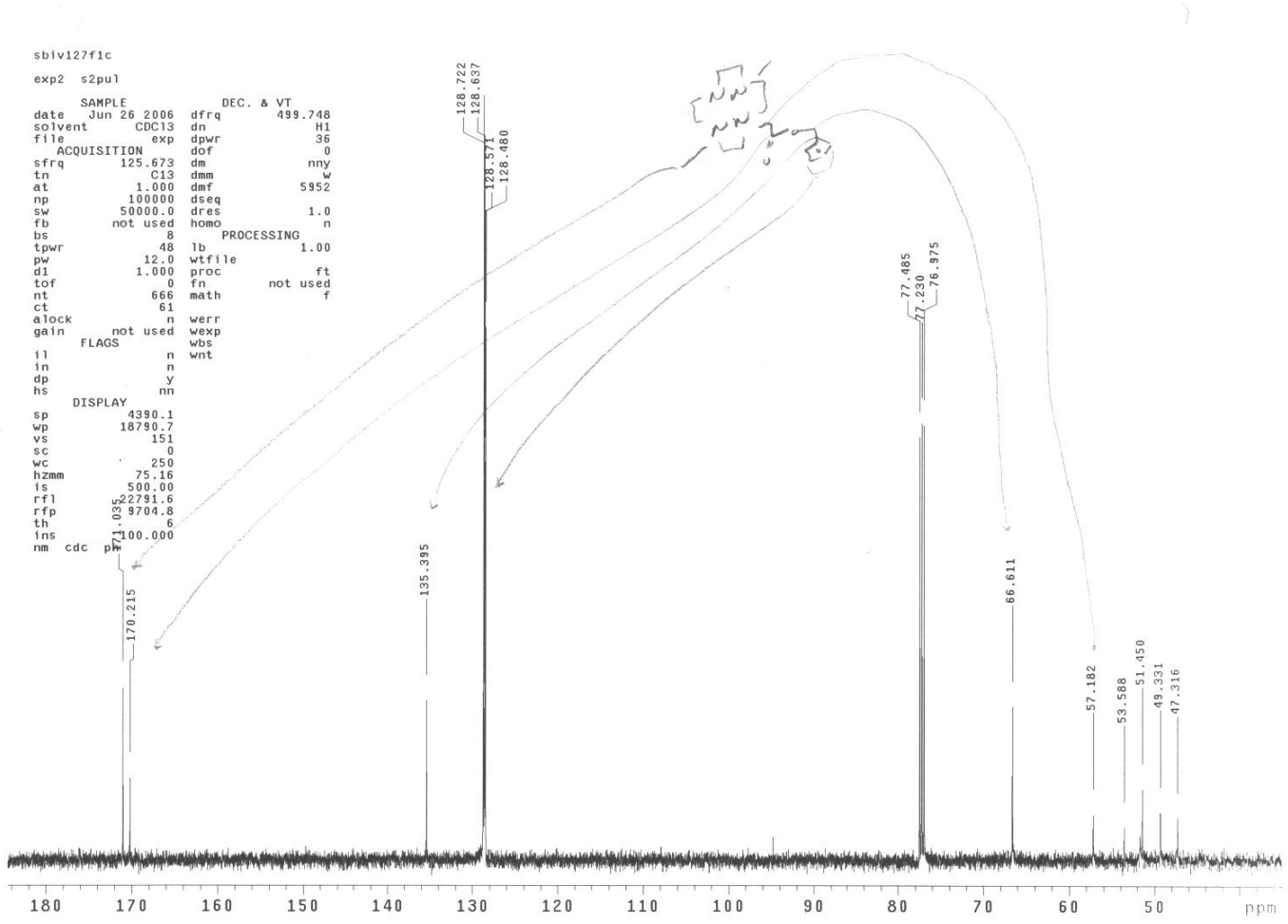
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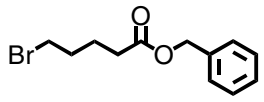
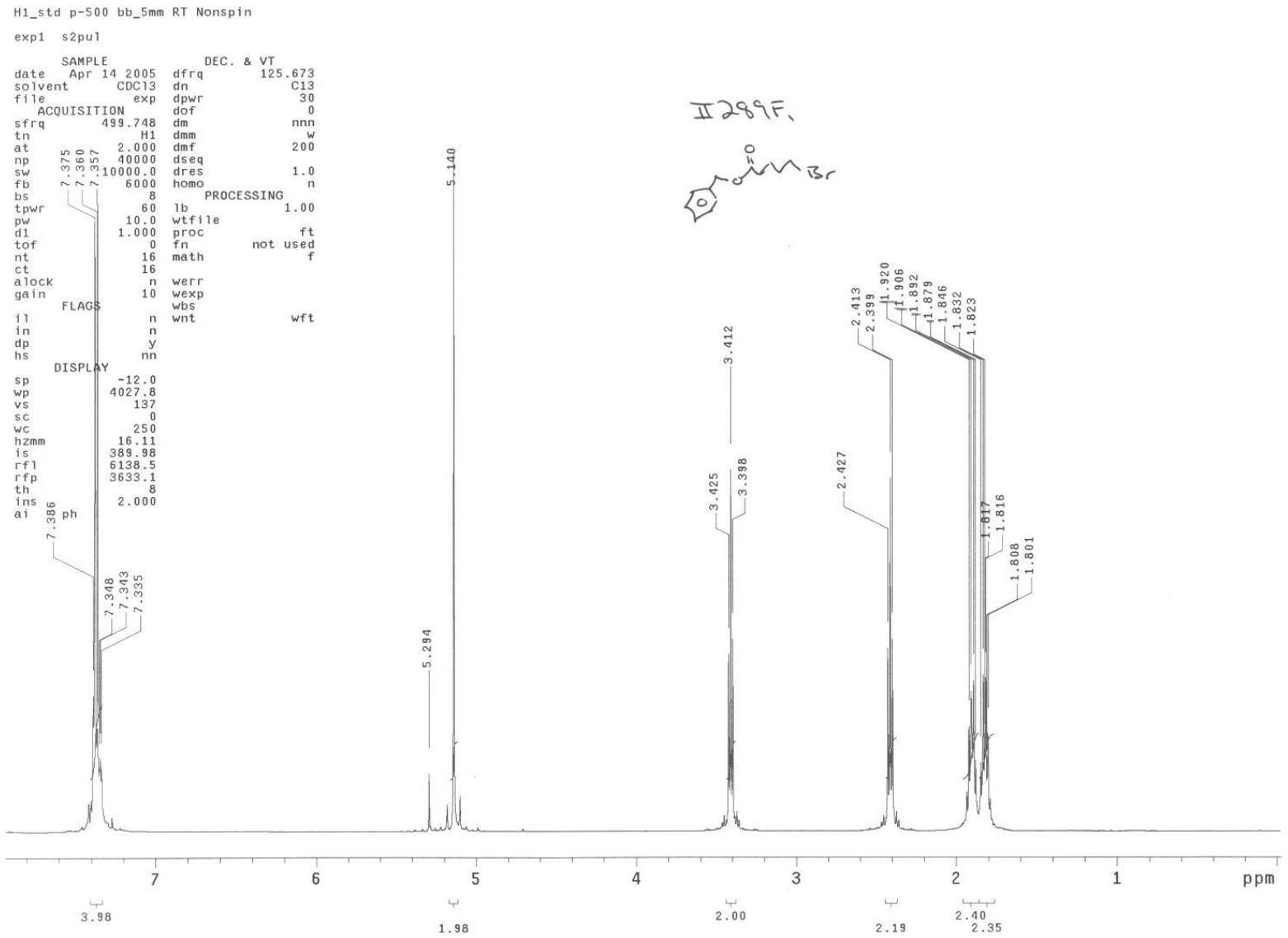


Synthesis of benzyl 5-bromopentanoate (**3**): In a dry roundbottom flask was combined 5-bromo valeric acid (2.00 g, 11 mmol), DTPS (3.56 g, 12.1 mmol), DIPC (1.81 g, 14.3 mmol) and DCM (200 mL). The solution was stirred for 5 minutes and a solution of benzyl alcohol (1.79 g, 16.6 mmol) and DCM (10 mL) was added dropwise. The reaction proceeded overnight then was diluted in DCM and washed with H_2O three times, dried over $MgSO_4$, the the solvent was removed by rotary evaporation. Flash chromatography was performed using 5/95 MeOH/DCM to afford a clear liquid (2.82 g, 95% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.36 (bs, 5H), 5.14 (s, 2H), 3.43 (t, *J*=7 Hz, 2H), 2.41 (t, *J*=7 Hz, 2H), 1.90 (q, *J*=7 Hz, *J*=14 Hz, 2H), 1.83 (q, *J*=7 Hz, *J*=14 Hz, 2H): ^{13}C (125 MHz, $CDCl_3$) δ 173.19, 136.19, 128.86, 128.54, 128.51, 66.55, 33.53, 33.33, 32.21, 23.74. ESI MS (methanol) Calcd. 270.03, found 271.23 M+H.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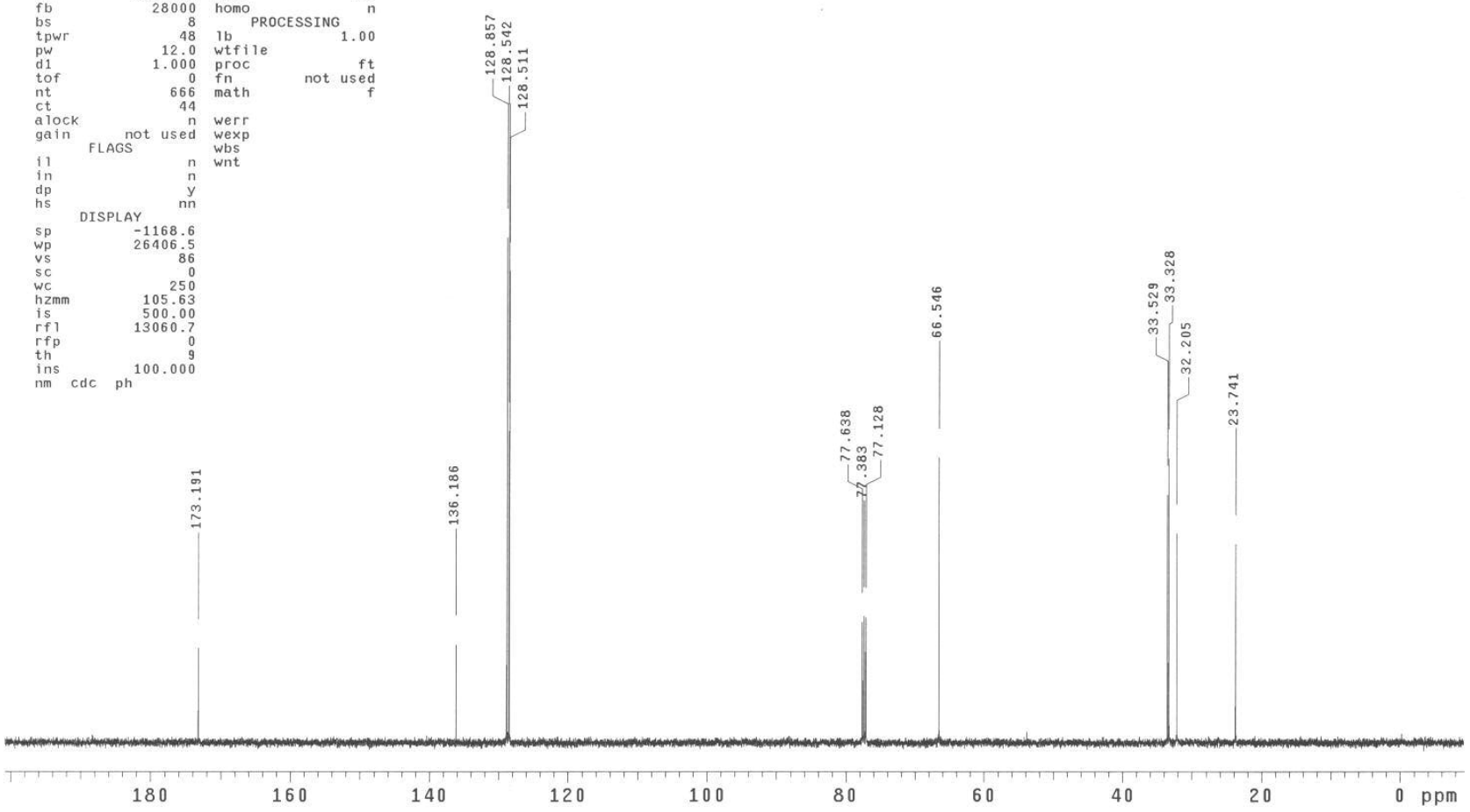
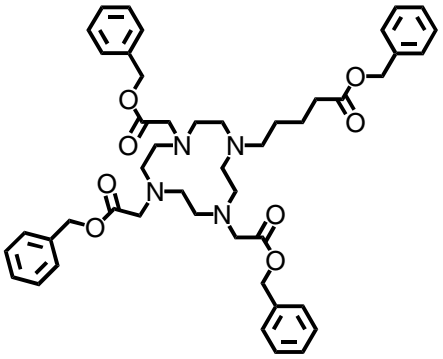
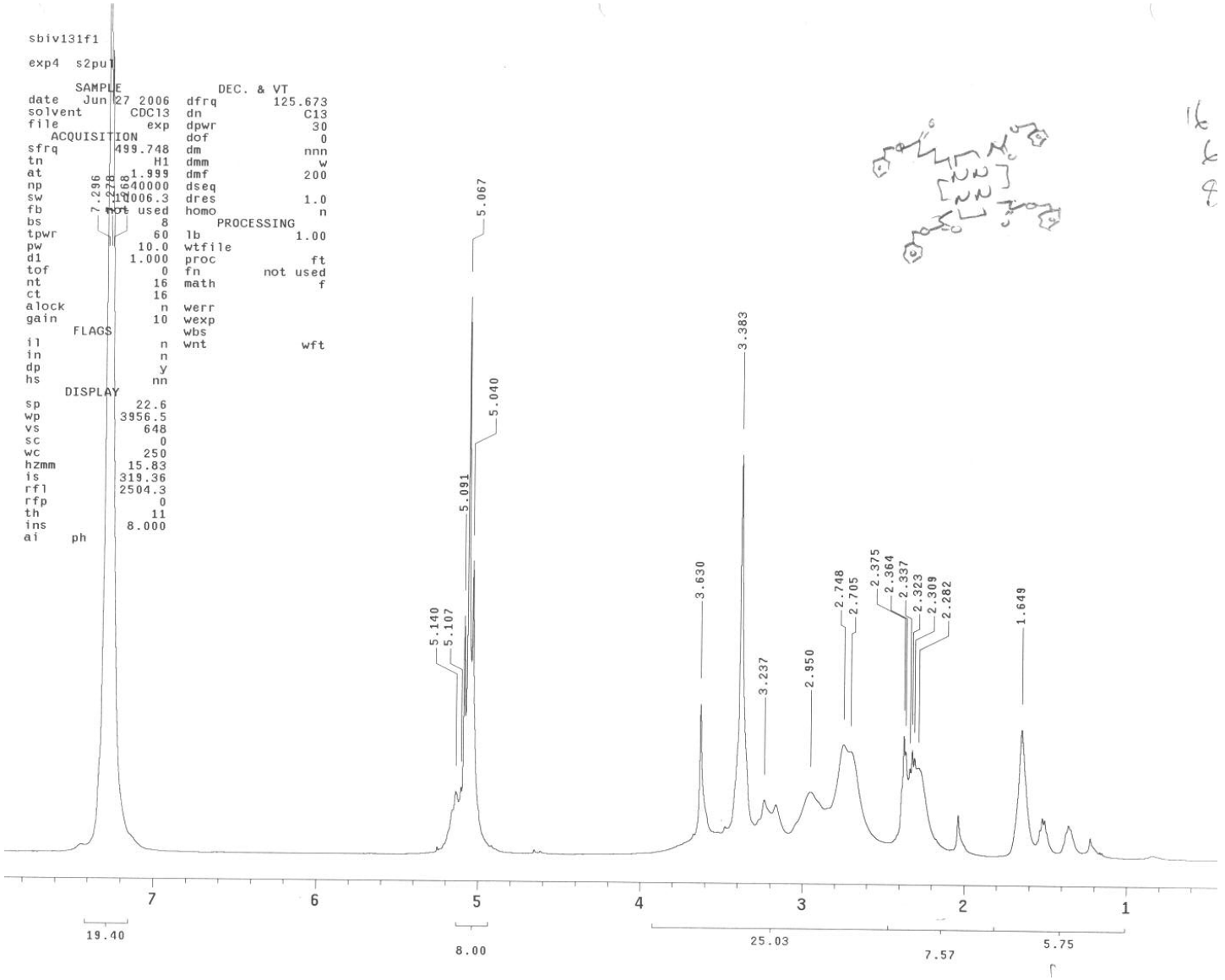




Synthesis of **4**: In a dry roundbottom flask was combined **2** (0.35 g, 0.57 mmol), potassium carbonate (0.235 g, 1.7 mmol) and dry acetonitrile (30 mL). The solution was stirred under N_2, for 5 minutes and a solution of dry acetonitrile (5 mL) and benzyl 5-bromopentanoate (**3**) (0.169 g, 0.74 mmol) was added dropwise. The reaction proceeded overnight then the solid was filtered, and the solvent was removed by rotary evaporation. Flash chromatography was performed using 1/9/90 KNO_3/H_2O/ACN to give a white hygroscopic solid (0.458 g, 70% yield). ^{1}H NMR (500 MHz, $CDCl_3$) δ 7.28 (bs, 20H), 5.07 (s, 8H), 3.63-2.28 (bm, 24H), 1.65 (s, 4H), 1.48 (m, 2H), 1.35 (m, 2H): ^{13}C (125 MHz, $CDCl_3$) δ 172.77, 170.98, 170.57, 135.83, 135.45, 135.22, 134.96, 128.66, 128.62, 128.57, 128.51, 128.41, 128.30, 128.24, 128.18, 66.67, 66.43, 66.26, 56.09, 53.00, 52.72, 52.44, 50.17, 47.53, 33.18. ESI MS (methanol) Calcd. 806.43, found 807.52 M+H.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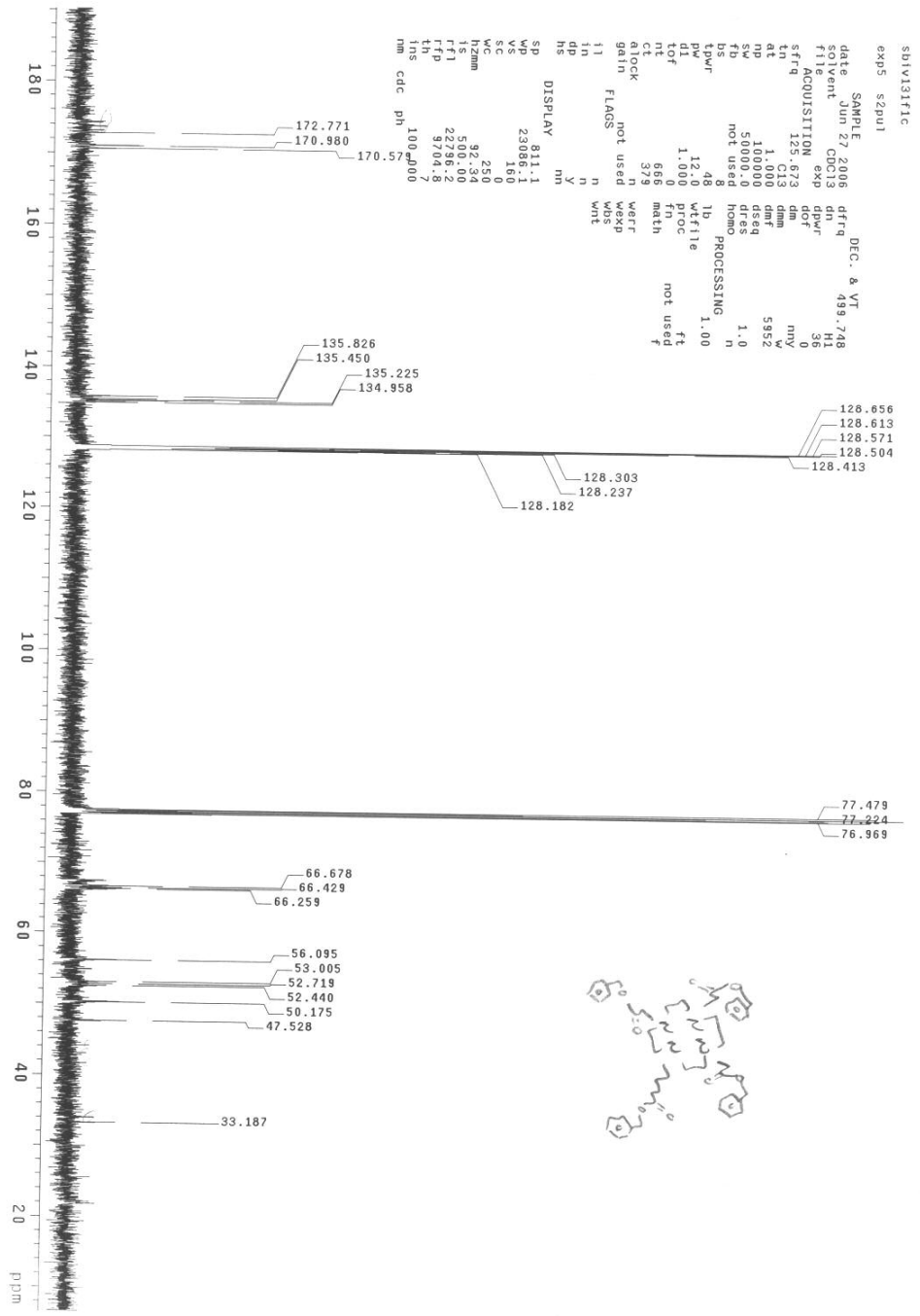
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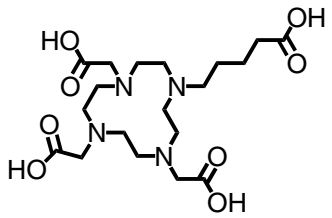
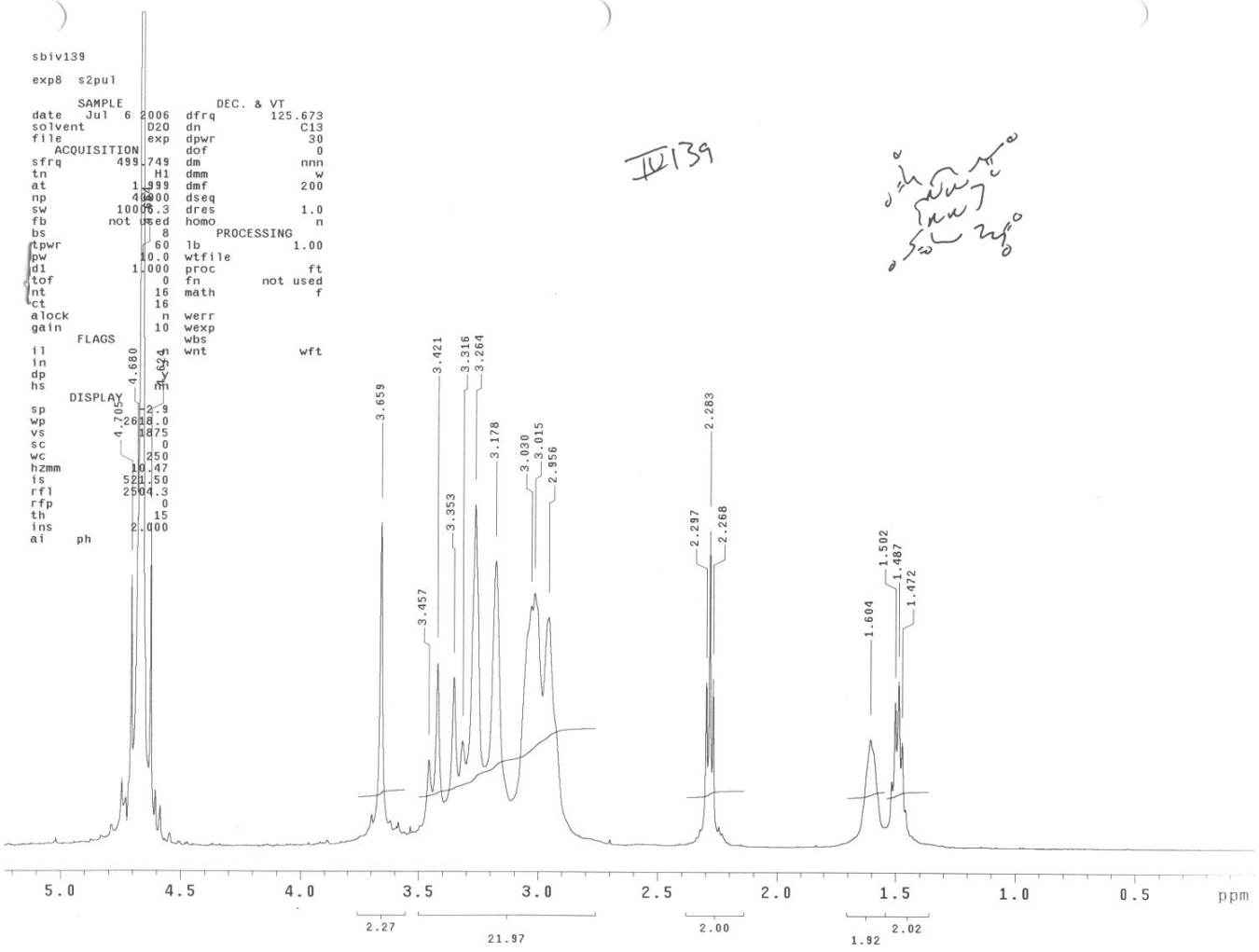


Synthesis of **5:** In a falcon tube was placed **4** (0.165 g, 0.70 mmol), MeOH (10 mL), DCM (10 mL), and Pd/C (0.040 g). The solution was stirred under a balloon of H_2 for two days. The solution was then filtered through celite, the solvent was removed by rotary evaporation providing a 99% yield. ^{1}H NMR (500 MHz, D_2O) δ 3.66 (s, 2H), 3.46-2.96 (bm, 22H), 2.28 (t, *J*=7.5 Hz, 2H), 1.60 (s, 2H), 1.48 (m, 2H): ^{13}C (125 MHz, $CDCl_3$) δ 177.86, 174.34, 169.40, 53.91, 53.11, 51.79, 49.93, 49.01, 48.47, 48.19, 33.12, 22.31, 21.34. ESI MS (methanol) Calcd. 446.24, found 469.42 M+Na.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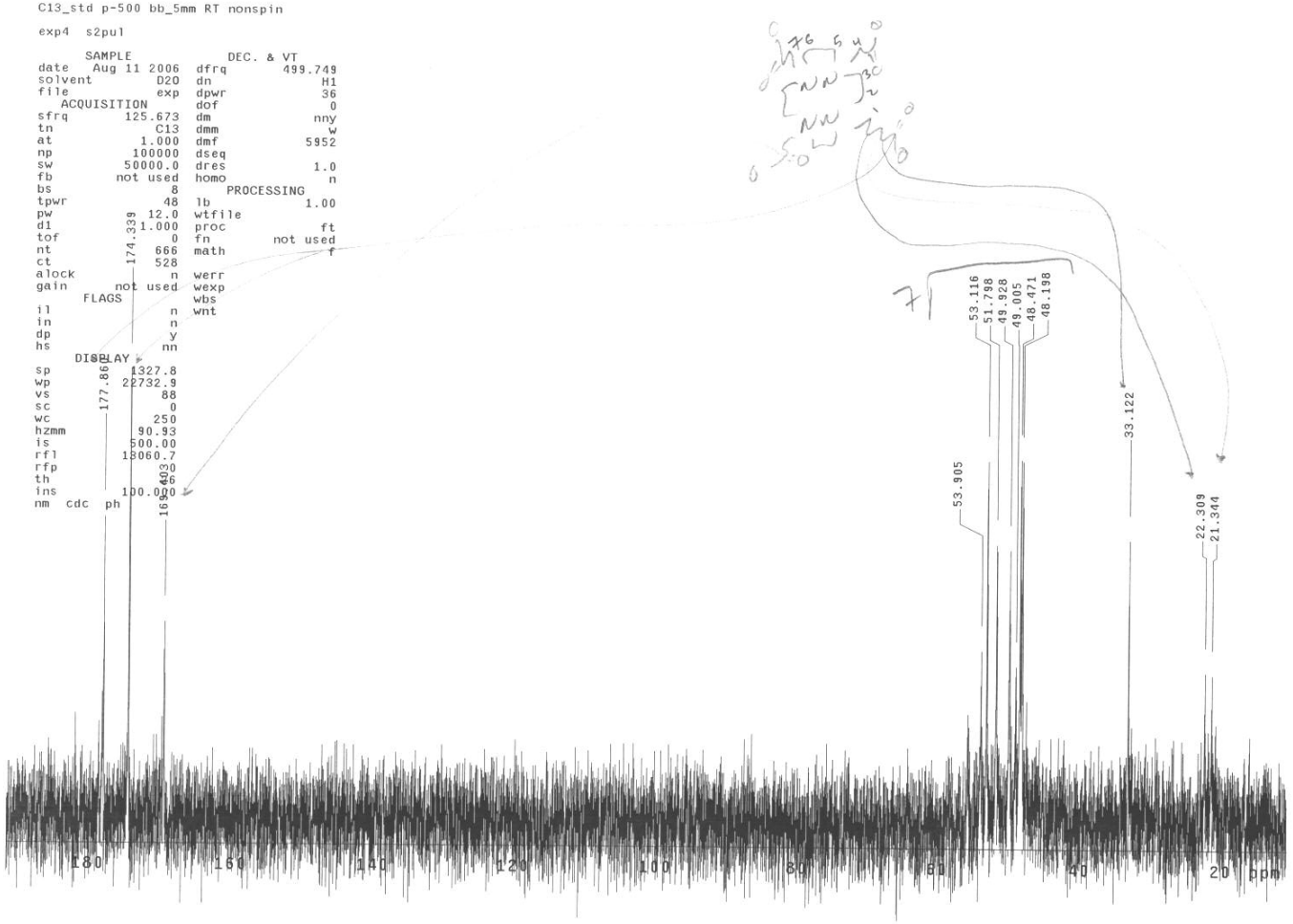

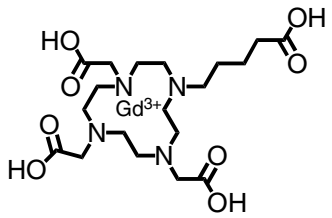

Synthesis of **6:** In a falcon tube was placed **5** (0.165 g, 0.37 mmol), milliQ H_2O (4 mL), and $GdCl_3$ (0.151 g, 0.4 mmol). The pH was adjusted to 6.5 using ammonium hydroxide and the solution was stirred for 2 days. The solution was then brought up to pH 10, the ppt was centrifuged at 4000 rpm for 10 minutes, and the liquid decanted. The water was removed by lyophilization giving a white powder (mass and yield inaccurate because of NaOH salt in the final product from the pH adjustment). ESI MS (negative mode) (methanol) Calcd. 600.16, found 600.21 M.

Synthesis and characterization of protein polymer-based CA.

The conjugation reaction between the primary amines on the lysine residues in the protein polymer and the carboxyl group on the Gd(III) chelator was performed in conical reaction vials (Chemglass) with "V"-shaped stir bars (Chemglass). The protein was dissolved at 1 mg/mL in a filtered 0.1 M MES, 0.5 M NaCl, pH 6.0 buffer. A 3.8-fold molar excess of the Gd(III) chelator per lysine residue on the protein was used. The peptide coupling agents, 1-Ethyl-3-[3-dimethylaminopropyl]carbodiimide Hydrochloride (EDC) (Pierce) and N-hydroxysulfosuccinimide (Sulfo-NHS) (Pierce) were added in 8-fold and 9-fold excess of the protein polymer by weight. The reaction stirred for ~36 hours and was then continuously dialyzed against deionized water for three days and

lyophilized. The dried conjugate was resuspended in deionized water, filtered with a 0.45 μm PTFE syringe filter, and lyophilized again. MALDI-TOF MS was used to analyze the conjugation reaction using a sinapinic acid matrix dissolved in a 1:1 solution of acetonitrile and deionized water. A mass of ~26,800 Da for the conjugate corresponds to approximately 8.6 Gd(III) chelators per protein molecule.

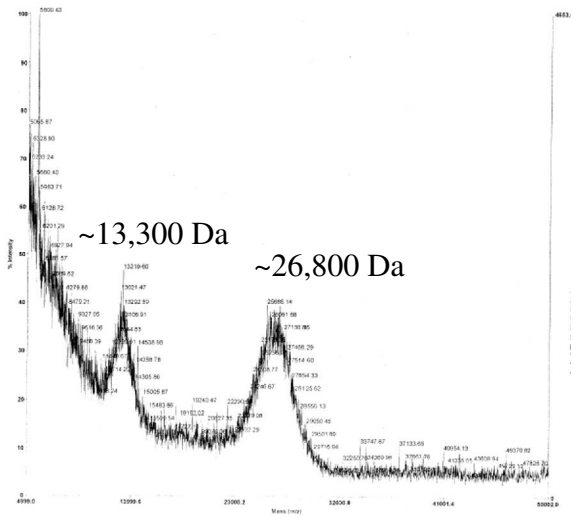

Figure S5: MALDI of conjugate. The mass for z=1 is ~26,800 Da.

Relaxivity

Relaxivity measurements were performed in triplicate using a Bruker mq60 NMR Analyzer (Bruker Canada, Milton, Ont., Canada) at 37 °C. Inductively coupled plasma mass spectrometry (ICP-MS) was used to determine Gd(III) concentration.

Synthesis of the hydrogels

A 5.8% (w/v) hydrogel was made in 5 mm NMR tubes. The hydrogel with **7** contained 5.30 x 10^{-7} mol of Gd(III). The remaining weight was made up of the

unconjugated protein polymer, **1**. The solids were dissolved in 650 μL of .01 M sodium phosphate, 0.15 M sodium chloride, pH 7.4 buffer and then put in the NMR tube. 45 μL of 25% aqueous glutaraldehyde (Calbiochem, San Diego, CA) was added, the NMR tube was sonicated, and then put on ice. The solution gelled within minutes.

MR Imaging of the hydrogels

The hydrogels were MR imaged on a 14.1 T/600 MHz Bruker Ultrashield Biospin with an 89 mm bore size. The imaging parameters are multiscan multiecho (MSME); repetition time (TR), 250.0 ms; echo time (TE), 10.2 ms; number of excitations (NEX), 2; slice thickness (SI), 1.00/1.25 mm; field of view (FOV), 1.5 cm; matrix size (MTX), 256.