

SUPPORTING INFORMATION FOR

Bisphosphonate tweezer and clickable pegylated PAMAM dendrons for the elaboration of functional iron oxide nanoparticles displaying renal and hepatobiliary eliminations

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I. Materials.

The synthesis of compounds **1-9** were performed under an argon atmosphere. The following solvents were distilled from the indicated drying agents: DCM (CaH_2), THF (Na), ACN (CaH_2) or dried over 4 Å molecular sieves. All commercially available reagents were used without further purification. Thin layer chromatography was performed on aluminum plates coated with Merck Silica gel 60 F254 and flash column chromatography was carried out using silica gel 60 and the specified eluent. Nuclear magnetic resonance spectra (^1H and ^{13}C) were recorded on 300 MHz spectrometer. Chemical shifts for ^1H and ^{13}C spectra are recorded in parts per million and are calibrated to solvent residual peaks (CHCl_3 : ^1H 7.26 ppm; ^{13}C 77.16 ppm; MeOH: ^1H 3.31 ppm; ^{13}C 49.00 ppm; H_2O : ^1H 4.79). Multiplicities are indicated by s (singlet), bs (broad singlet), d (doublet), t (triplet), q (quartet), quin (quintuplet) and m (multiplet). Coupling constants, J , are reported in Hertz. Fourier transform infrared (FTIR) spectra were performed on Digilab FTS 3000 spectrometer (samples were gently ground and diluted in non-absorbent KBr matrices) and are reported in reciprocal centimetres (cm^{-1}). The samples were compressed into KBr pellets. Matrix assisted laser desorption ionization time of flight (MALDI-TOF) mass spectra were acquired using a Bruker spectrometer. High resolution mass spectra were performed on Waters-QTOF spectrometer with electrospray ionization mode. Compound **2** was purchased from Quanta BioDesign and was used without further purification.

ACN=acetonitrile, TMSBr=bromotrimethylsilane, CDCl_3 =deuterated chloroform, MeOD=deuterated methanol, D_2O =deuterated water, DCM=dichloromethane, DIPEA=diisopropylethyl amine, DIBALH=diisobutylaluminium hydride, EDCI=*N*-(3-Dimethylaminopro-propyl)-*N'*-ethylcarbodiimide hydrochloride, EtOAc=ethylacetate, HCl=hydrochloric acid, HOBt=hydroxybenzotriazol, MeOH=methanol, PAMAM=poly(amido amine), KBr=potassium bromide, NaOH=sodium hydroxide, NaCl=sodium chloride, THF=tetrahydrofuran, NP@OA=nanoparticles coated with oleic acid, r1 and r2=relaxivities, D_H =hydrodynamic diameter.

II. Synthesis of magnetite nanoparticles (MNPs-OA)

Spherical iron oxide nanoparticles (NPs) were synthesized by the thermal decomposition of iron stearate in octyl ether which leads to the formation of NPs in-situ coated by fatty acids. 1.38 g (2.22 mmol) of $\text{Fe}(\text{stearate})_2$ (Strem Chemicals) and 1.24 g (4.44 mmol) of oleic acid (99%, Alfa Aesar) were added in 20 mL of octyl ether (99%, Fluka, b.p. 287°C). The mixture was heated and kept at 110°C under stirring for 30 min in order to dissolve the reactants. The solution was heated and maintained to 250°C for 30 min with a heating rate of $5^\circ\text{C}/\text{min}$ without stirring and then heated to 287°C to reflux for 120 min under air. The resultant black solution was then cooled down to room temperature and the NPs were

washed 3 times by addition of ethanol and by centrifugation (8000 rpm, 10 min.). The as-synthesized NPs, named NP@OA, were then easily suspended in hexane.

III. Synthesis and characterization of compounds 1-9.

Compound 1. 1 M solution of sodium trimethylsilanolate in dichloromethane (66 mmol; 66 ml) was added at room temperature to a solution of G0.5 PAMAM dendron (22 mmol) in DCM (90 ml). The mixture was stirred for 16 hrs after which a gel was formed. The solvent was then evaporated and the residue triturated in EtOAc. The solid was filtered and compound **1** was obtained quantitatively as yellow solid; ^1H NMR (300 MHz, D_2O) δ 4.21 (m, 2H), 3.65 (t, $J = 6$ Hz, 4H), 3.17 (m, 1H), 2.98 (t, $J = 6$ Hz, 4H) ppm; ^{13}C NMR (75 MHz, D_2O) δ 181.1, 78.0, 74.4, 49.8, 41.1, 34.9 ppm; ES-HRMS: 242.288.

Compound 3. EDCI (2.8 mmol), HOBT (0.5 mmol) and DIPEA (2.6 mmol) were added to a suspension of **1** (1.2 mmol) and Amino-dPEG₄-*t*-butyl ester **2** (2.6 mmol) in ACN (15 ml). The mixture was stirred at room temperature for 16 hrs then the solvent evaporated. The crude was purified by silica gel chromatography (DCM/MeOH 90/10) to yield compound **3** (67%) as yellow oil; ^1H NMR (300 MHz, CDCl_3) δ 3.70 (t, $J = 6.7$ Hz, 4H), 3.66-3.60 (m, 24H), 3.54 (t, $J = 5.3$ Hz, 4H), 3.41 (m, 6H), 2.83 (t, $J = 6.3$ Hz, 4H), 2.49 (t, $J = 6.7$ Hz, 4H), 2.36 (t, $J = 6.3$ Hz, 4H), 2.23 (t, $J = 2.3$ Hz, 1H), 1.44 (s, 18H) ppm; ^{13}C NMR (75 MHz, CDCl_3) δ 171.8, 170.6, 80.2, 73.6, 70.4, 70.35, 70.32, 70.3, 70.1, 70.0, 69.7, 66.7, 49.3, 41.3, 38.9, 36.1, 33.6, 27.9 ppm; IR (KBr): 3504, 3290, 3070, 2972, 2871, 1728, 1655, 1544, 1458, 1365, 1112, 947, 846, 654, 590 cm^{-1} ; MALDI-TOF $[\text{M}+\text{H}]^+ = 806.551$.

Compound 4. Synthesized according to literature procedure¹ (40%) as yellow solid. The experimental data were identical to those reported in reference 1.

Compound 5. A mixture of DIBALH (38.6 mmol) and toluene (25 ml) was cooled to 0 °C under argon. A solution of **4** (18.4 mmol) in toluene (45 ml) was added dropwise and the reaction mixture was stirred for 3 hrs at 0°C. A solution of HCl 1 N was added until pH = 1. The phases were separated; the organic phase was washed with water, dried over magnesium sulphate, filtered and concentrated *in vacuo*. The crude was purified by silica gel chromatography (cyclohexane/EtOAc 70/30) to yield compound **5** as a white solid (90%); the experimental data were identical to those reported in reference 2.

Compound 6. The dibromide **5** (9.7 mmol) was heated at 140 °C in the presence of triethylphosphite (38.8 mmol) for 2 h. The excess of triethylphosphite was then removed *in vacuo* to yield a white solid **6**

quantitatively, which was used in the next step without further purification; the experimental data were identical to those reported in reference 3.

Compound 7. Thionyl chloride (2.3 ml) was added dropwise to a solution of compound **6** (15.8 mmol) in chloroform (6 ml). The solution was refluxed under argon for 1 hr. The organic phase was washed with a solution of NaOH 0.1 N and brine then dried over magnesium sulfate, filtered and concentrated *in vacuo* to yield compound **7** quantitatively. The crude was used in the next step without further purification. An aliquot (500 mg) was purified by silica gel flash chromatography (DCM/MeOH 90/10) to yield yellow oil **7** (310 mg) for analytical measurements; ^1H NMR (300 MHz, CDCl_3) δ 7.22 (m, 2H), 7.19 (m, 1H), 4.55 (s, 2H), 4.02 (quin, $J = 7.3$ Hz, 8H), 3.17 (s, 2H), 3.09 (s, 2H), 1.25 (t, $J = 7.0$ Hz, 12H) ppm; ^{13}C NMR (75 MHz, CDCl_3) δ 137.8, 132.4, 131.05, 128.4, 62.05, 45.6, 34.2, 32.3, 16.2 ppm; ^{31}P NMR (81 MHz, CDCl_3) δ 26.45 ppm; IR (KBr): 2982, 2910, 1605, 1458, 1392, 1248, 1164, 1026, 960, 785, 711, 531 cm^{-1} ; ES-HRMS $[\text{M}+\text{H}]^+ = 427.065$.

Compound 8. Sodium azide (14.6 mmol) was added to a solution of compound **7** (2.4 mmol) in ACN (12 ml) and the resulting mixture was refluxed for 16 hrs. The solvent was then removed under pressure and the residue taken in DCM. The organic phase was washed with water, dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude was purified by silica gel chromatography to yield quantitatively compound **8** as yellow oil (DCM/MeOH 95/5); ^1H NMR (300 MHz, CDCl_3) δ 7.21 (m, 1H), 7.16 (m, 2H), 4.31 (s, 2H), 4.02 (quin, $J = 7.3$ Hz, 8H), 3.18 (s, 2H), 3.11 (s, 2H), 1.25 (t, $J = 7$ Hz, 12H) ppm; ^{13}C NMR (75 MHz, CDCl_3) δ 135.4, 132.2, 130.6, 127.6, 61.5, 53.8, 33.8, 32.0, 15.8 ppm; ^{31}P NMR (81 MHz, CDCl_3) δ 26.49 ppm; IR (KBr): 2984, 2912, 2098, 1604, 1454, 1393, 1250, 1053, 1027, 962 cm^{-1} ; ES-HRMS $[\text{M}+\text{H}]^+ = 434.108$.

Compound 9. a) A mixture of azide **8** (0.1 mmol) and propargylic PEGylated G0.5-PAMAM dendron **3** (0.1 mol) in THF/ H_2O (0.8:0.2 ml) in the presence of 5% mol of $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ and 10% mol of sodium ascorbate was stirred at room temperature for 16 hrs. The reaction mixture was then quenched with brine and the aqueous phase extracted with EtOAc. The organic phase was then dried over magnesium sulfate, filtered and concentrated *in vacuo*. The crude was purified by silica gel chromatography (DCM/MeOH 90/10) to yield the desired protected compound **9a** in 75% yield as yellow oil; ^1H NMR (300 MHz, CDCl_3) δ 7.52 (bs, 1H), 7.35 (m, 2H) 7.21 (m, 1H), 7.10 (m, 2H), 5.47 (s, 2H), 4.0 (quin, $J = 7.3$ Hz, 8H), 3.78 (s, 2H), 3.69 (t, $J = 6.6$ Hz, 4H), 3.60 (m, 24H), 3.53 (t, $J = 5.3$ Hz, 4H), 3.38 (m, 4H), 3.14 (s, 2H), 3.07 (s, 2H), 2.75 (t, $J = 6.4$ Hz, 4H), 2.49 (t, $J = 6.6$ Hz, 4H), 2.39 (t, $J = 6.4$ Hz, 4H), 1.43 (s, 18H), 1.23 (t, $J = 7.3$ Hz, 12H) ppm; ^{13}C NMR (75 MHz, CDCl_3) δ 171.8, 170.5, 143.8, 135.2,

132.7, 131.1, 127.6, 122.7, 80.1, 70.2, 70.14, 70.1, 70.0, 69.8, 69.5, 66.5, 61.8, 53.3, 49.2, 47.6, 38.7, 35.9, 33.9, 33.4, 32.1, 27.8, 16.1 ppm; ^{31}P NMR (81 MHz, CDCl_3) δ 25.60 ppm; MALDI-TOF $[\text{M}+\text{H}]^+ = 1239.58$; b) TMSBr (1.3 mmol) was added at room temperature to a solution of bisphosphonate ester **9a** (0.04 mmol) in DCM (0.5 ml). The reaction mixture was stirred for 2 hrs then quenched with MeOH. The solvent was then evaporated under vacuum to give the desired compound **9** quantitatively which was used without further purification; ^1H NMR (300 MHz, MeOD) δ 8.34 (m, 1H), 7.25 (s, 1H), 7.19 (s, 2H), 5.65 (s, 2H), 4.62 (s, 2H), 3.75-3.39 (m, 40H), 3.17 (m, 2H), 3.10 (m, 2H), 2.86 (m, 4H), 2.58 (m, 4H) ppm; ^{13}C NMR (75 MHz, MeOD) δ 173.4, 171.9, 170.3, 135.4, 134.7, 134.4, 133.8, 130.7, 127.0, 126.5, 69.6, 69.5, 69.3, 68.4, 65.9, 65.7, 53.0, 50.3, 49.2, 45.6, 38.6, 34.6, 33.9, 32.8, 27.8 ppm; ^{31}P NMR (81 MHz, MeOD) δ 24.92 ppm; IR (KBr): 3425, 3081, 2877, 1732, 1653, 1559, 1242, 1198, 1109, 941 cm^{-1} ; MALDI-TOF $[\text{M}+\text{H}]^+ = 1015.36$.

IV. Functionalization process.

10 ml of a NP@OA suspension in hexane (1 mg/ml) were put into contact with a suspension of the dendritic bisphosphonate (13 mg of **9**, 5 ml of water and 2 ml of methanol) at pH 3.5. Both immiscible suspensions were magnetically stirred for one night. A ligand exchange and phase transfer occurs leading to a water suspension of dendronized NPs. The grafted NPs were then separated from the ungrafted dendrons by ultrafiltration. This technique, well adapted to purify all functionalized NPs water suspensions, involves regenerated cellulose membranes with a nominal molecular weight limit (NMWL) of 30 kDa. After, at least, 4 purification steps by ultrafiltration, the pH of the NPs suspension was 6. The so-obtained grafted nanoparticles display a particle size distribution centered at about 30 nm. To obtain dendronized NPs with a lower average hydrodynamic size in water suspension, the concentration in NPs has been lowered to half of the previous concentration. Such a ligand exchange and phase transfer process in diluted conditions leads to a particle size distribution centered at about 15 nm.

Alexa coupling: Alexa-647 grafting onto the surface of NP@9

To a stirring suspension of NP@9 (5 mg of NP@9 in 10 ml distilled water (pH 6.5)) were added 25 mg of EDC.HCl at 0°C. After 30 min., Alexa-647-amine (1 mg, Dyomics) was added and the suspension was stirring at RT overnight. To eliminate the fluorophore excess, the purification was done several times by ultrafiltration in order to obtain washing solutions without Alexa-647-Amine. At each step, UV-visible spectra were done (λ Alexa 647 = 650 nm) to verify the elimination of the fluorophore from the supernatant of the suspension. Then, the purified suspension of NP@9@Alexa647 was dried by lyophilization for in vivo biological tests.

V. Characterization techniques.

The NP@OA, before and after grafting, were characterized by transmission electron microscopy (TEM) with a TOPCON 002B microscope operating at 200 kV, (point resolution 0.18 nm) and equipped with a GATAN GIF 200 electron imaging filter.

The grafting step was confirmed by Infra Red spectroscopy using a Fourier Transform Infrared (FTIR) spectrometer (Digilab FTS 3000) (samples were gently ground and diluted in non-absorbent KBr matrices)) and chemical analyses.

The stability of the water suspensions was assessed by measuring the particle size distribution in water, at pH 7, or in isoosmolar conditions or in PBS buffer and their zeta potential using a nano-size MALVERN (nano ZS) zetasizer.

Hysteresis cycles at room temperature and ZFC/FC measurements between 5 K and 300 K under a field of 75 G, of both as-synthesized and dendronized NPs, were performed with a Superconducting Quantum Interference Device (SQUID) magnetometer (Quantum Design MPMS-XL model).

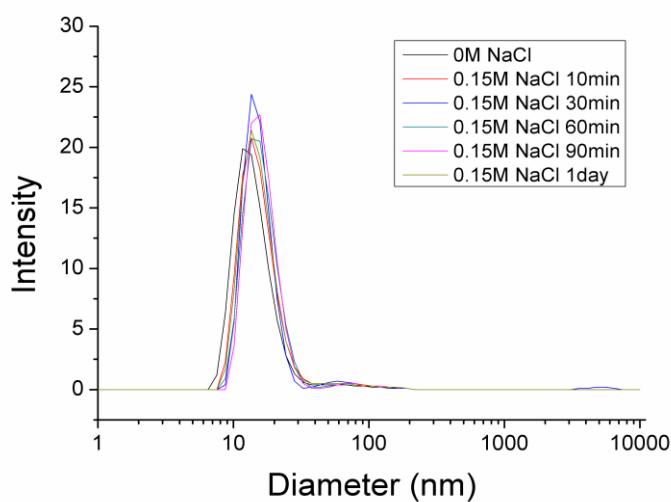


Figure S1. Particle size distribution of NP@**9d** in water (0M NaCl) and in iso-osmolar media as function of time.

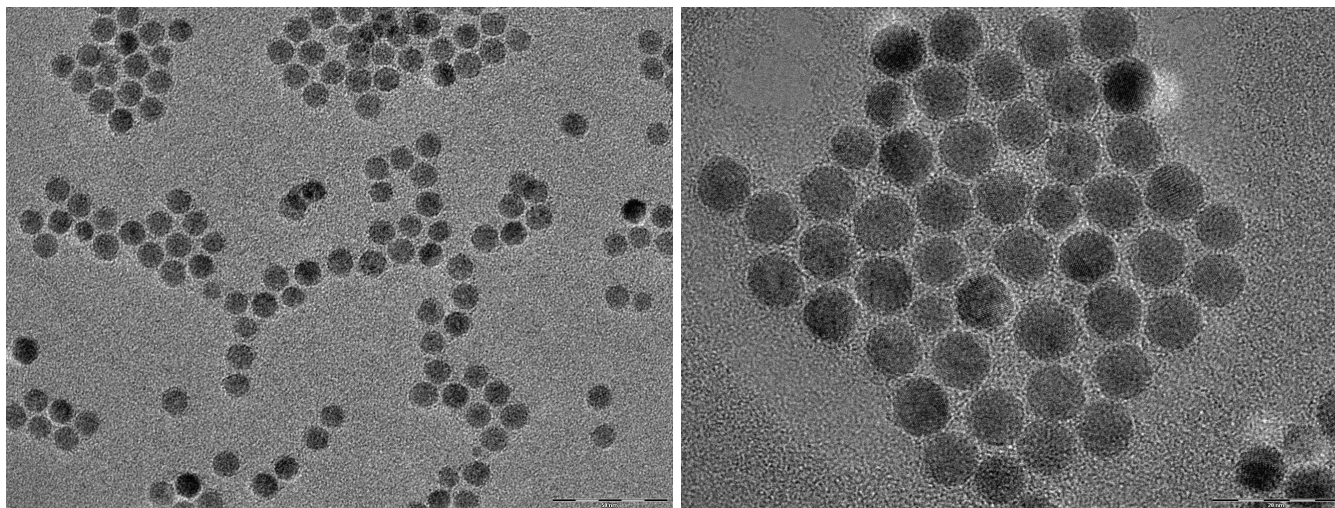


Figure S2. TEM images of NPs functionalized with **9**.

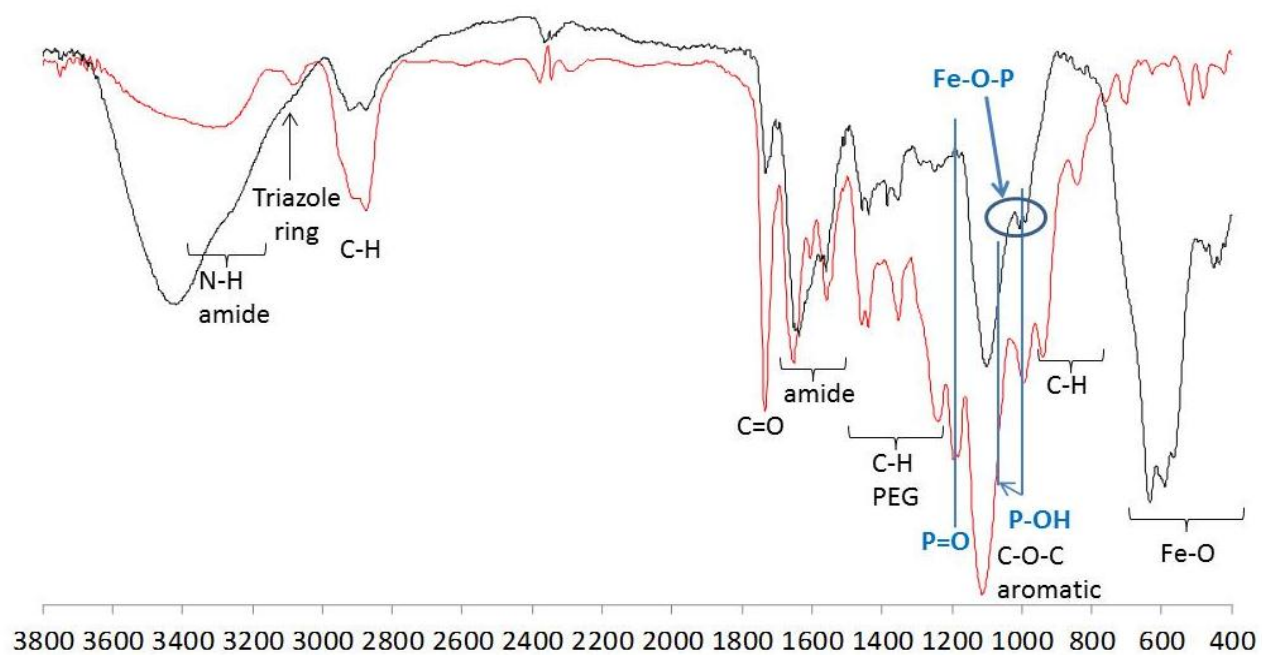


Figure S3. IR spectra of **9** (red line) and NP@**9** (black line).

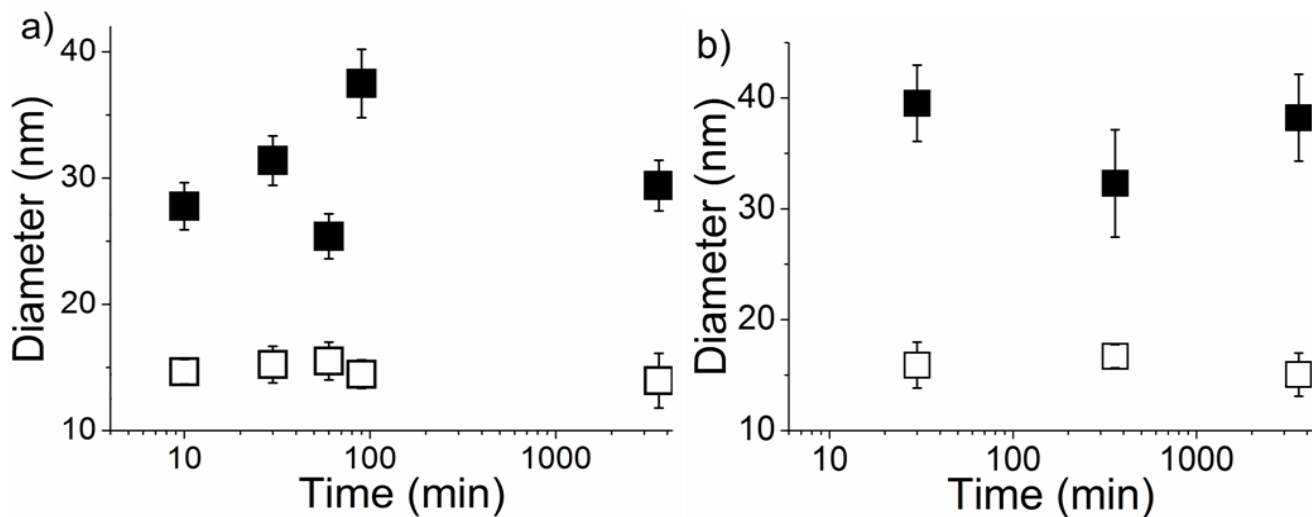


Figure S4. Evolution of the size distribution of NP@9 (■) and NP@9d (□) in sodium chloride medium (0.15 M NaCl) after 24h (a) and in PBS (b)

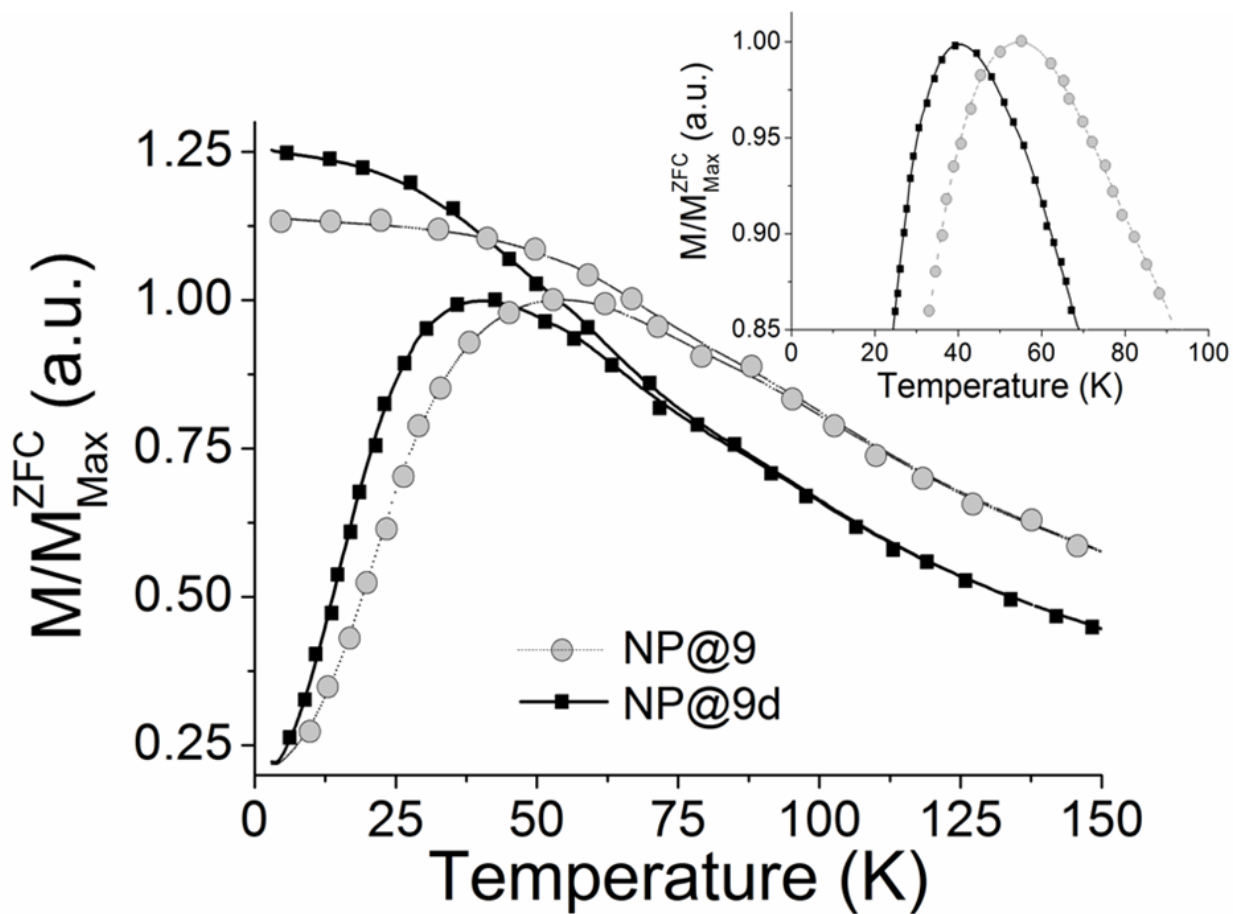


Figure S5. Magnetization curves (ZFC/FC) for NP@9 and NP@9d. The inset graph represents a zoom for a better view of T_B .

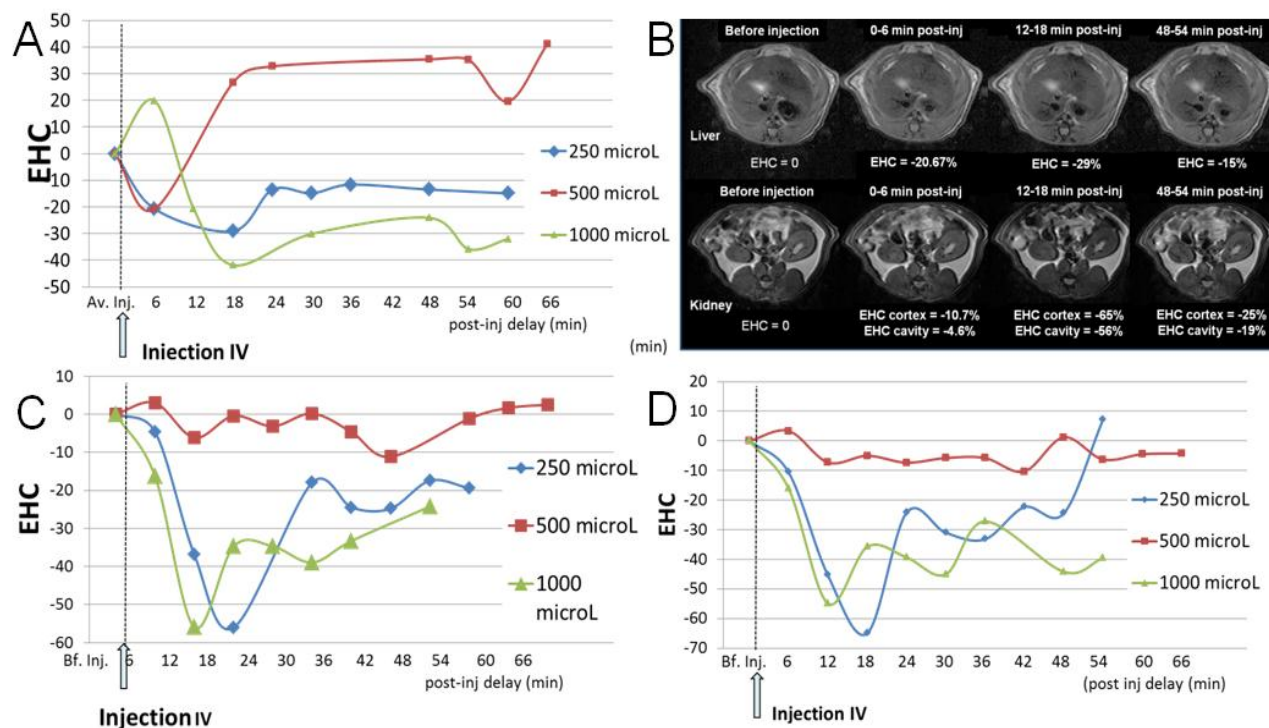


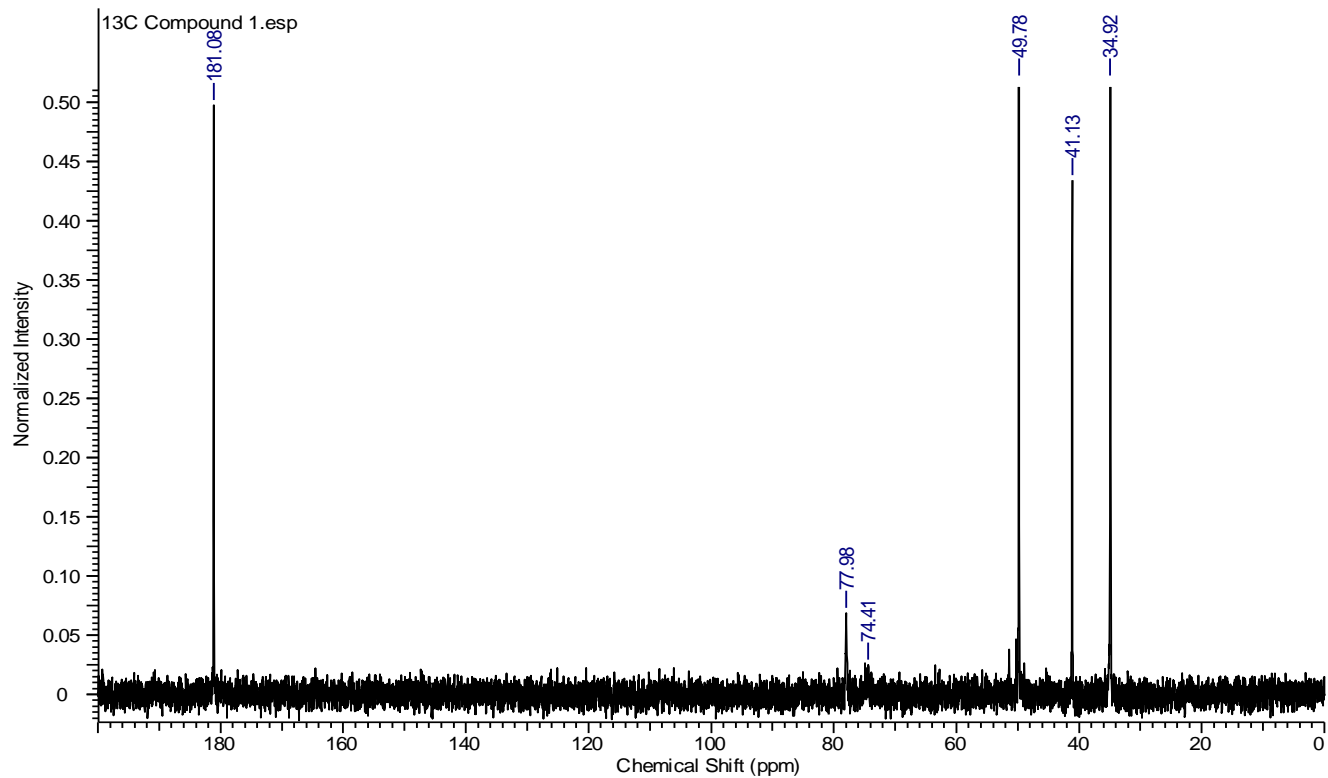
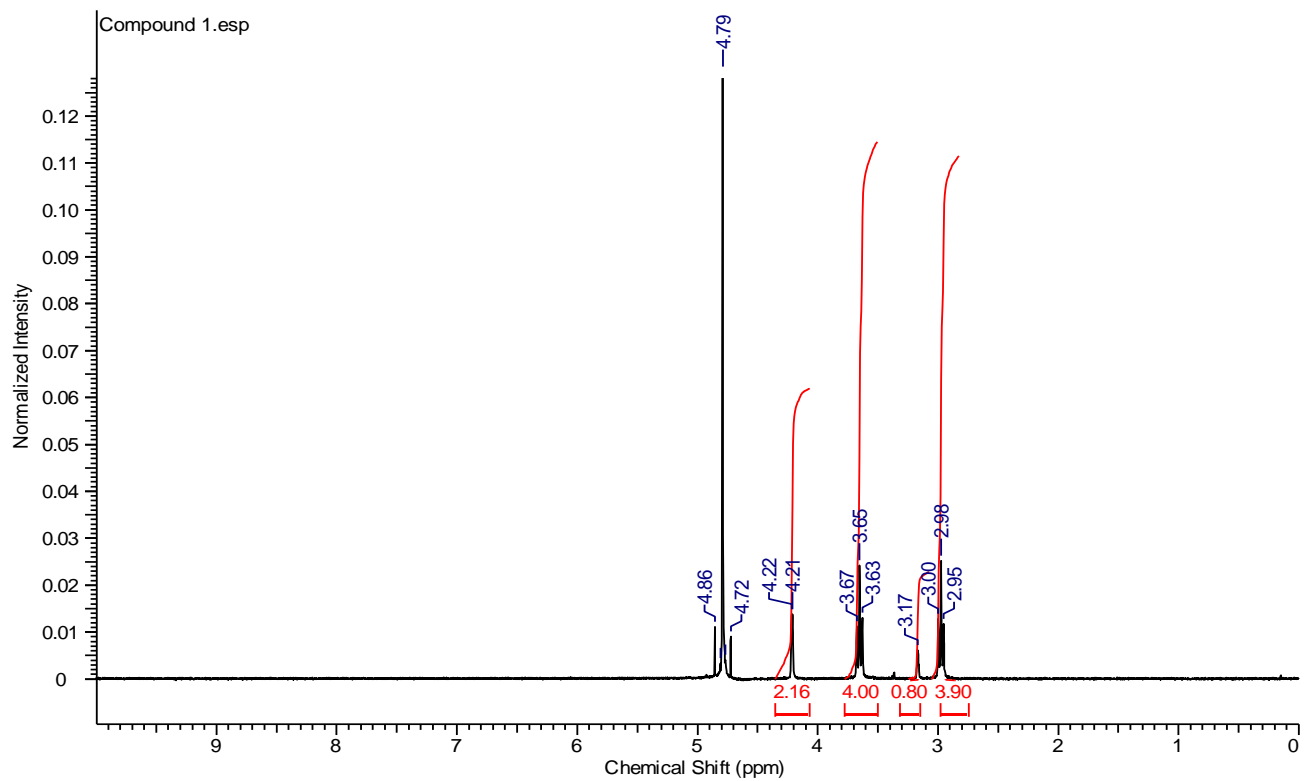
Figure S6. Enhanced contrast (EHC %) evolution in liver (A), cortex (C) and pelvic (excretory renal cavity) after 250 µl, 500 or 1000 µl i.v. injection of NPs@9 at the equivalent iron concentration of 0,152 mM. Coronal T2w. MR images (RARE triggered sequence, TR/TE (ms)= 3000/38,1, FA= 180°). EHC (%) = $[S_{\text{norm}}(t) - S_{\text{norm}}(\text{before inj.})]/S_{\text{norm}}(\text{before inj.}) \times 100$

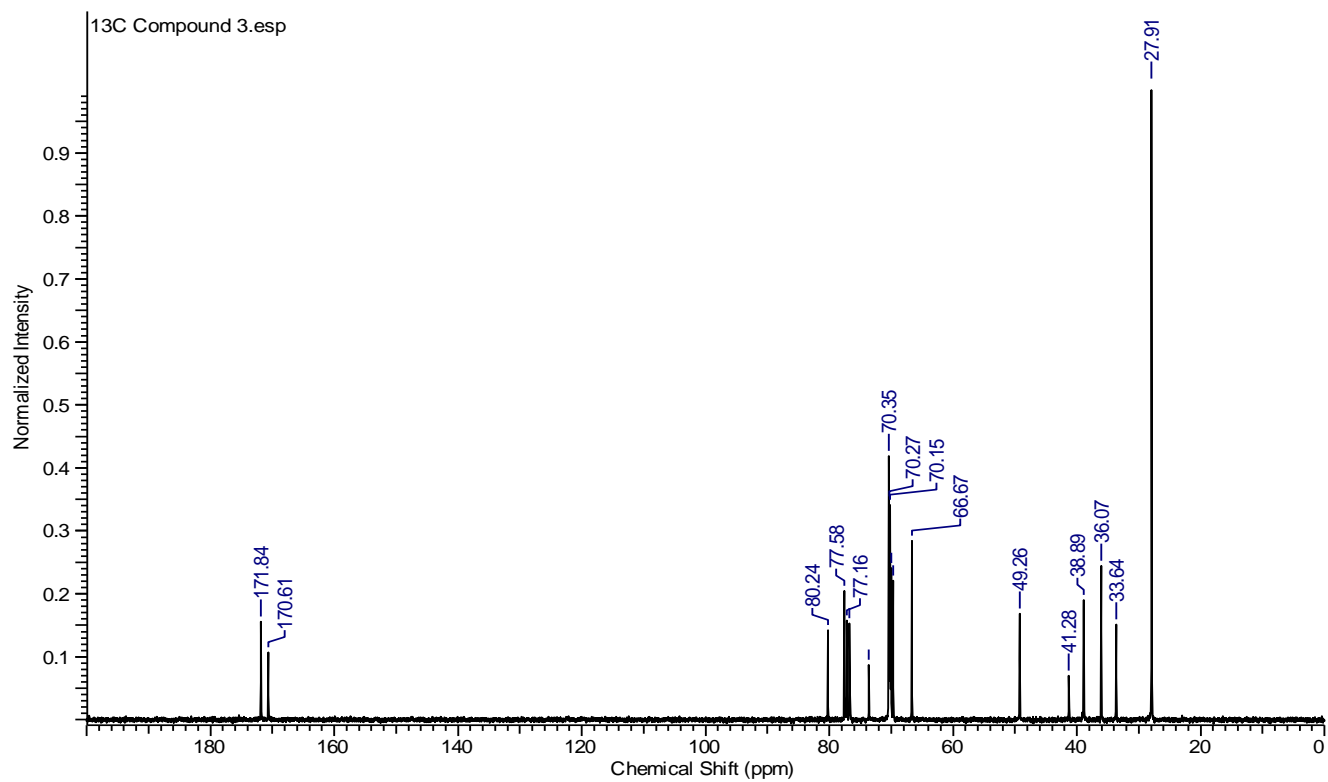
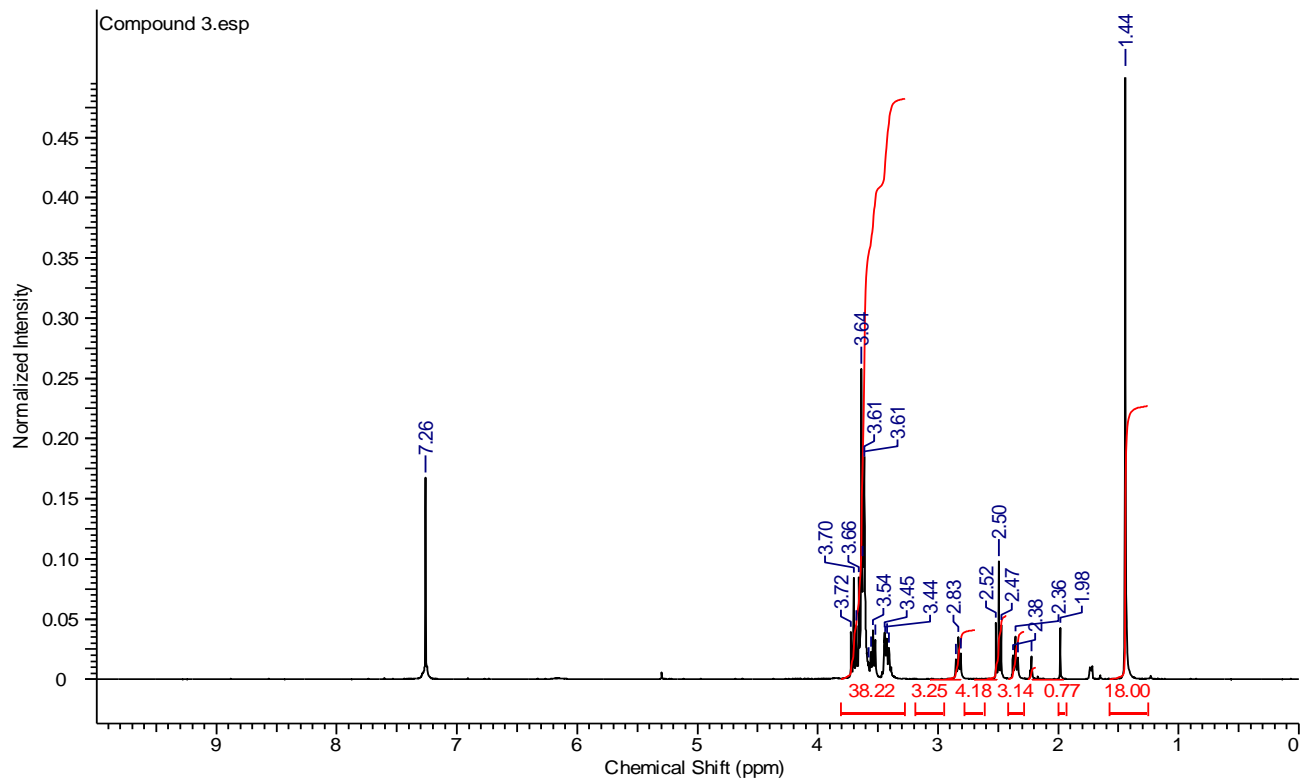
Table 1. *In vitro* relaxivity studies (1.5T, room temperature) of dendronized nanoparticles, compared to commercially available polymer-coated nanoparticles displaying similar average hydrodynamic diameter (D_H). *The characteristics of dendrimer D2 are reported in a previous paper.⁴

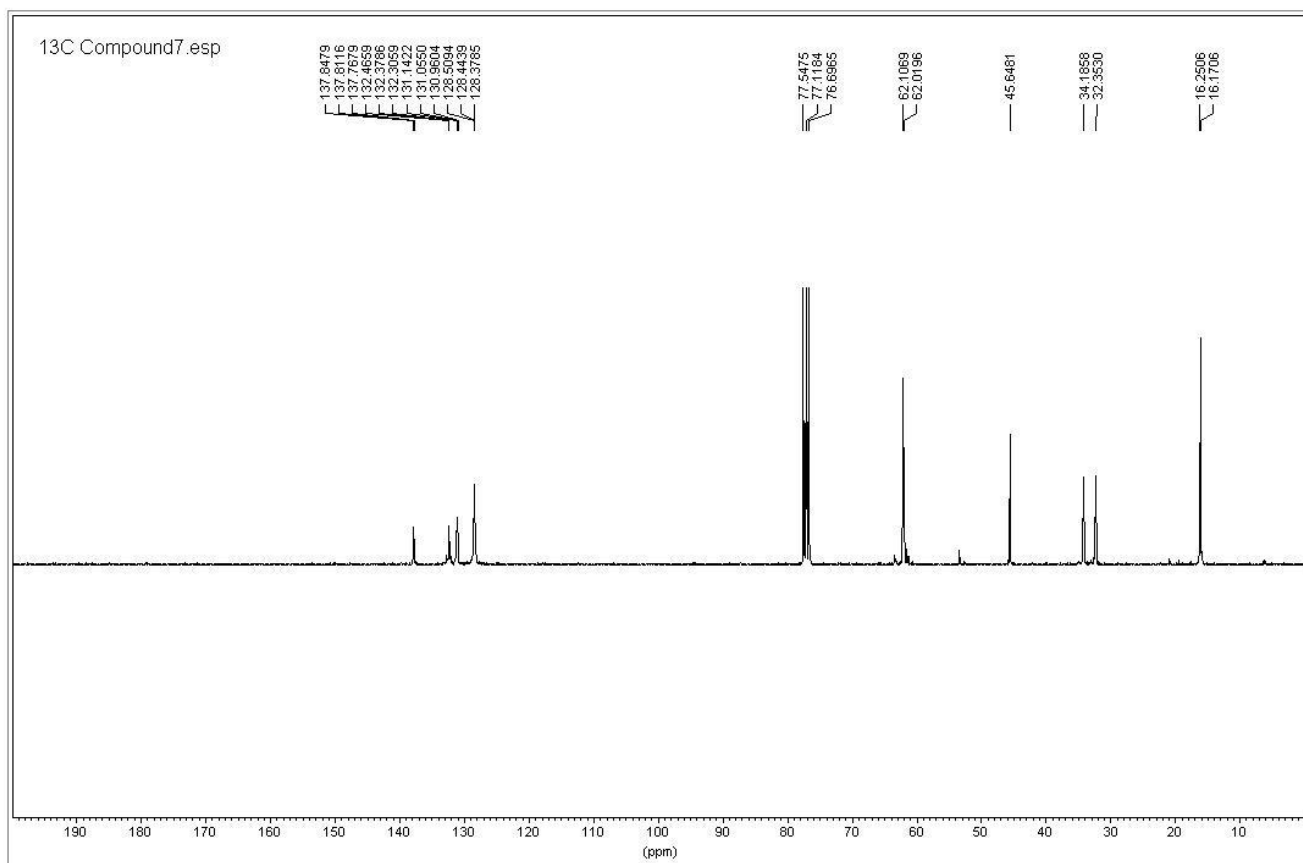
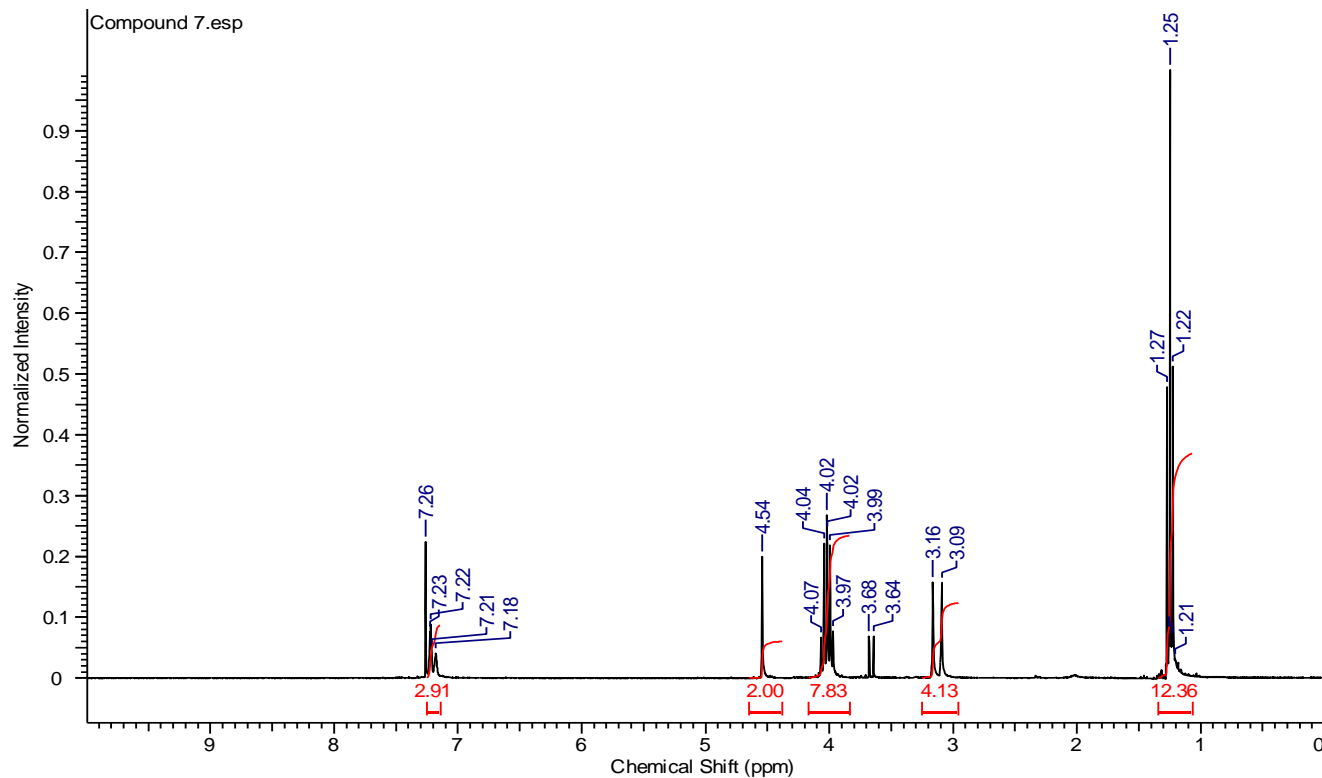
Compound name (company)	Coating agent	D_H (nm)	r_1 ($\text{mM}^{-1}\text{s}^{-1}$)	r_2 ($\text{mM}^{-1}\text{s}^{-1}$)	r_2/r_1
Endoterm (Guebert)	Dextran T10	120-180	10.1	120	11.9
Resovist (Schering)	Carboxydextran	60	9.7	189	19.5
Ferumoxytol (Adv. Magnetics)	Carboxymethyldextran T10	30	15	89	5.9
Sinerem (Guebert)	Dextran T10, T1	15-30	9.9	65	6.7
Supravist (Schering)	Carboxydextran	21	10.7	38	3.6
Abdoscan	Citrate	7	17	33.4	2.4
Our study					
Endorem	Dextran T10	120-180	10.7	141	13.2

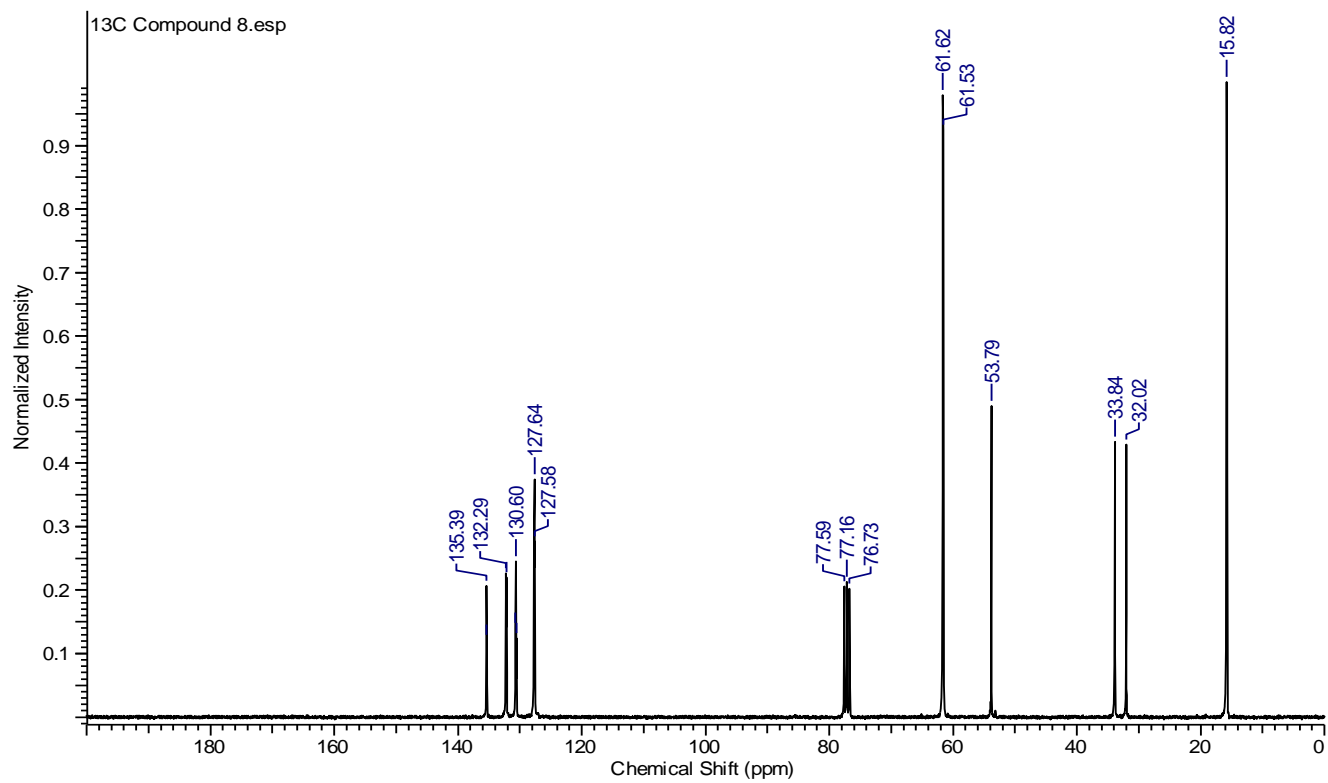
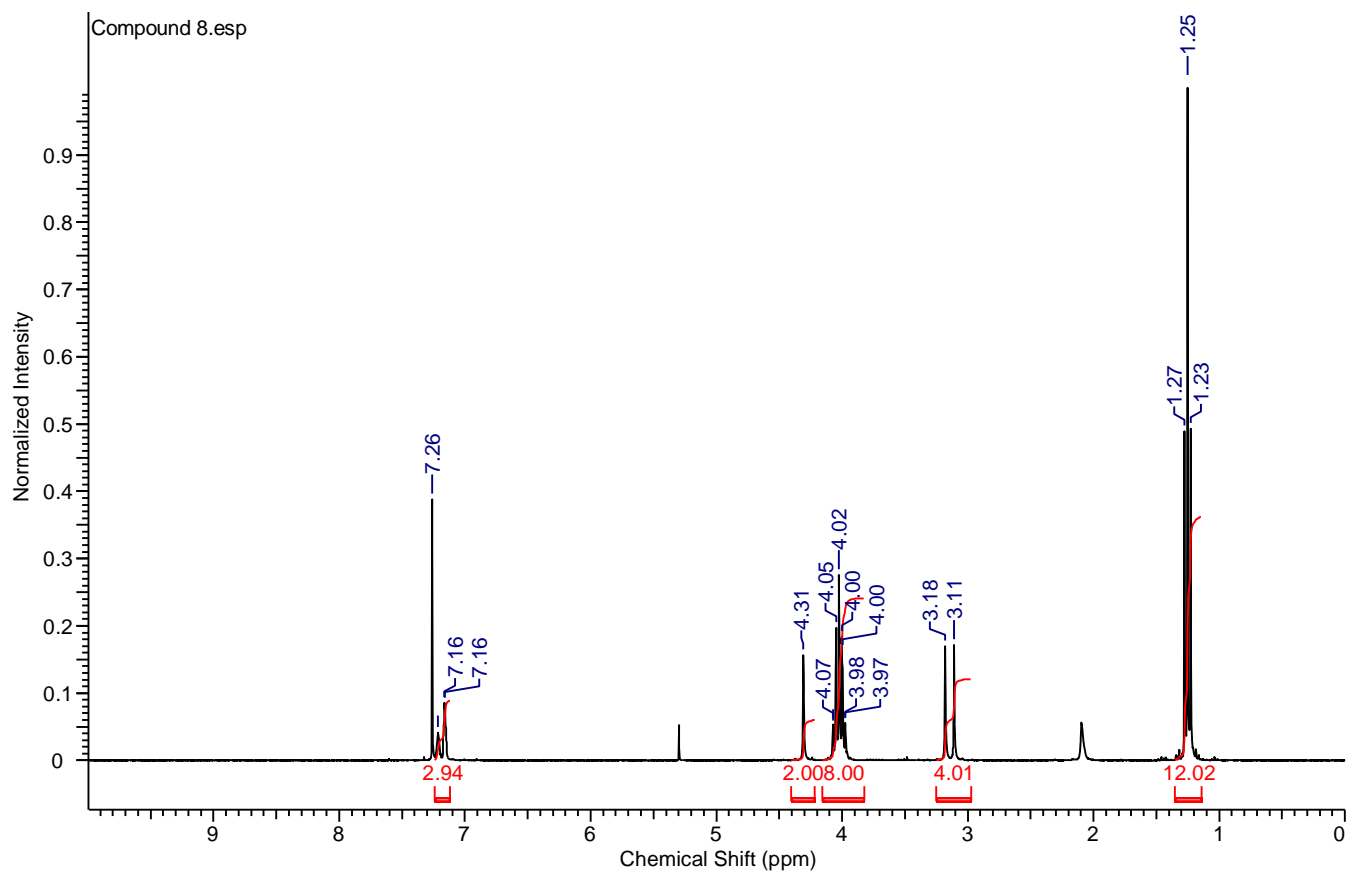
NP@D2*	Dendron monophosphonate	30	6.2	91.2	14.7
NP@9	G0.5-PAMAM-Dendron bisphosphonate	30	7.7	53.8	7.0
NP@9d	G0.5-PAMAM-Dendron bisphosphonate	15	9.4	101.6	10.8

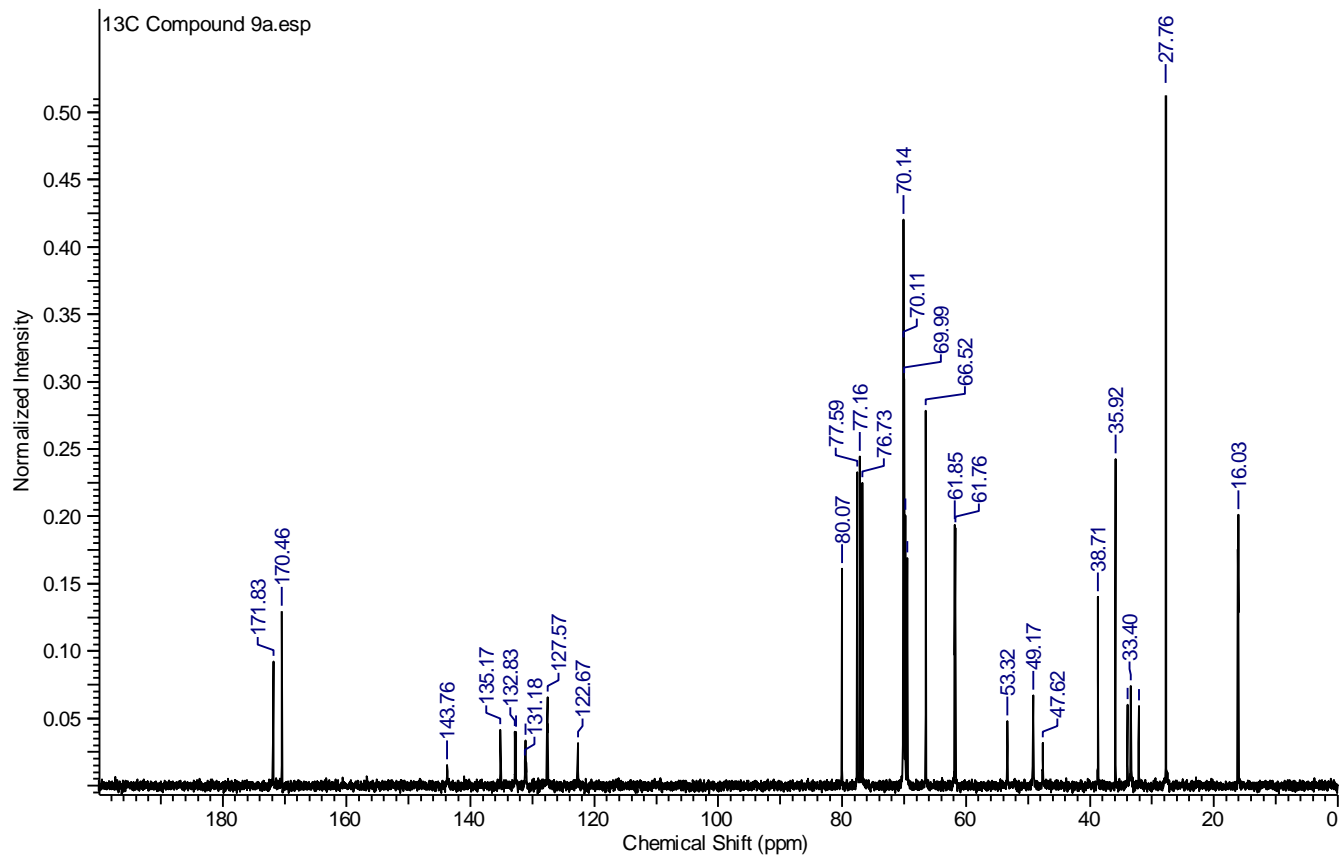
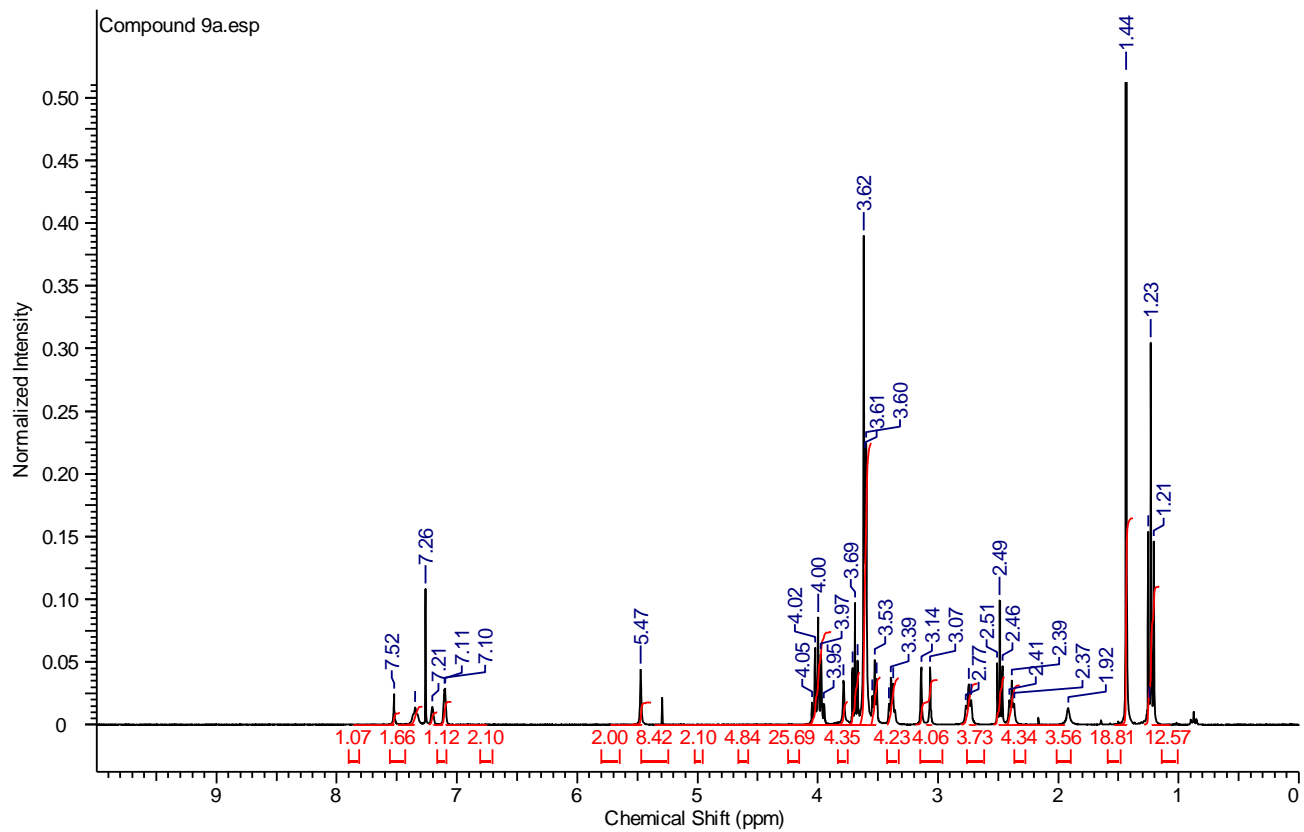
VI. NMR Spectra

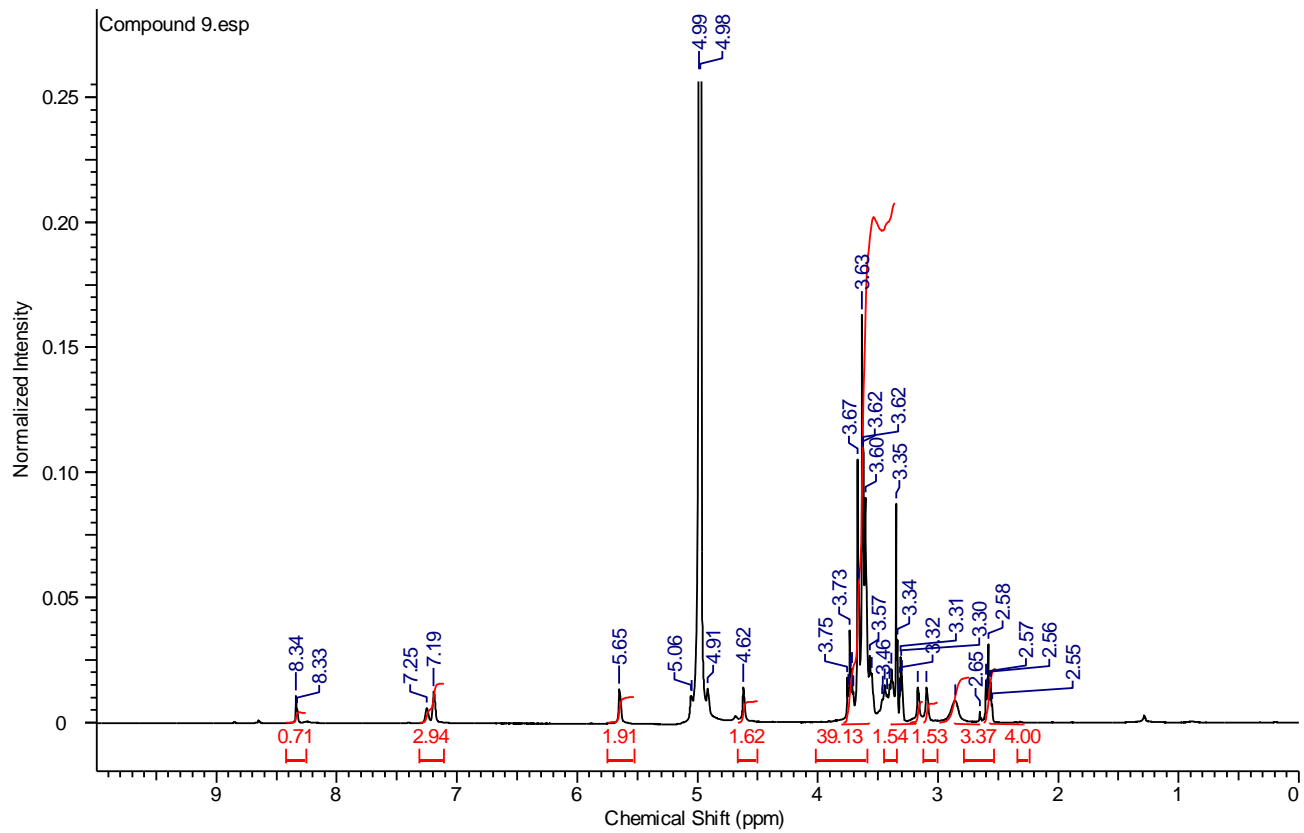




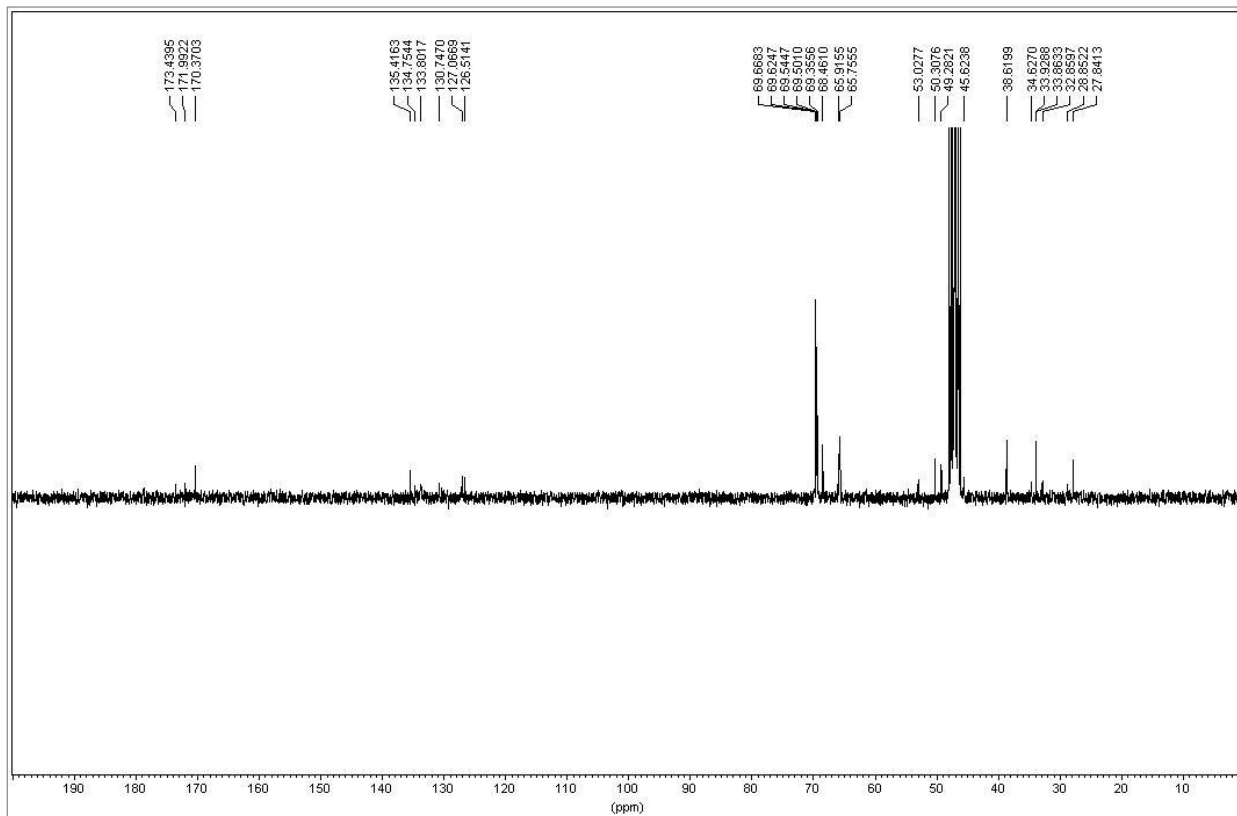








¹³C Compound 9.esp



VII. References.

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