Synthesis and in vivo MRI Evaluation of Biocompatible Branched Copolymer Nano-Contrast Agents

Electronic Supporting Information

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Materials and Synthesis:

Oligoethylene glycol methyl methacrylate monomethyl ether ($M_w \approx$ 300 g/mol) and *n*-butyl methacrylate were passed through a basic alumina column prior to use. Deionized water was prepared using an AquaMAX-Basic 321 DI water purification system. ^{1}H and ^{13}C NMR spectra were recorded on Bruker 400 Ultra Shield spectrometer. High resolution mass spectrometry was performed on a Thermo Finnigan MAT 95XP HRSM spectrometer. Gel permeation chromatography (GPC) was conducted on a Waters 717 plus autosampler equipped with a Waters 515 pump and a Waters 2414 refractive index (RI) detector. Three columns; Styragel HR0.5 (0-1,000), Styragel HR3 (500-30,000) and a Styragel HR5E (2,000-4,000,000) were applied in sequence for separation. Tetrahydrofuran was used as the eluent at 0.3 ml/min. Dynamic light scattering analysis was carried out on a Malvern Zetasizer Nano-ZS machine. Cyro-TEM analysis was performed on Tecnai G2 Spirit - T12 with 120kV acceleration made by FEI Company, Hillsboro, OR. Camera is Gatan UltraScan 4kx4k, made by Gatan Inc., Pleasanton, CA. USA. The relevant vitrobot model is Mark IV, made by FEI Company, Hillsboro, OR. USA. A known amount of polymer nanoparticles chelated with gadolinium was taken in a clean glass vial and digested using 70% concentrated nitric acid (200 μL). The vial was heated at 80 ºC for 45 min; the sample was finally made up to 20 mL using DI water. The diluted sample was further filtered through a 0.45 μM disposable sterile PTFE syringe filter unit. ICP-MS was calibrated for Gadolinium of different concentration prepared from serial dilution of Gadolinium ICP standard solution (Sigma-Aldrich, FLUKA 356220) in 1M nitric acid. The detection limit of the instrument was determined from the standard error of regression. The sample was analyzed using an ICP-MS system (Agilent 7500). Gadolinium at molecular weight of 156 and 157 were chosen for detection.

4-[2-(2-Hydroxyethoxy)ethoxy]benzoic acid (1)

4-Hydroxybenzoic acid (10.66 g, 77.20 mmol, 1 eq), 2-(2-chloroethoxy)ethanol (28.85 g, 231.6 mmol, 3 eq), potassium hydroxide (12.99 g, 231.6 mmol, 3 eq) and a catalytic amount of potassium iodide were dissolved in EtOH (90 mL) and H_2O (30 mL). The reaction was heated under reflux conditions for 72 h. EtOH was removed on the rotary evaporator and H_2O (50 mL) added. The aqueous solution was washed with diethyl ether (3 × 100 mL) to remove impurities. 6M HCl (aq) was added to the aqueous phase at 50 ºC until the pH reached 1. After cooling to 0 ºC a white solid was isolated by filtration and dried under high vacuum. The desired product 4-[2-(2-hydroxyethoxy)ethoxy]benzoic acid (**1**) was obtained via recrystallization from acetone: diethyl ether (1: 4) as a white solid (5.20 g, 30 %). ^{1}H NMR (DMSO-*d*$_6$): δ 12.60 (br, 1H), 7.88 (d, 2H, J = 8.9 Hz), 7.02 (d, 2H, J = 8.9 Hz), 4.63 (br, 1H), 4.16 (t, 2H, J = 4.7 Hz), 3.75 (t, 2H, J = 4.7 Hz), 3.50 (m, 4H). ^{13}C NMR (400 Hz, DMSO-*d*$_6$): δ 167.0, 162.1, 131.4, 123.0, 114.3, 72.5, 68.8, 67.5, 60.3.

4-Methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic acid (2)

4-[2-(2-Hydroxyethoxy)ethoxy]benzoic acid (**1**, 2.06 g, 9.11 mmol, 1 eq) was suspended in toluene (40 mL). *p*-Toluenesulfonic acid monohydrate (0.69 g, 3.64 mmol, 0.4 eq) and methacrylic acid (11.76 g, 136.65 mmol, 15 eq) were then added. The reaction was heated under Dean-Stark conditions for 24 h. After this time the reaction was cooled to 0 ºC and filtered. $CHCl_3$ (100 mL) was added to the filtrate and the organic layer washed with H_2O (2 × 100 mL), dried with Na_2SO_4 and evaporated to dryness to afford a crude solid, which was purified by column chromatography [SiO_2, Hexane–EtOAc (1:1)] to afford the desired product 4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic acid (**2**) as a white solid (2.01 g, 75 %). ^{1}H NMR ($CDCl_3$): δ 11.58 (br, 1H), 8.04 (d, 2H, J = 8.9 Hz), 6.94 (d, 2H, J = 8.9 Hz), 6.12 (s, 1H), 5.56 (m, 1H), 4.33 (t, 2H, J = 4.8 Hz), 4.19 (t, 2H, J = 4.8 Hz), 3.89 (t, 2H, J = 4.8 Hz), 3.82 (t, 2H, J = 4.8 Hz), 1.93 (s, 3H). ^{13}C NMR (400 Hz, $CDCl_3$): δ 171.9, 167.5, 163.3, 136.1, 132.4, 126.0, 122.0, 114.4, 69.5, 69.4, 67.7, 63.8, 18.4. HRMS (ESI) *m*/*z*: $[M + H]^+$ calculated for $C_{15}H_{19}O_6$, 295.1176; found: 295.1175 (ppm 0.32).

4-Methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic succinimide ester (3)

N,*N*'-Dicyclohexylcarbodiimide (1.00 g, 4.85 mmol, 1.1 eq) in CH_2Cl_2 (10 mL) was added to a stirred solution of 4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic acid (**2**, 1.30 g, 4.40 mmol, 1 eq) and *N*-hydroxysuccinimide (0.55 g, 4.85 mmol, 1.1 eq) in CH_2Cl_2 (20 mL). After stirring for 2 h the dicyclohexylurea byproduct was removed by filtration and the solution obtained evaporated to dryness to obtain a crude clear oil which was purified by column chromatography [SiO_2, Hexane–EtOAc (3:1)] to afford the desired product 4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic succinimide ester (**3**) as a clear oil (0.24 g, 70 %). ^{1}H NMR ($CDCl_3$): δ 8.02 (d, 2H, J = 8.9 Hz), 6.94, (d, 2H, J = 8.9 Hz), 6.07 (s, 1H), 5.53 (m, 1H), 4.28 (t, 2H, J = 4.8 Hz), 4.16 (t, 2H, J = 4.8 Hz), 3.84 (t, 2H, J = 4.8 Hz), 3.76 (t, 2H, J = 4.8 Hz), 2.84 (s, 4H), 1.89 (s, 3H). ^{13}C NMR ($CDCl_3$): δ 169.6, 167.3, 164.1, 161.1, 136.0, 132.8, 125.8, 117.2, 114.8, 69.4, 69.3, 67.8, 63.7, 25.7, 18.3. HRMS (ESI) *m*/*z*: $[M + H]^+$ calculated for $C_{19}H_{22}NO_8$, 392.1345; found: 392.1348 (ppm 0.76).

5-Amino-(1,3-ethylmethacrylate dibenzamide) (4)

5-Aminoisophthalic acid (5.08 g, 28.04 mmol, 1 eq), 2-aminoethylmethacrylate (11.15 g, 67.30 mmol, 2.4 eq) and Et_3N (7.34 g, 72.91 mmol, 2.6 eq) were dissolved in DMF (70 mL). After stirring for 15 mins the reaction was filtered to remove the $Et_3N{\cdot}HCl$ precipitate. To the solution obtained DMAP (2.74 g, 22.43 mmol, 0.8 eq) was added followed by the dropwise addition of DCC (13.88 g, 67.30 mmol, 2.4 eq) in DMF (30 mL) over 30 mins. The reaction was stirred overnight at room temperature. The DCU precipitate was then removed by filtration. The solution obtained was evaporated to dryness to afford a crude oil which was purified by column chromatography [SiO_2, Hexane–EtOAc (50:50)] to afford the desired product 5-amino-(1,3-ethylmethacrylate dibenzamide) (**4**) as a white solid (2.52 g, 22 %). ^{1}H NMR (DMSO-*d*$_6$): δ8.49 (t, 2H, J = 5.6 Hz), 7.35 (s, 1H), 7.11 (s, 2H), 6.05 (s, 2H), 5.67 (s, 2H), 5.45 (s, 2H), 4.20 (t, 4H, J = 5.6 Hz), 3.52 (q, 4H, J = 5.7 H), 1.86 (s, 6H). ^{13}C NMR (400 Hz, DMSO-*d*$_6$): δ167.1, 166.6, 148.8, 135.9, 135.6, 126.0, 115.1, 113.2, 63.0, 38.3, 18.0. HRMS (ESI) *m*/*z*: $[M + H]^+$ calculated for $C_{20}H_{26}O_6N_3$, 404.1618; found, 404.1629 (ppm 3.19).

1,3-Ethylmethacrylate-5-methylbromide tribenzamide (5)

5-Amino-(1,3-ethylmethacrylate dibenzamide) (**4**, 0.95 g, 2.36 mmol, 1 eq) and Et_3N (0.26, 2.59 mmol, 1.1 eq) were dissolved in THF (20 mL). Bromoacetyl bromide (0.50 g, 2.47 mmol, 1.05 eq) was then added dropwise in THF (20 mL) over 20 mins at 0 ºC. The reaction was allowed to warm to room temperature and left to stir for 2 h. The reaction was then filtered and the solvent obtained evaporated to dryness to afford a crude oil. The crude oil obtained was purified by column chromatography [SiO_2, Hexane–EtOAc (50:50)] to afford the desired product 1,3-ethylmethacrylate-5-methylbromide tribenzamide (**5)** as a white solid (0.88 g, 71 %). ^{1}H NMR (DMSO-*d*$_6$): δ10.69 (s, 1H), 8.75 (t, 2H, J = 5.7 Hz), 8.14 (s, 2H), 7.96 (s, 1H), 6.06 (s, 2H), 5.66 (s, 2H), 4.22 (t, 4H, J = 5.6 Hz), 4.06 (s, 2H), 3.56 (q, 4H, J = 5.7 Hz), 1.86 (s, 6 H). ^{13}C NMR (400 Hz, DMSO-*d*$_6$): δ166.6, 166.2, 165.3, 138.8, 135.9, 135.6, 126.0, 121.2, 120.9, 62.9, 38.4, 30.2, 18.1. HRMS (ESI) *m*/*z*: $[M + Na]^+$ calculated for $C_{22}H_{26}O_7N_3BrNa$, 546.0846; found, 546.0849 (ppm 0.57).

1,3-Ethylmethacrylate-5-methyDO3A tribenzamide (6)

1,3-Ethylmethacrylate-5-methylbromide tribenzamide (**5**, 0.78 g, 1.48 mmol, 1 eq) and DO3A-*tert*-butyl-ester (0.76 g, 1.48 mmol, 1 eq) were dissolved in CH_3CN (20 mL) and potassium carbonate (0.26 g, 1.92 mmol, 1.3 eq) added in one portion. The reaction was left to stir overnight at room temperature. The reaction was then cooled to 0 ºC in an ice bath and filtered, the desired product 1,3-ethylmethacrylate-5-methyDO3A tribenzamide (**6**) was isolated as a white solid (1.22 g, 95 %). ^{1}H NMR (DMSO-*d*$_6$): δ10.54 (s, 1H), 8.72 (t, 2H, J = 5.7 Hz), 8.17 (s, 2H), 7.95 (s, 1H), 6.06 (s, 2H), 5.66 (s, 2H), 4.22 (t, 4H, J = 5.6 Hz), 3.55 (q, 4H, J = 5.7 Hz), 3.45-1.95 (br, 24H), 1.86 (s, 6H), 1.37 (s, 27H). ^{13}C NMR (400 Hz, DMSO-*d*$_6$): δ186.9, 170.4, 166.5, 166.2, 138.7, 135,7, 135.3, 125.9, 121.2, 81.2, 80.8, 62.8, 58.2, 56.0, 55.8, 38.3, 27.7, 17.9. HRMS (ESI) *m*/*z*: $[M + Na]^+$ calculated for $C_{48}H_{75}O_{13}N_7Na$, 980.5315; found, 980.5295 (ppm 2.07).

Poly(oligoethylene glycol methyl methacrylate monomethyl ether)-c-(4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic acid) (P1-P3)

A typical copolymerization was conducted as follows, 4-cyano-4-(phenylcarbonothioylthio)pentanoic acid (43.3 mg, 0.155 mmol, 1 eq) and AIBN (5.1 mg, 31.0 μmol, 0.2 eq) were added to a small schlenk tube. Oligoethylene glycol methacrylate monomethyl ether ($M_w \approx$ 300 g/mol, 4.42 g, 14.73 mmol, 95 eq) and 4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy]benzoic succinimide ester (**3**, 0.30 g, 0.775 mmol, 5 eq) were then added followed by DMF (15 mL). The reaction mixture was degassed and the vessel was backfilled with N_2. The reaction mixture was then placed in an oil bath at 70 °C, and the polymerization was quenched after 20 h. The reaction mixture was dissolved in a minimal amount of THF and added dropwise to a large excess of ice cold hexane. The precipitation was repeated twice before the polymer (**P3**) was obtained as pink oil (3.30 g). ^{1}H NMR ($CDCl_3$): δ 8.01 (br, Ar), 6.99 (br, Ar), 4.06 (br, $C(O)OCH_2$), 3.64 (br, CH_2OCH_2), 3.54 (br, CH_2OCH_3), 3.36 (br, CH_2OCH_3), 1.76 (br, $C(CH_3)CH_2$), 0.86 (br, $C(CH_3)CH_2$).

DO3A Branched copolymer nanoparticles (N1(^{t}Bu)-N3(^{t}Bu))

A typical branched copolymerization was conducted as follows, **P3** (0.95 g, 42.4 μmol, 1 eq) and AIBN (1.4 mg, 8.48 μmol, 0.2 eq) were added to a small schlenk tube. Butyl methacrylate (0.42 g, 2.97 mmol, 70 eq) and 1,3-ethylmethacrylate-5-methyDO3A tribenzamide (**5**, 0.12 g, 0.127 mmol, 3 eq) were then added followed by DMF (15 mL). The reaction mixture was degassed and the vessel was backfilled with N_2. The reaction mixture was then placed in an oil bath at 70 °C, and the polymerization was quenched after 20 h. The reaction mixture was dissolved in a minimal amount of THF and added dropwise to a large excess of ice cold hexane. The precipitation was repeated twice before the branched copolymer nanoparticle (**N3(^{t}Bu)**) was obtained as clear oil (0.97 g). ^{1}H NMR ($CDCl_3$): δ 8.06 (br, Ar), 7.03 (br, Ar), 4.06 (br, $C(O)OCH_2$), 3.93 (br, $CH_2CH_2CH_2CH_3$), 3.64 (br, CH_2OCH_2), 3.54 (br, CH_2OCH_3), 3.36 (br, CH_2OCH_3), 2.89 (br, $C(O)CH_2CH_2(O)C$) 1.78 (br, $C(CH_3)CH_2$), 1.59 (br, $CH_2CH_2CH_2CH_3$), 1.42 (br, $C(CH_3)_3$), 1.39 (br, $CH_2CH_2CH_2CH_3$), 0.93 (br, $CH_2CH_2CH_2CH_3$), 0.85 (br, $C(CH_3)CH_2$).

Deprotection of branched copolymer nanoparticle tert-butyl groups (N1-N3)

A typical branched copolymer nanoparticle deprotection was conducted as follows, **N2(^{t}Bu)** (1.18 g) was dissolved in CH_2Cl_2 (4 mL), the solution was cooled to 0 ºC in an ice bath. TFA (4 mL) was added and the reaction was stirred at 0 ºC for 2 h. After this time the reaction was evaporated to dryness. The oil obtained was dissolved in THF (10 mL) and added dropwise to ice cold hexane. The precipitation was repeated twice before the branched copolymer (**N2**) was obtained as clear oil (0.90 g). ^{1}H NMR ($CDCl_3$): δ 8.05 (br, Ar), 7.02 (br, Ar), 4.06 (br, $C(O)OCH_2$), 3.92 (br, $CH_2CH_2CH_2CH_3$), 3.63 (br, CH_2OCH_2), 3.53 (br, CH_2OCH_3), 3.35 (br, CH_2OCH_3), 2.88 (br, $C(O)CH_2CH_2(O)C$) 1.78 (br, $C(CH_3)CH_2$), 1.58 (br, $CH_2CH_2CH_2CH_3$), 1.38 (br, $CH_2CH_2CH_2CH_3$), 0.92 (br, $CH_2CH_2CH_2CH_3$), 0.84 (br, $C(CH_3)CH_2$).

Chelation of gadolinium within the core of branched copolymer nanoparticle (N2(Gd))

A typical chelation was conducted as follows, **N2** (5.35 g, possessing approximately 90 mg and 0.23 mmol of DO3A units) was dissolved in isopropanol (30 mL). $GdCl_3.6H_2O$ (2.11 g, 5.68 mmol, approximately 25 eq relative to DO3A units) was then added in 10 mM ammonium acetate (70 mL). The reaction was heated under reflux for 24 h at 55 °C. After this time the solution obtained was purified extensively by dialysis (M_w cut-off 7 kDa) for 2 days. The water was then removed by freeze drying and the branched copolymer dissolved in THF (10 mL) and added dropwise to ice cold hexane. The **N2(Gd)** was obtained as clear oil (3.37 g). ^{1}H NMR ($CDCl_3$): δ 8.04 (br, Ar), 7.01 (br, Ar), 4.04 (br, $C(O)OCH_2$), 3.90 (br, $CH_2CH_2CH_2CH_3$), 3.62 (br, CH_2OCH_2), 3.51 (br, CH_2OCH_3), 3.34 (br, CH_2OCH_3), 2.87 (br, $C(O)CH_2CH_2(O)C$), 1.77 (br, $C(CH_3)CH_2$), 1.57 (br, $CH_2CH_2CH_2CH_3$), 1.35 (br, $CH_2CH_2CH_2CH_3$), 0.91 (br, $CH_2CH_2CH_2CH_3$), 0.83 (br, $C(CH_3)CH_2$).

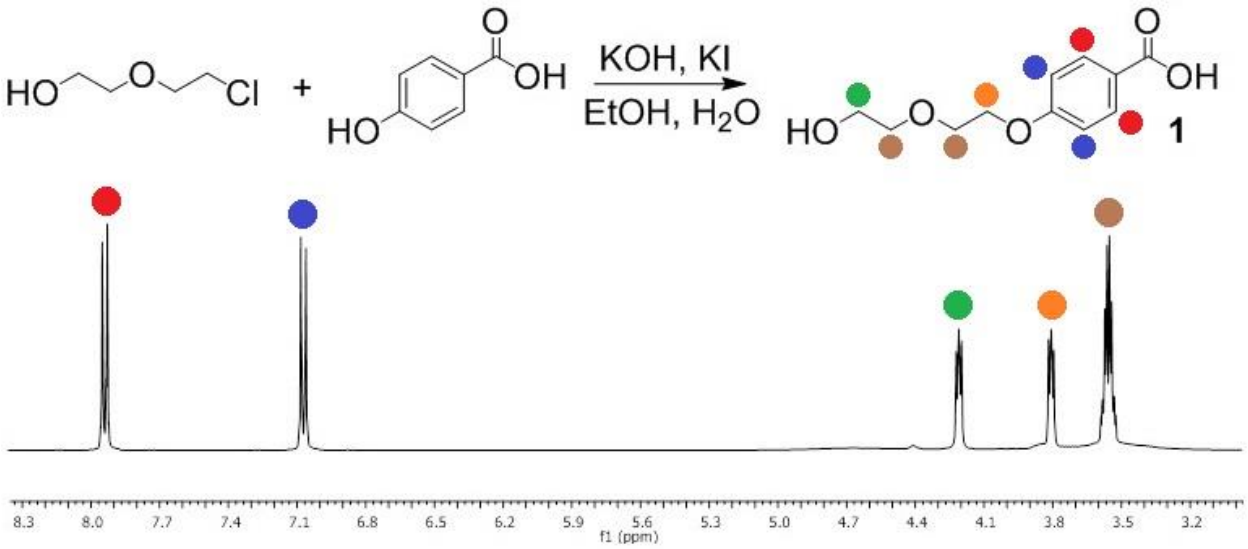

Figure S1. ^{1}H NMR (DMSO-d_6) spectrum of 4-[2-(2-hydroxyethoxy)ethoxy]benzoic acid (**1**).

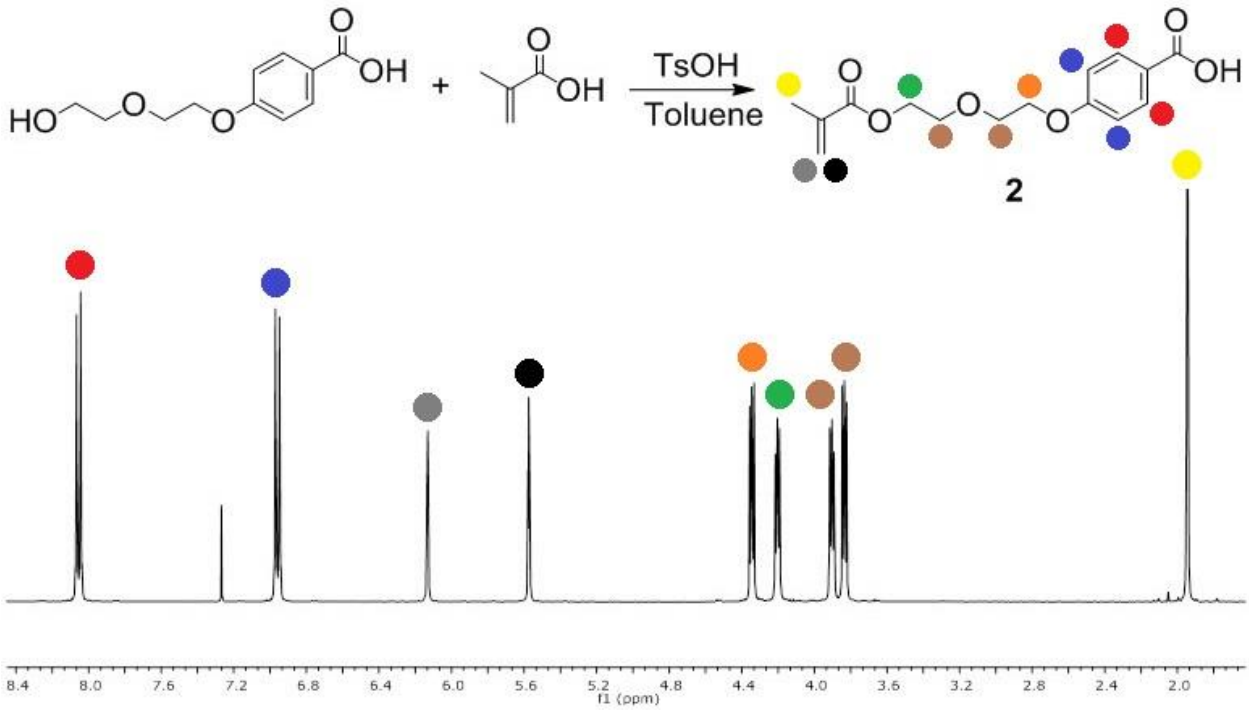

Figure S2. ^{1}H NMR ($CDCl_3$) spectrum of 4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy] benzoic acid (**2**).

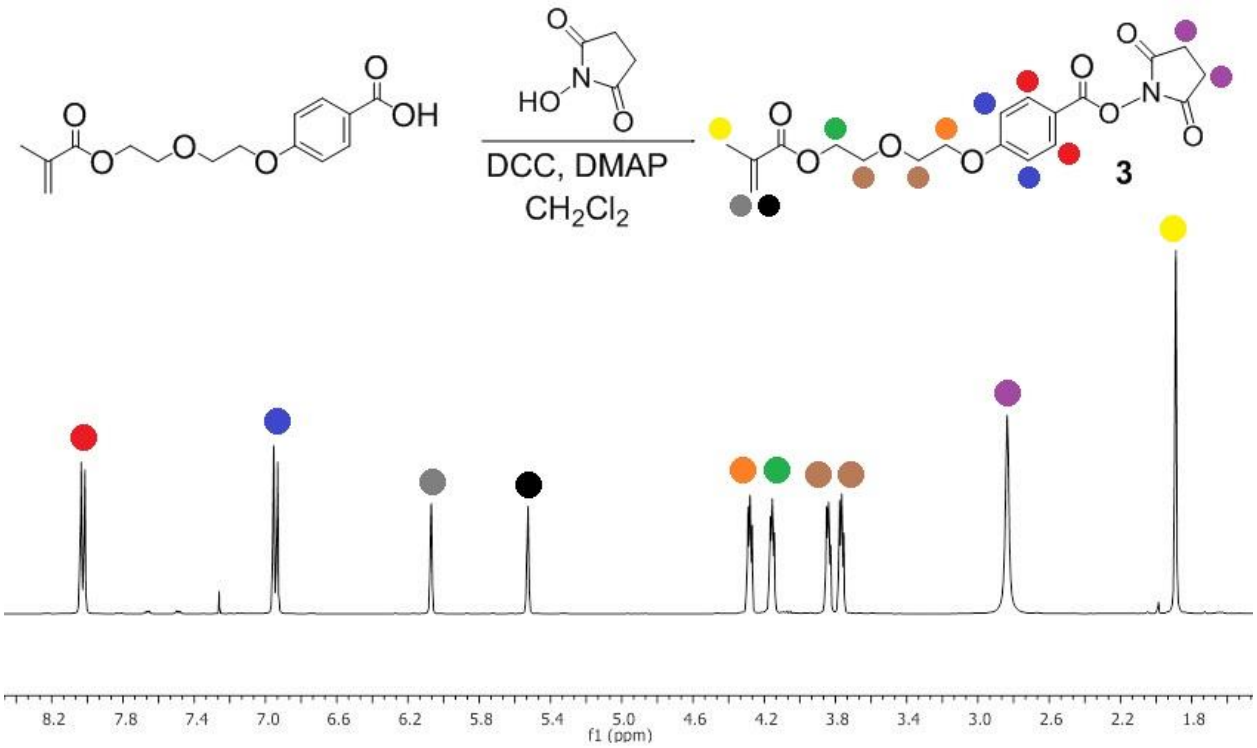

Figure S3. ^{1}H NMR ($CDCl_3$) spectrum of 4-methyl methacrylate[2-(2-hydroxyethoxy)ethoxy] succinimide ester benzoate (**3**).

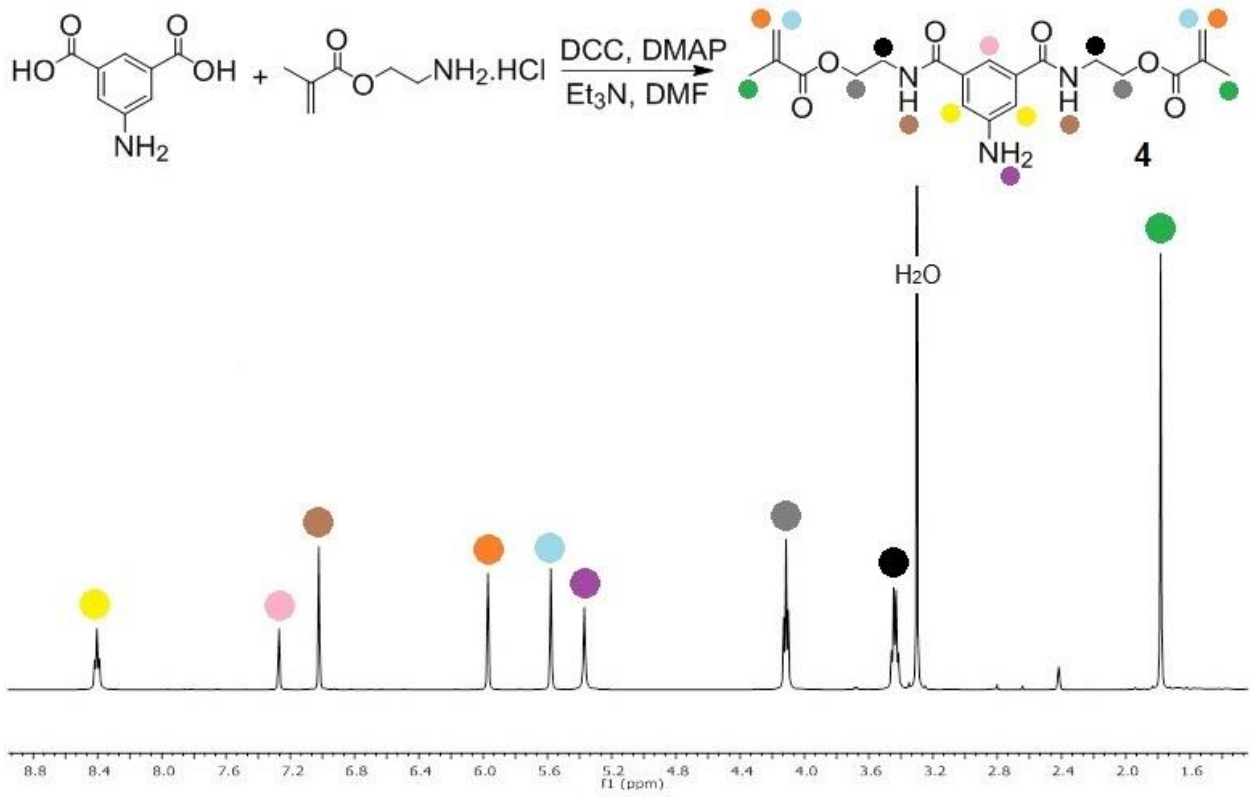

Figure S4. ^{1}H NMR (DMSO-d_6) spectrum of 5-amino-(1,3-ethylmethacrylate dibenzamide) (**4**).

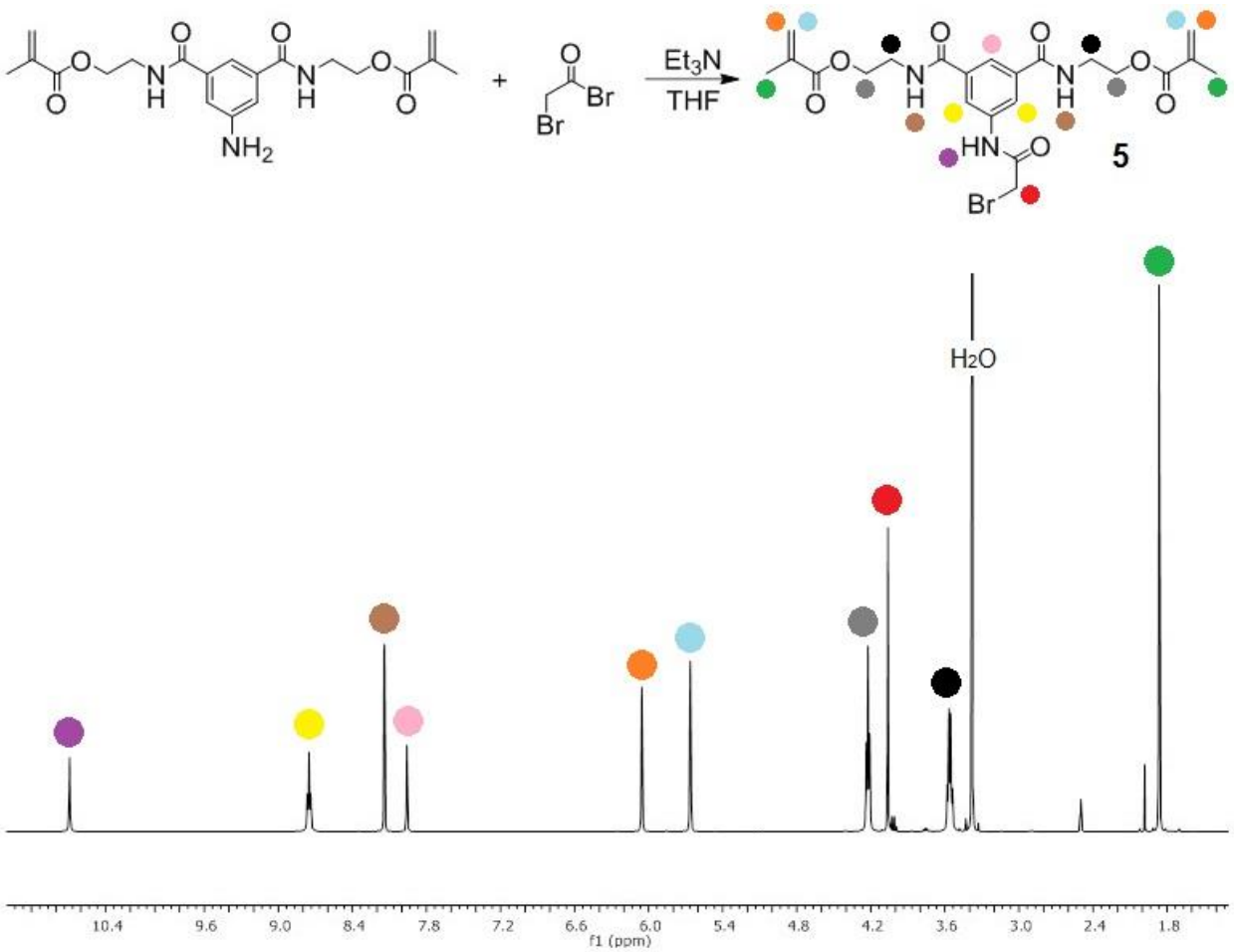

Figure S5. ^{1}H NMR (DMSO-d_6) spectrum of 1,3-ethylmethacrylate-5-methylbromide tribenzamide (**5**).

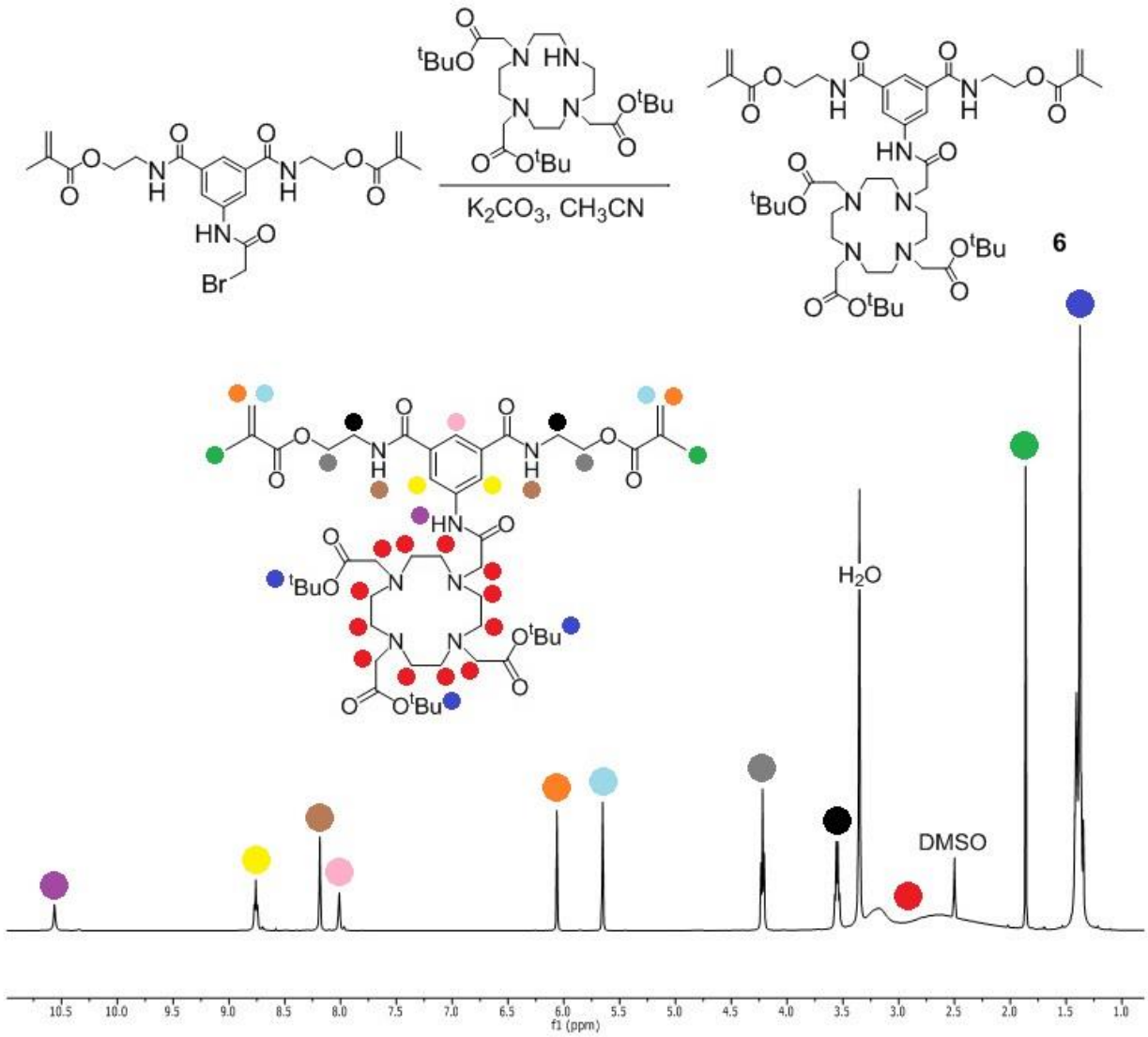

Figure S6. ^{1}H NMR (DMSO-d_6) spectrum of 1,3-ethylmethacrylate-5-methyDO3A tribenzamide (**6**).

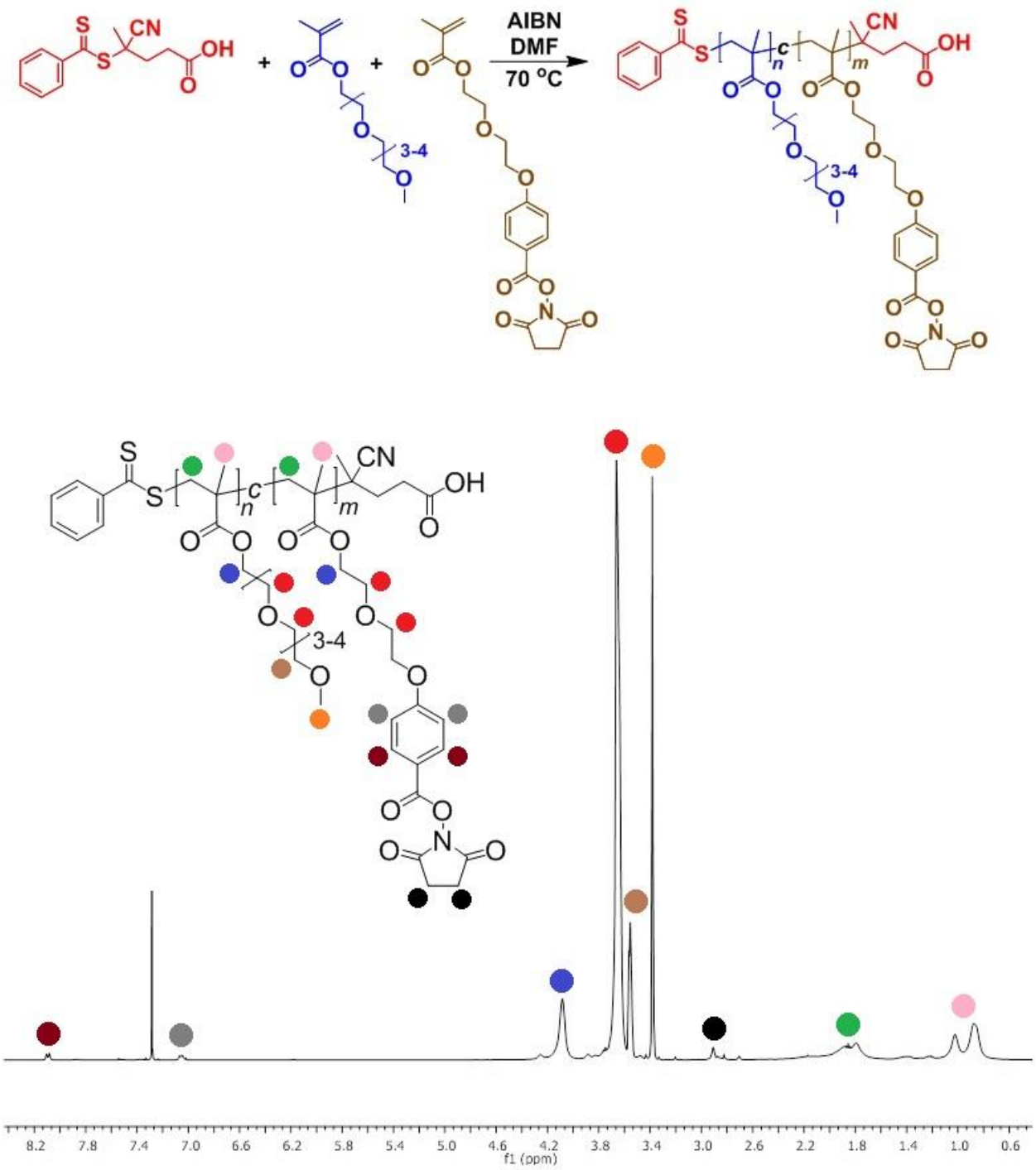

Figure S7. ^{1}H NMR ($CDCl_3$) spectrum of **P3**.

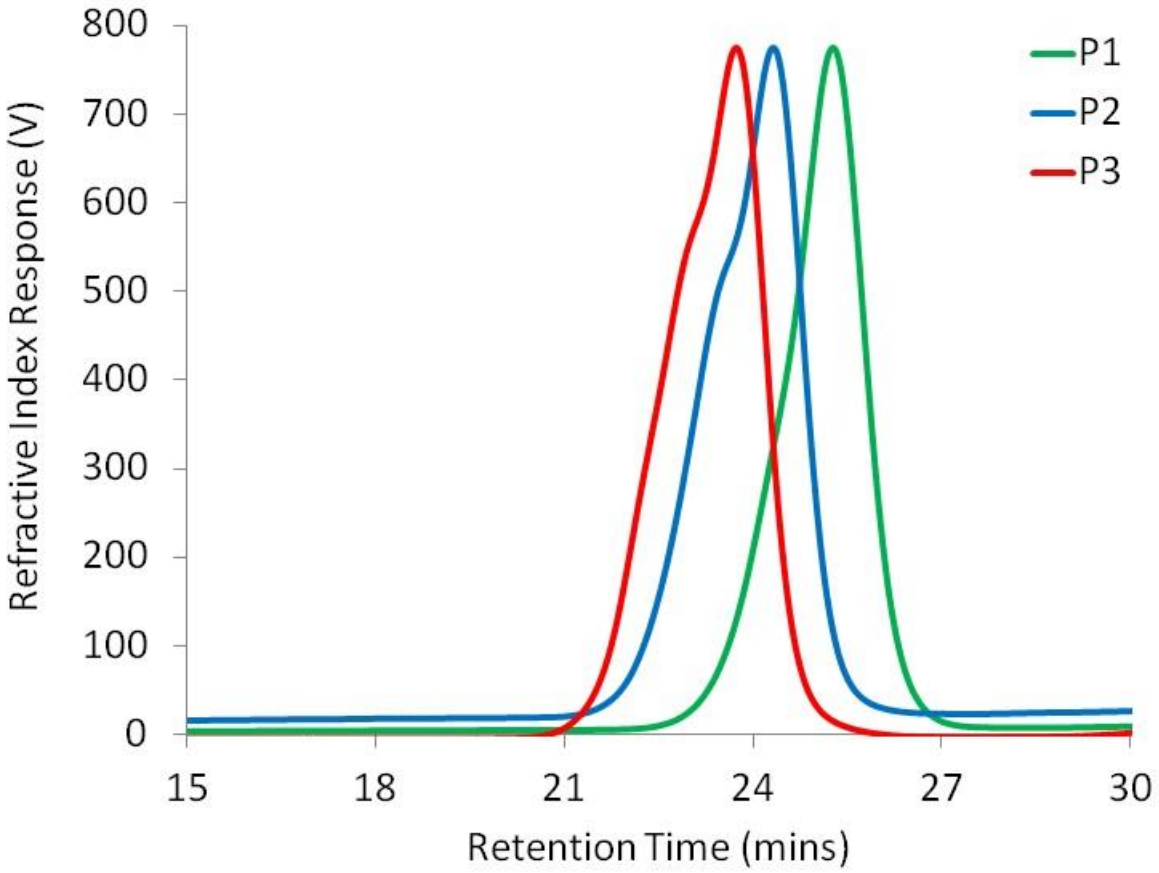

Figure S8. Gel permeation chromatography (GPC) traces in THF of linear copolymers **P1**-**P3**.

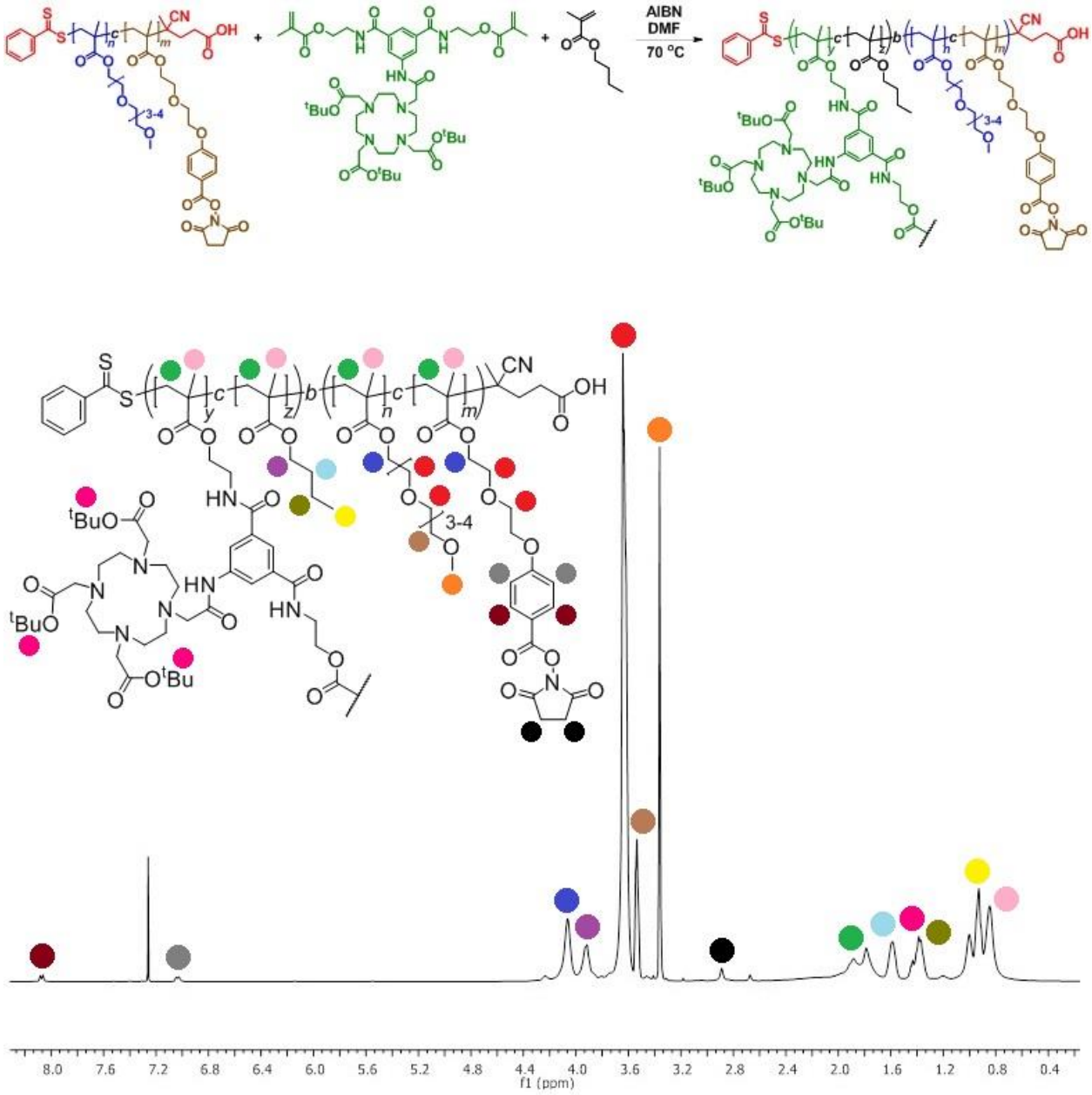

Figure S9. ^{1}H NMR ($CDCl_3$) spectrum of **N3(ᵗBu)**.

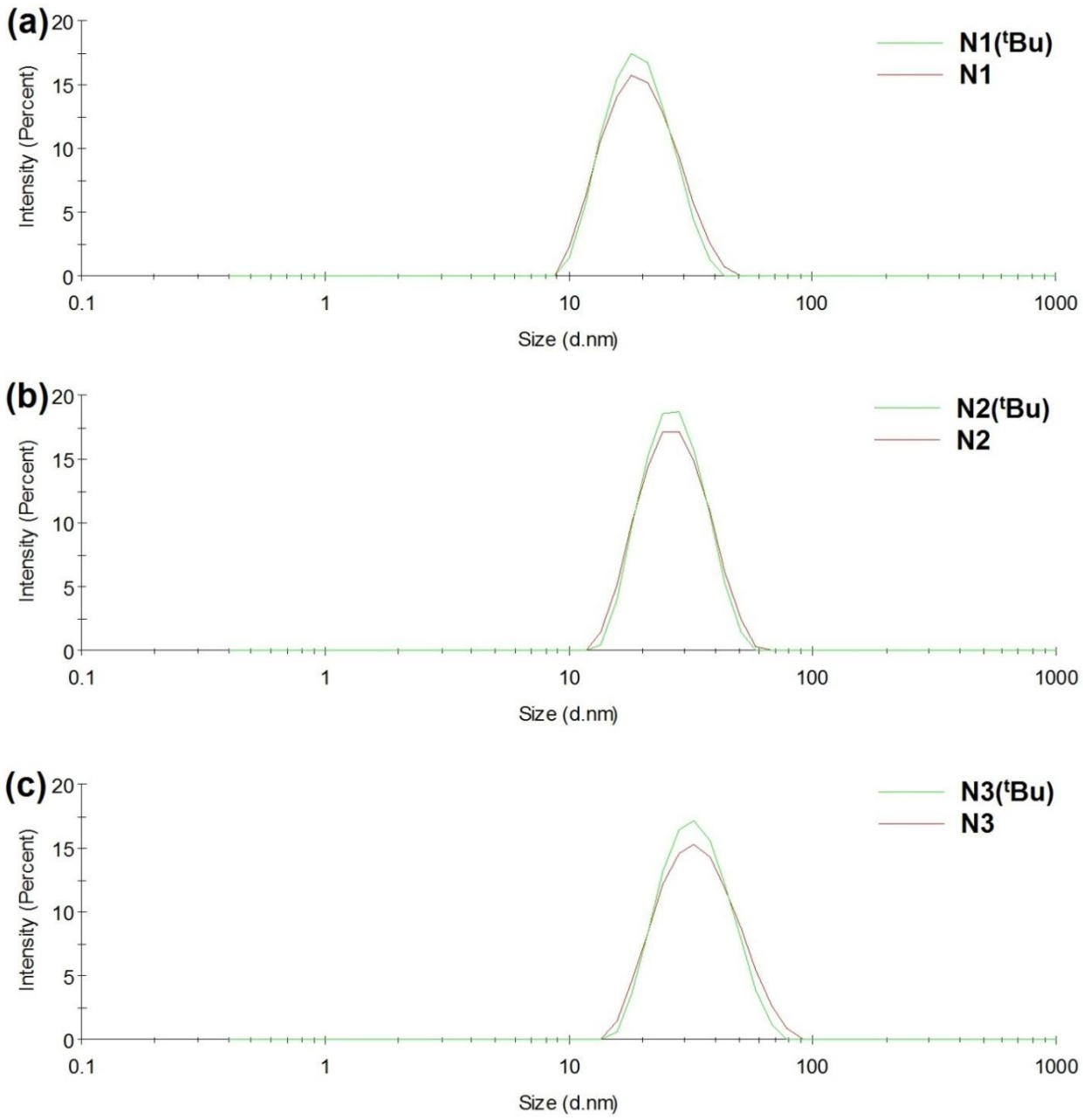

Figure S10. Dynamic light scattering analysis of nanoparticles in H_2O at 25 ºC of (a) **N1(ᵗBu)** - **N1**, (b) **N2(ᵗBu)** - **N2** and (c) **N3(ᵗBu)** - **N3**.

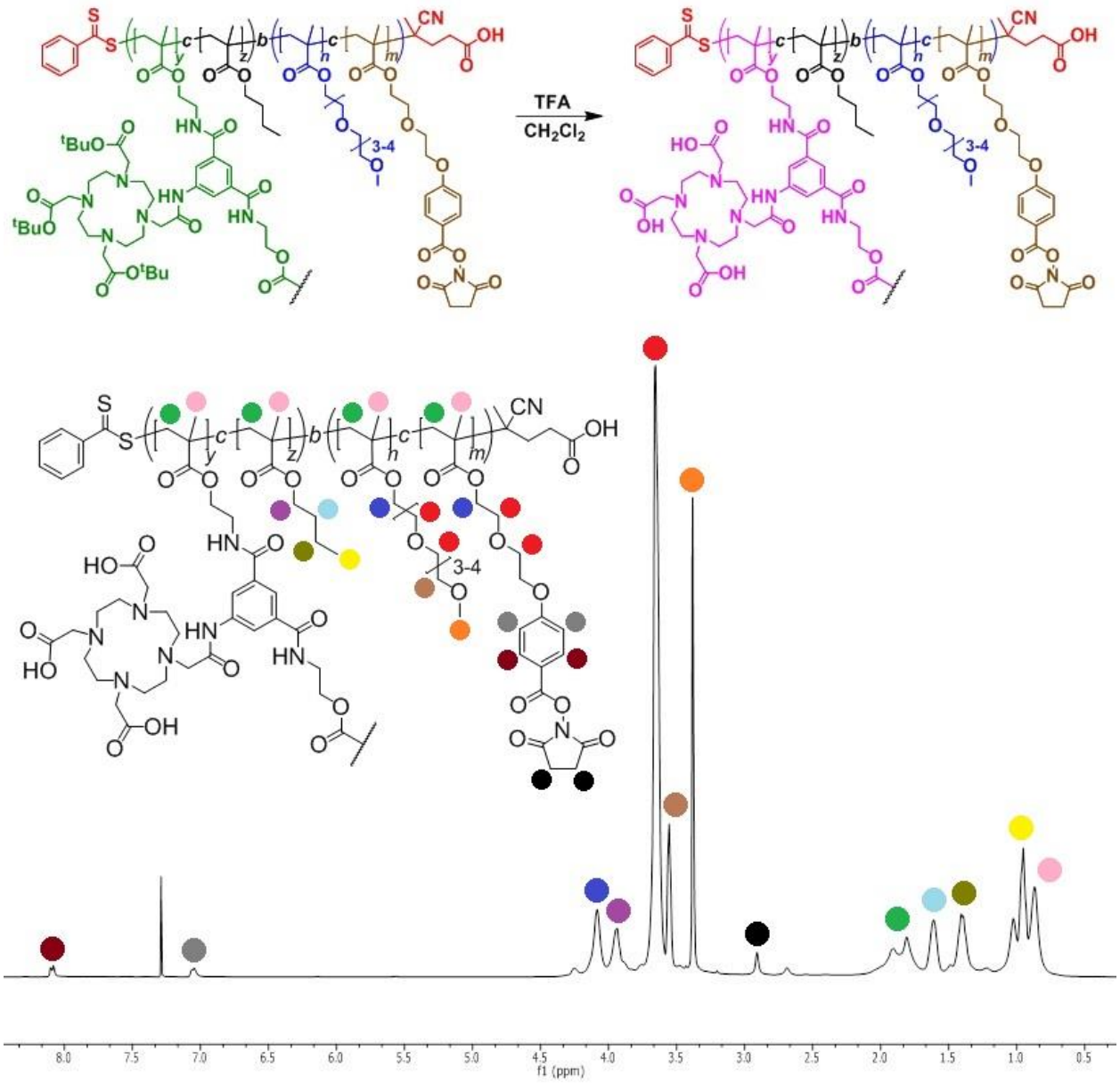

Figure S11. ^{1}H NMR ($CDCl_3$) spectrum of **N2**.

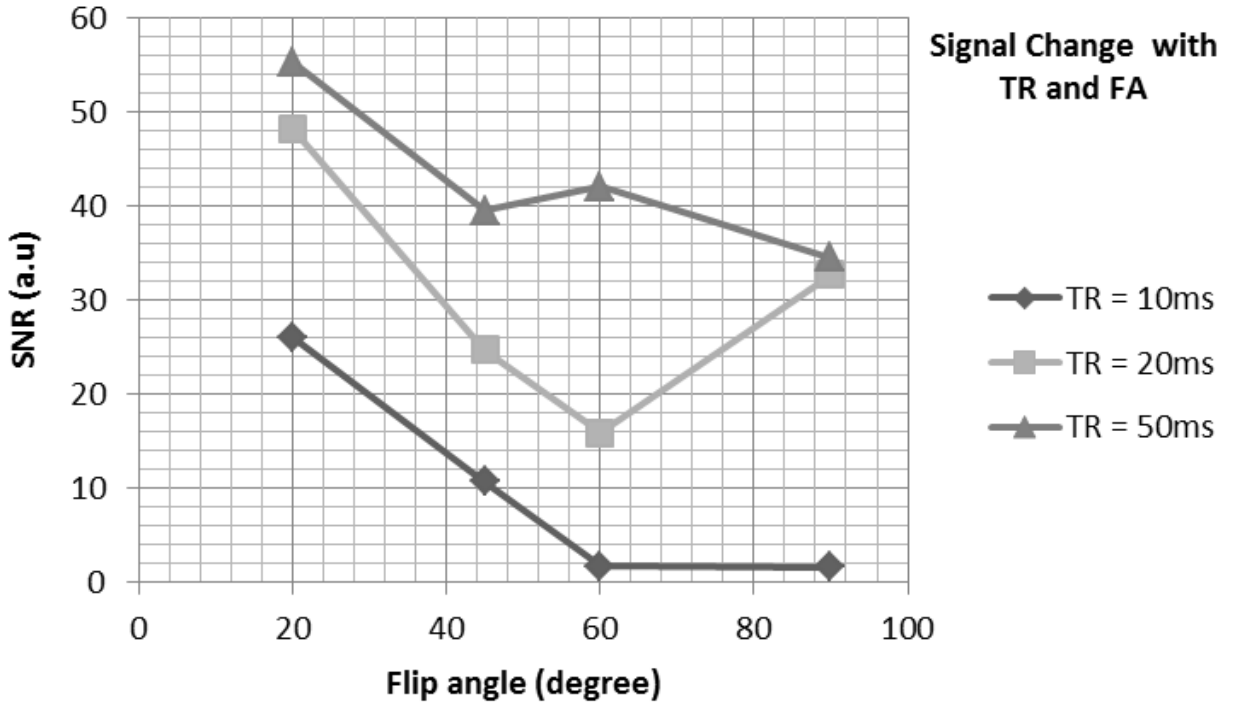

Figure S12. Optimization of flip-angle with respect to TR.

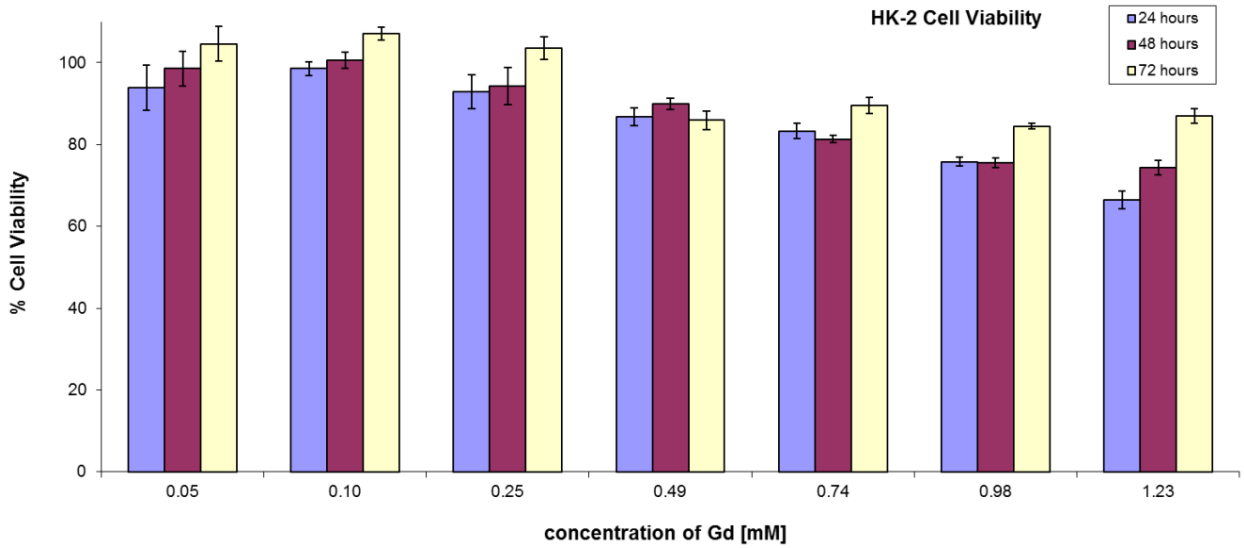

Figure S13. Cell viability assay of **N2(Gd)** branched copolymer nanoparticles on HK-2 human proximal tubule cells at 24, 48 and 72 h incubation.

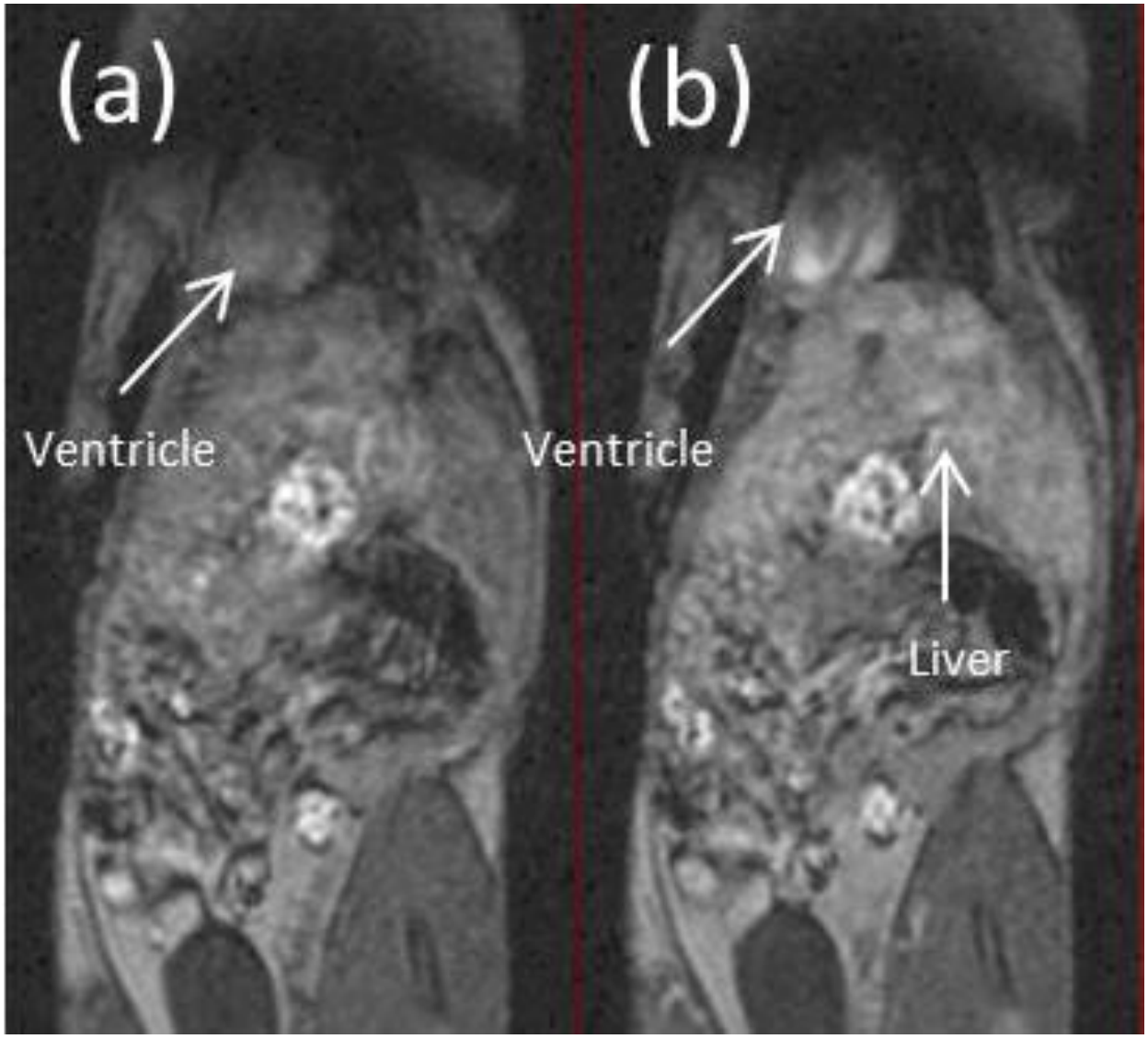

Figure S14. Images acquired by FLASH sequence a) before and b) after 10 min of administration of **N2(Gd)** branched copolymer nanoparticles. The ventricular space of heart appears bright 10 min after administration of the nanoparticles suggesting long circulation behavior of the nanoparticles.