A multimodal, β-amyloid-targeted contrast agent

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Experimental procedures

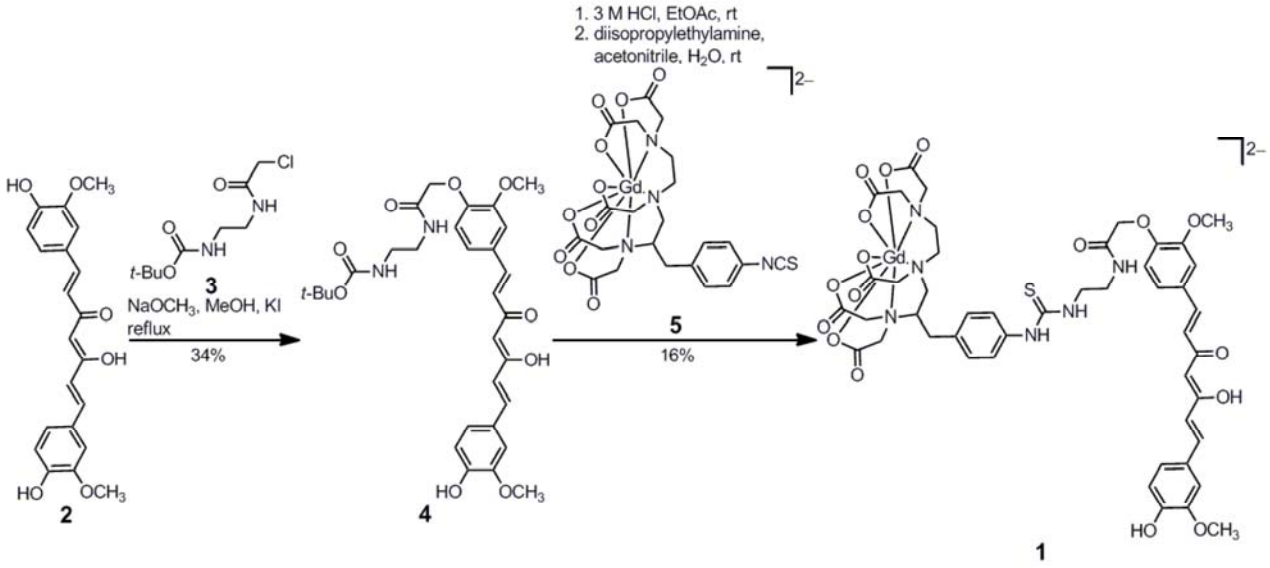
Commercially available chemicals were of reagent-grade purity or better and were used without purification unless otherwise noted. β-Amyloid peptide (1–42) was obtained from American Peptide Company Inc. Gd^{III}diethylenetriaminepentaacetate (Gd^{III}DTPA) was obtained as a 0.5 M aqueous solution from Bayer HealthCare Pharmaceutical Inc. Water was purified using a PURELAB Ultra Mk2 water purification system (ELGA). Phosphate buffered saline (PBS) (1×, 11.9 mM phosphate, 137 mM NaCl, and 2.7 mM KCl, pH = 7.4) was used for β-amyloid aggregate formation and relaxation time measurements. Compounds **3** and **5** were synthesized using previously published procedures.[1,2]

Analytical thin-layer chromatography (TLC) was carried out on ASTM TLC plates precoated with silica gel 60 F_{254} (250 μm layer thickness). Visualization of TLC was accomplished using a UV lamp followed by charring with potassium permanganate stain (3 g $KMnO_4$, 20 g K_2CO_3, 5 mL 5% w/v aqueous NaOH, 300 mL H_2O). Flash chromatography was performed using silica gel 60, 230–400 mesh (EMD Chemicals).[3] Preparative reverse-phase chromatography was performed using RP-tC18 SPE Sep-Pak columns (Waters) and a high-performance liquid chromatography (HPLC) system (Shimadzu) equipped with a C18 column (Restek International, Viva C18, 5 μm, 250 × 10.0 mm). Analytical HPLC analyses were performed with a C18 column (Restek International, Viva C18, 5 μm, 250 × 4.6 mm). Both preparative and analytical HPLC used a binary gradient method (pump A: water, pump B: acetonitrile; 5% B for 5 min, 5→30% B over 1 min, 30% B for 10 min, 30→95% B over 1 min, 95% B for 2 min). The flow rates used for preparative and analytical columns were 5 and 1 mL/min, respectively. Detection was carried out with a photodiode array detector and fluorescence detector (λ_{ex} = 395 nm, and λ_{em} = 521 nm).

^{1}H NMR spectra were obtained using a Varian Mercury 400 (400 MHz) spectrometer, and ^{13}C NMR spectra were obtained using a Varian Mercury 400 (101 MHz) spectrometer. Chemical shifts are reported relative to residual solvent signals ($CDCl_3$: ^{1}H: δ 7.27, ^{13}C: δ 77.23). NMR data are assumed to

be first order, and the apparent multiplicity is reported as "s" = singlet, "d" = doublet, "m" = multiplet, and "brs" = broad singlet. Italicized elements are those that are responsible for the chemical shifts. High-resolution electrospray ionization mass spectra (HRESIMS) were obtained on an electrospray time-of-flight high-resolution Waters Micromass LCT Premier XE mass spectrometer. Dynamic light scattering measurements were obtained on a Zetasizer nanoparticle analyzer equipped with 633 nm helium-neon laser (NanoZS, Malvern Instruments Ltd.). Transmission electron microscopy (TEM) was performed on a JEOL-2010 FasTEM Transmission Electron Microscope.

Longitudinal relaxation time (T_1) measurements were performed using a Bruker mq60 minispec NMR spectrometer at 60 MHz and 37 °C. Fluorescence spectra were obtained using a HORIBA Jobin Yvon Fluoromax-4 spectrofluorometer. Sonication was performed using a FS60H sonicator (Fisher Scientific). Centrifugation was performed using a mini-centrifuge (05-090-100, Fisher Scientific) at 6600 rpm. Vortexing was done using a vortex mixer (Fisher Scientific). Freeze drying was performed using a Freezone 2.5 freeze dryer (LABCONCO). Spin filtration of amyloid protein was performed using SpinX 8161 (Costar) spin filters (0.22 μm). Rotating of β-amyloid samples was done using a Thermo Labquake Tube Shaker/Rotator. Inductively coupled plasma mass spectrometry (ICP–MS) analyses were performed by Columbia Analytical Services Inc, Tucson, Arizona, USA.



***tert*-Butyl-2-(2-(4-((1E,4Z,6E)-5-hydroxy-7-(4-hydroxy-3-methoxyphenyl)-3-oxohepta-1,4,6-trienyl)-2-methoxyphenoxy)acetamido)ethylcarbamate (4):**

To a solution of commercially available curcumin, **2**, (205 mg, 0.557 mmol, 1 equiv) in anhydrous methanol (7 mL) at 50 °C was added a solution of sodium methoxide (0.81 mmol, 1.5 equiv) in anhydrous methanol (1.5 mL) dropwise over a period of 20 min under Ar. The resulting reaction mixture was stirred for 40 min at 50 °C under Ar. A mixture of amine linker **3** (323 mg, 1.36 mmol, 2.5 equiv) and KI (186 mg, 1.12 mmol, 2 equiv) in anhydrous methanol (1 mL) was added to the reaction mixture dropwise over 15 min. Anhydrous toluene (3 mL) was added, and the resulting reaction mixture was stirred at 80 °C under Ar for 22 h in the dark. Volatiles were removed under reduced pressure, and the resulting solid was purified using silica gel column chromatography (stepwise gradient of 2:3→4:1→1:0 ethyl acetate/hexanes) to yield **4** (0.11 g, 34%) as an orange oil. ^{1}H NMR (400 MHz, $CDCl_3$, δ): 1.43 (s, C*H*$_3$, 9H), 3.18–3.58 (m, C*H*$_2$, 4H), 3.95 (s, C*H*$_3$, 6H), 4.57 (s, C*H*$_2$, 2H), 4.87 (brs, N*H*, 1H), 5.83 (s, C*H*, 1H), 5.96 (brs, O*H*, 1H), 6.49 (d, *J* = 15.98 Hz, C*H*, 1H), 6.52 (d, *J* = 15.58 Hz, C*H*, 1H), 6.80–7.19 (m, Ar*H*, 6H), 7.22 (brs, N*H*, 1H), 7.59 (d, *J* = 15.58 Hz, C*H*, 1H), 7.61 (d, *J* = 15.98 Hz, C*H*, 1H), 16.01 (brs, O*H*, 1H); ^{13}C NMR (101 MHz, $CDCl_3$, δ): 28.5 (*C*H$_3$), 39.6 (*C*H$_2$), 40.6 (*C*H$_2$), 56.1 (*C*H$_3$), 56.2 (*C*H$_3$), 69.2 (*C*H$_2$), 79.8, 101.6 (*C*H), 109.8 (*C*H), 110.9 (*C*H), 114.9 (*C*H), 115.1 (*C*H), 121.9 (*C*H), 122.2 (*C*H), 123.2 (*C*H), 123.3 (*C*H), 127.7, 130.3, 139.8 (*C*H), 141.1 (*C*H), 147.0, 148.2, 148.8, 149.9, 156.5, 169.1, 182.6, 184.2; TLC: R_f = 0.23 (4:1 ethyl acetate/hexanes); HRESIMS (*m*/*z*): [M + H]$^{+}$calcd for $C_{30}H_{37}N_2O_9$, 569.2499; found, 569.2504.

Curcumin-conjugated Gd^{III}DTPA complex (1):

Compound **4** (34 mg, 0.060 mmol) was added to a solution of HCl (3 M) in ethyl acetate (2.5 mL), and the resulting mixture was stirred at ambient temperature for 30 min. Solvents were removed under reduced pressure, and the product was triturated with diethyl ether (2 × 3 mL) to obtain a brown solid (26 mg, 0.056 mmol, 1 equiv) that was dissolved in water (0.2 mL). To the resulting solution was added a solution of diisopropylethylamine (DIEA) (39 μL, 0.22 mmol, 4 equiv) in acetonitrile (0.2 mL) dropwise over 5 min while stirring. At the end of the addition, water (0.2 mL) was added to the reaction mixture followed by complex **5** (55 mg, 0.079 mmol, 1.4 equiv) as a solid in 4 equal portions. Upon complete addition of **5**, water (1.1 mL) was added to the reaction mixture that was subsequently sonicated (1 min). The reaction mixture was stirred in the dark at ambient temperature for 6.5 h then purified by reverse-phase chromatography using RP-tC18 SPE Sep-Pak (stepwise gradient of 3:7→1:1 acetonitrile/water) followed by HPLC. Volatiles were removed under reduced pressure, and the resulting residue was dissolved in water (1 mL) and freeze dried to obtain **1** (0.010 g, 16%) as a fluffy yellow powder. HRESIMS (*m*/*z*): $[M]^{2-}$ calcd for $C_{47}H_{51}N_6O_{17}SGd$, 579.1129; found, 579.1102. HPLC chromatograms are on page S7 (Fig S1).

Relaxivity measurements of 1 and Gd^{III}DTPA:

Longitudinal relaxation times (T_1) were measured using a standard inversion-recovery method with a Bruker Minispec mq 60 at 1.4 T and 37 °C in PBS. The slope of a plot of $1/T_1$ vs concentration of Gd^{III} was used to obtain relaxivity. Four to six different concentrations of Gd^{III} (0–1 mM) were used. Measurements were repeated 3 times with independently prepared samples, and Gd concentrations were determined by ICP–MS.

Preparation of β-amyloid fibrils:

β-Amyloid peptide (1–42) (1 mg, 0.2 μmol) was dissolved in PBS (1 mL) and stirred gently (350 rpm) at 37 ºC for 20 h to obtain a 0.2 mM stock solution of β-amyloid aggregates following published procedures.[4,5]

Transmission electron microscopy (TEM) studies:

Aggregated β-amyloid peptide was centrifuged (0.2 mM, 100 μL), and the resulting sediment was suspended in water (20 μL) and mixed by vortexing. Aggregated protein solution (5 μL) was applied to a TEM substrate grid (200-mesh copper grid coated with Formvar/carbon film) and incubated for 10 min. Excess solution was removed by wicking with filter paper. Phosphotungstic acid (1% (w/v), 3 μL) was applied to the grid containing protein. After incubation for 3 min, excess stain was washed with water (20–40 μL), and excess water was removed by wicking with filter paper. The resulting sample was air dried for 3 h. TEM was performed at 100 kV (60,000×). The TEM image (Fig S2) of aggregated fibrils is similar to the appearance of other reported TEM images of aggregated fibrils.[5]

Dynamic light scattering (DLS) studies of fibril formation:

A 100 µL aliquot was taken from the β-amyloid stock while stirring and filtered through a 0.22 µm spin filter to remove large aggregates. The filtered solution was transferred to a disposable microcuvette (40 µL) and measured for size distribution by collecting data at a 173º measurement angle on a Zetasizer NanoZS instrument at 24.9 ºC (dispersant refractive index = 1.332, and viscosity = 0.9128 cP). Size distribution data obtained from DLS based on intensity (Fig S3) provide evidence for the presence of fibril aggregates with diameters of 200–600 nm that are comparable to the sizes of aggregates reported in other studies.[6]

Binding studies between conjugate 1 and amyloid fibrils:

Aliquots (0, 25, 50, and 100 µL) were taken from the stirring stock solution of β-amyloid aggregates and mixed with conjugate **1** (0.1 mM, 100 µL) in PBS. The total volume of each sample was brought to 200 µL with PBS. Samples were vortexed (~5 s) and incubated at ambient temperature with rotation for 3 h. β-Amyloid (0, 25, 50, and 100 µL) was mixed with PBS to a total volume of 200 µL, vortexed (~5 s), incubated at ambient temperature with rotation for 3 h, and used as the control. Gd^{III}DTPA (0.1 mM, 100 µL) in PBS was used as the non-specific control, and samples with Gd^{III}DTPA were prepared following the same procedure as for conjugate **1**.

After 3 h, T_1 measurements of the samples were performed using standard inversion-recovery methods with a Bruker Minispec mq 60 at 1.4 T and 37 °C. Measurements were replicated (n = 3 for conjugate **1** and n = 2 for controls) using independently prepared samples.

Fluorescence emission spectra of samples were obtained using 385 nm excitation wavelength, a 10 nm excitation slit width, and a 5 nm emission slit width.

Gd concentrations were determined by ICP–MS.

References

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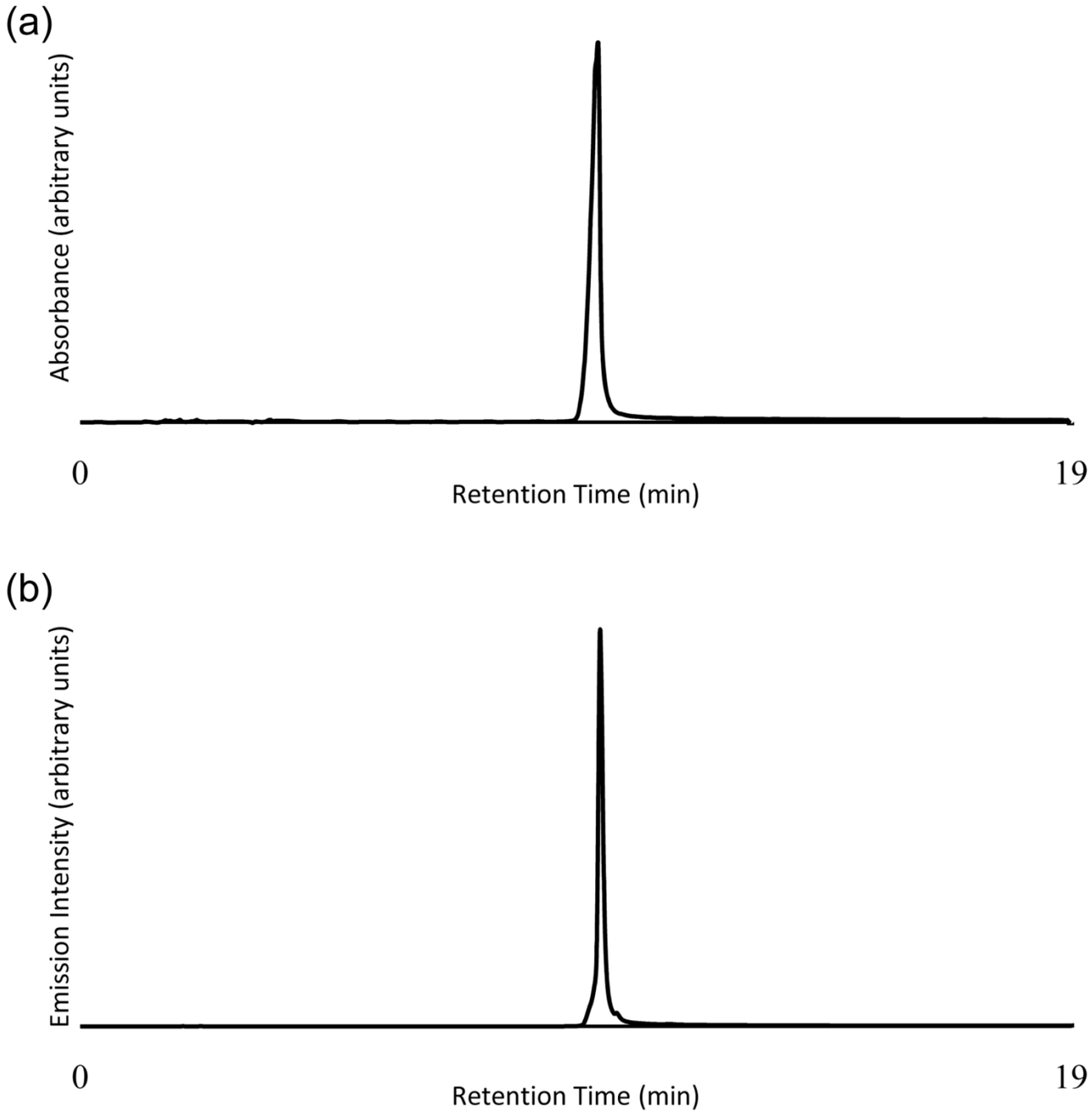


Fig S1. HPLC chromatograms of conjugate **1** detected using (a) a photodiode array (254 nm) and (b) a fluorescence detector (λ_{ex} = 395 nm, and λ_{em} = 521 nm).

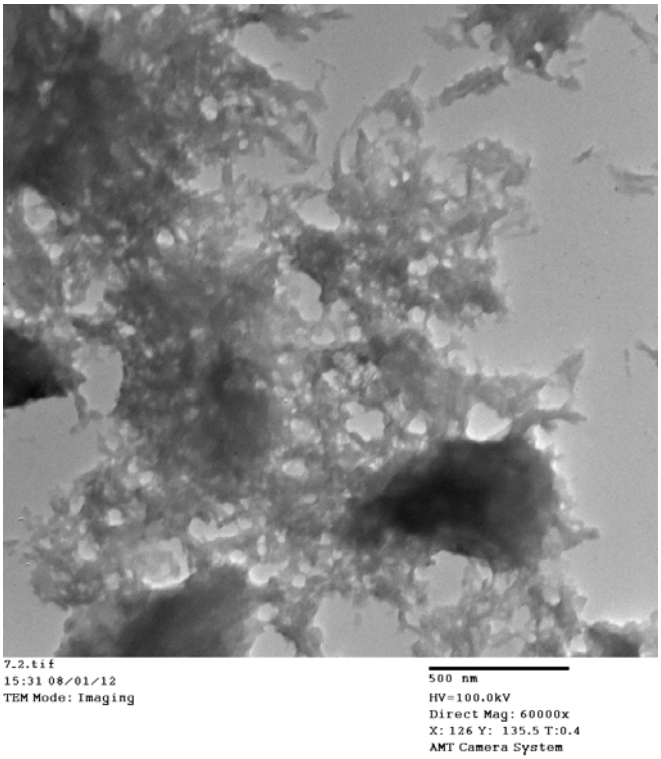


Fig S2. TEM image of β-amyloid aggregates.

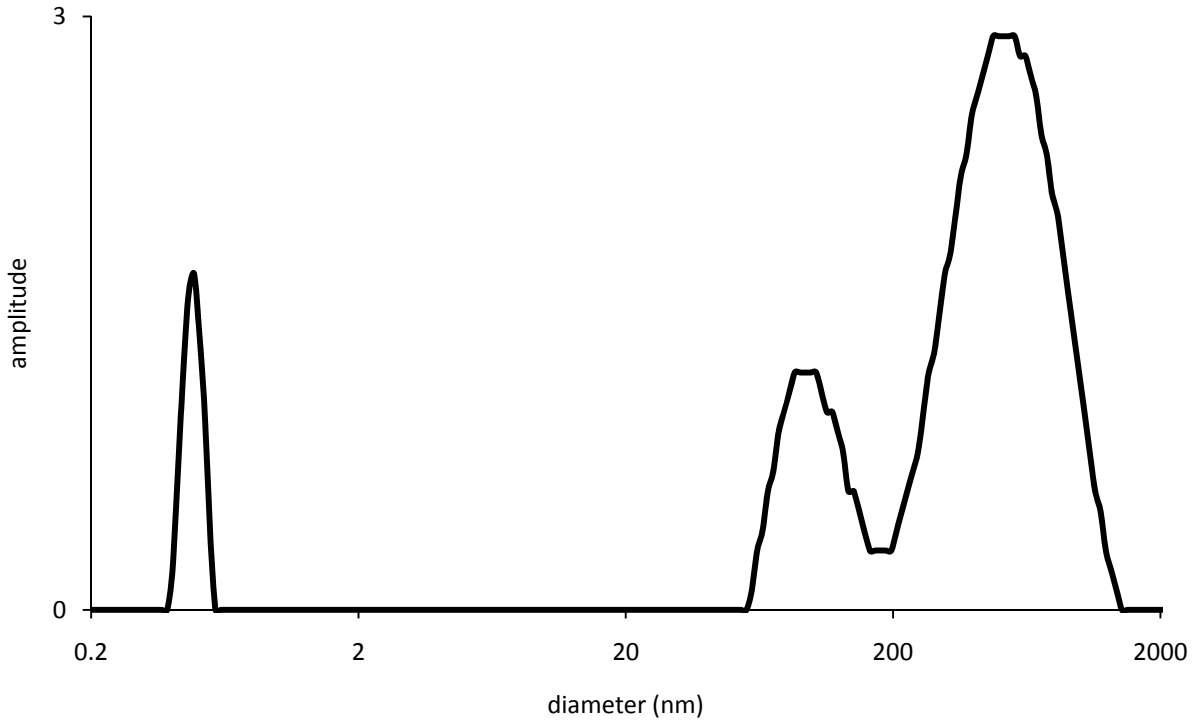
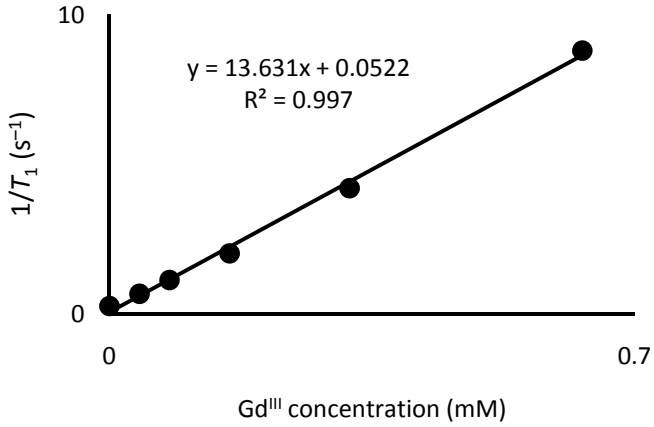


Fig S3. DLS distribution data of β-amyloid aggregates (intensity distribution).

Relaxivity data of conjugate **1**.

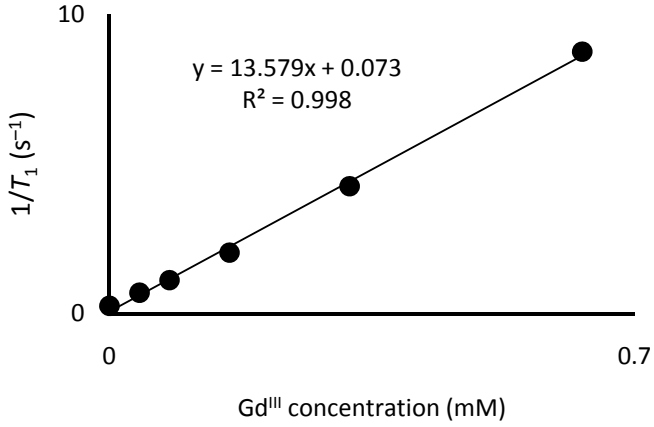
Sample 1

Concentration (mM)	T_1 (s)	$1/T_1$ (s^{-1})
0.63	0.1137	8.80
0.32	0.2380	4.20
0.16	0.4959	2.02
0.08	0.8838	1.13
0.04	1.5010	0.67
0.00	3.8600	0.26



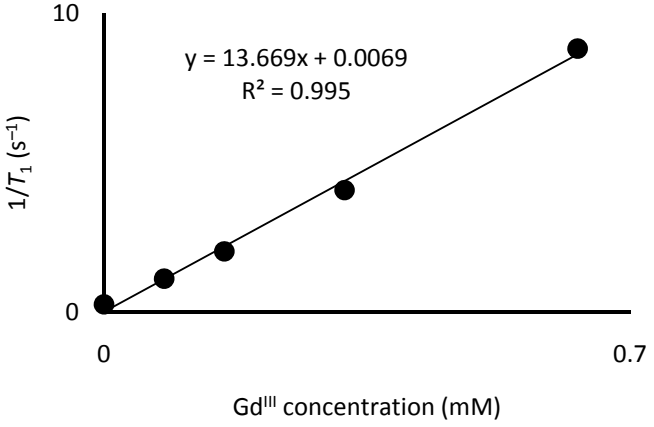
Sample 2

Concentration (mM)	T_1 (s)	$1/T_1$ (s^{-1})
0.63	0.1141	8.76
0.32	0.2350	4.26
0.16	0.4902	2.04
0.08	0.8915	1.12
0.04	1.4350	0.70
0.00	3.8460	0.26



Sample 3

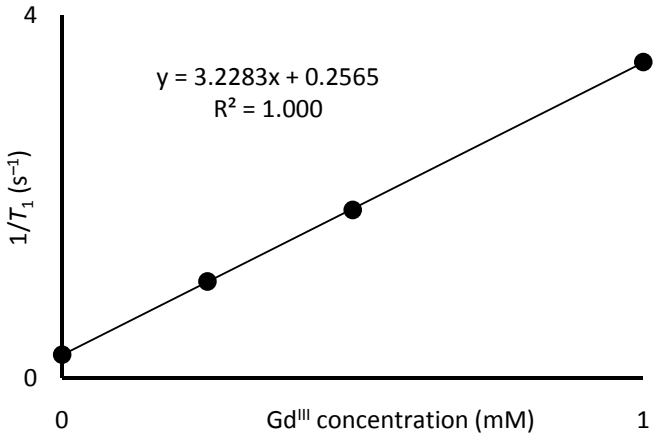
Concentration (mM)	T_1 (s)	$1/T_1$ (s^{-1})
0.63	0.1135	8.81
0.32	0.2450	4.08
0.16	0.4915	2.03
0.08	0.8898	1.12
0.00	3.8650	0.26



Relaxivity data of $Gd^{III}DTPA$.

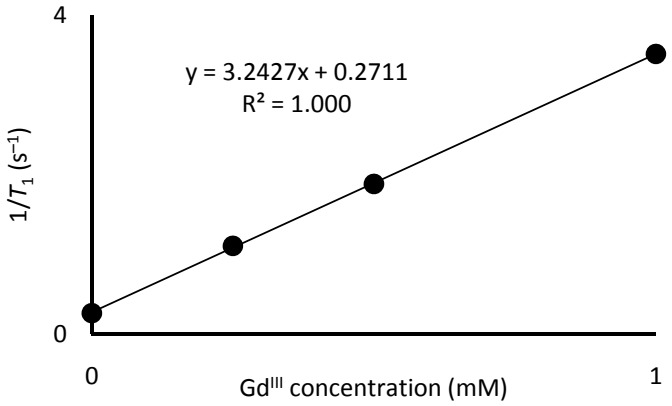
Sample 1

Concentration(mM)	T_1 (s)	$1/T_1$ (s^{-1})
1.00	0.2865	3.49
0.50	0.5384	1.86
0.25	0.9366	1.07
0.00	3.8460	0.26



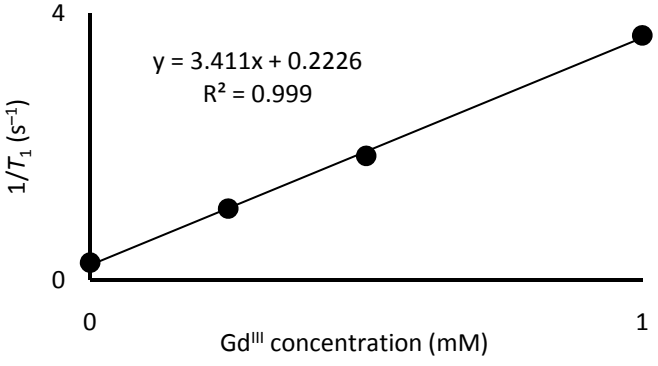
Sample 2

Concentration(mM)	T_1 (s)	$1/T_1$ (s^{-1})
1.00	0.2846	3.51
0.50	0.5315	1.88
0.25	0.9055	1.10
0.00	3.8500	0.26



Sample 3

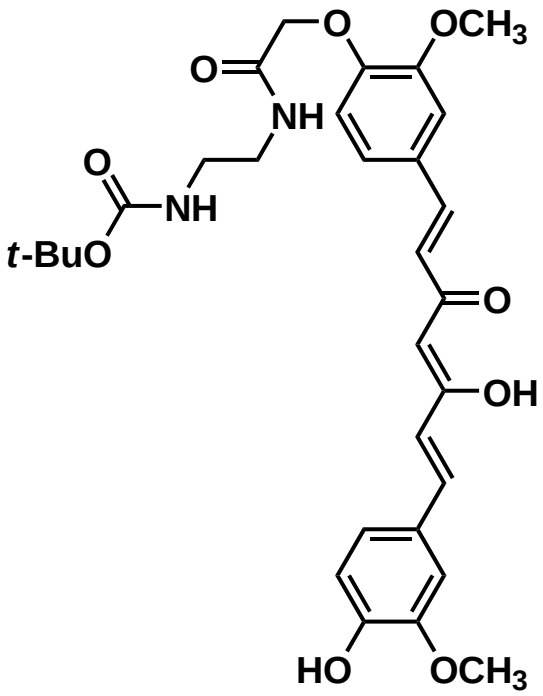
Concentration(mM)	T_1 (s)	$1/T_1$ (s^{-1})
1.00	0.2726	3.67
0.50	0.5373	1.86
0.25	0.9344	1.07
0.00	3.8470	0.26



β-Amyloid (βA) binding T_1 measurements

Sample	T_1 (ms) at the following ratios of complex to βA* (1:2)	(1:1)	(1:0.5)	(1:0)
1/βA (trial 1)	1267	1302	1339	1388
1/βA (trial 2)	1265	1298	1395	1375
1/βA (trial 3)	1262	1321	1328	1380
GdDTPA/βA (trial 1)	2225	2243	2247	2253
GdDTPA/βA (trial 2)	2232	2247	2243	2253
βA control (trial 1)	3826	3822	3835	3865
βA control (trial 2)	3815	3850	3830	3860

* The concentration of βA in the βA control trials is the same as in the samples containing **1** and GdDTPA at the same ratios.



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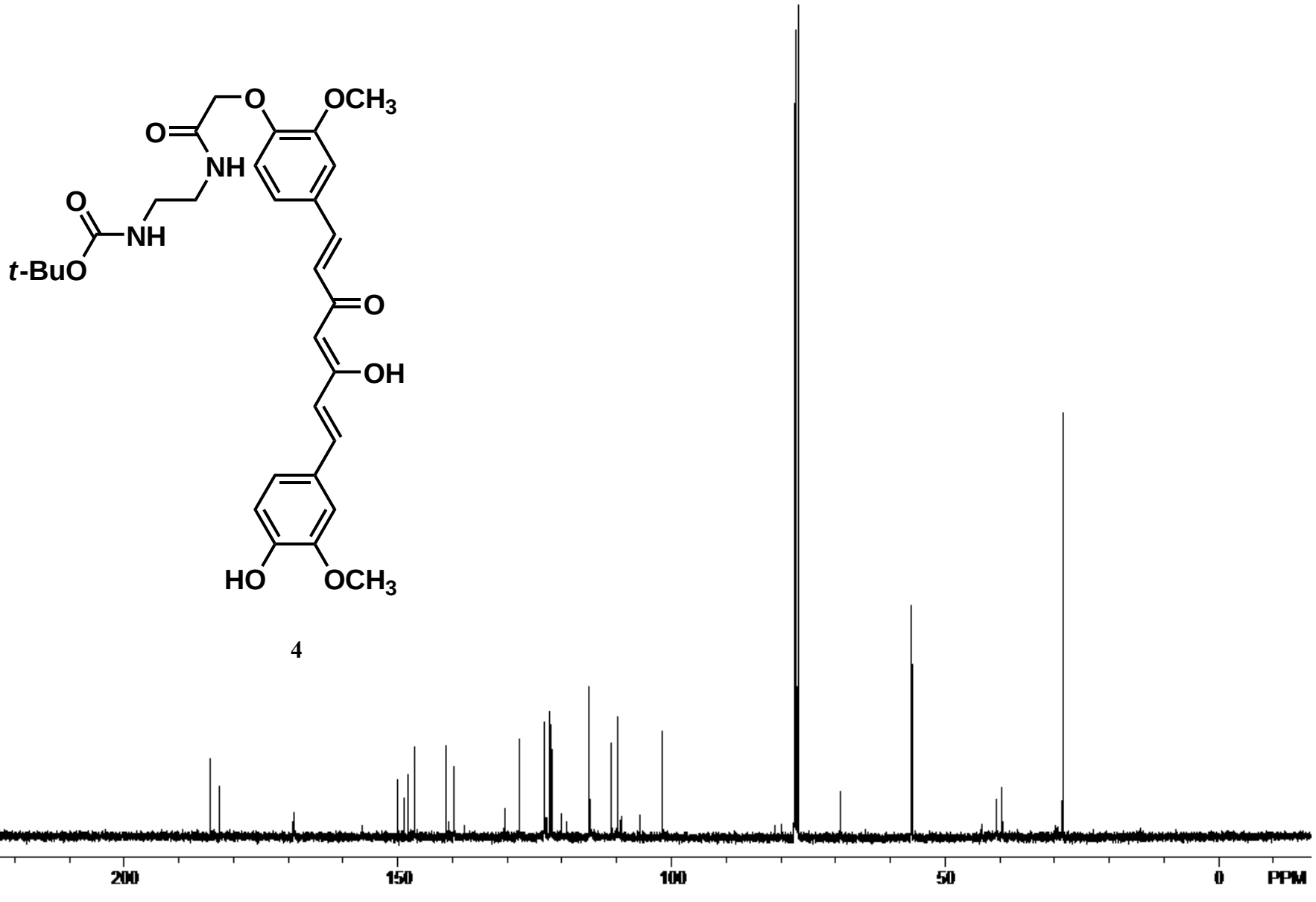

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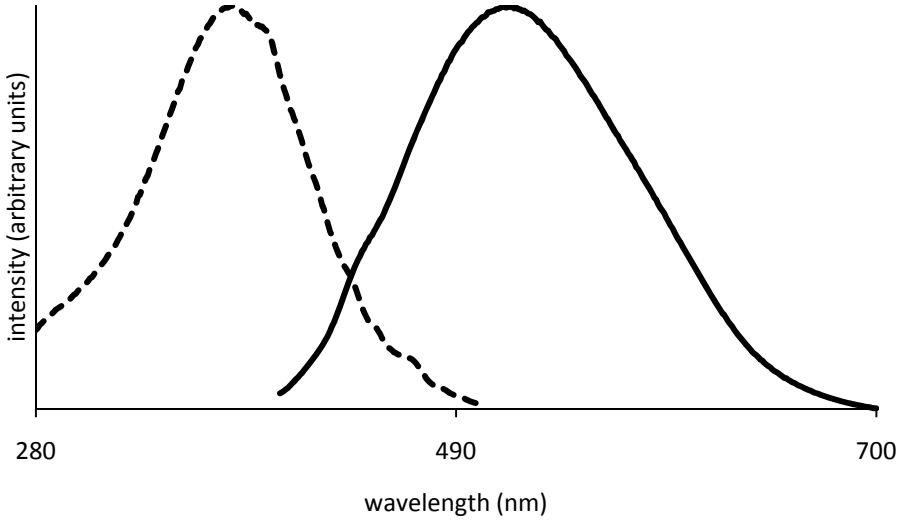

Fig S4. (a) Excitation (- -, λ_{em} = 516 nm) and (b) emission (—, λ_{ex} = 385 nm) spectra for conjugate **1**.