A Gd(III)-labelled self-assembling peptide as a potential pH-responsive MRI contrast agent

Electronic Supplementary Information

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Contents

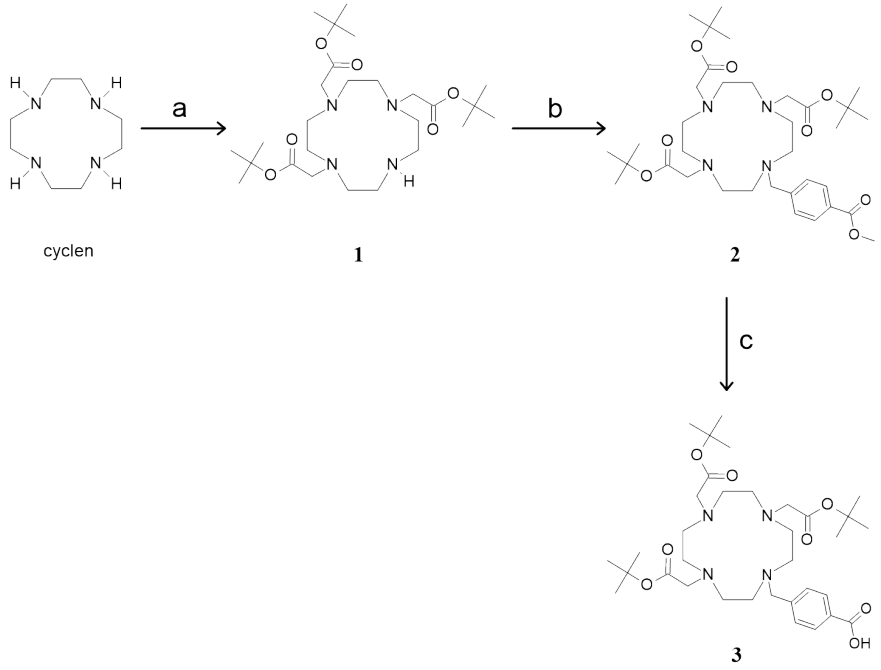
1 Methods

1.1 Synthesis

1.1.1 Materials

All chemicals were acquired from these sources Sigma-Aldrich, GL Biosciences, JT-Baker, and Duksan. Dotarem was acquired by a generous donation from Guerbet LLC.

1.1.2 Chelator synthesis



Scheme S1: Synthesis scheme for chelator **3**, (**a**) 3.3 eq tert−butylbromoacetate, 3.3 eq $NaHCO_3$, ACN; (**b**) 1.1 eq methyl4−(bromomethyl)benzoate, 1.5 eq DIPEA, ACN; (**c**) i. 3 parts MeOH, ii. 5 eq LiOH in 1 part water, iii. quench to pH 4

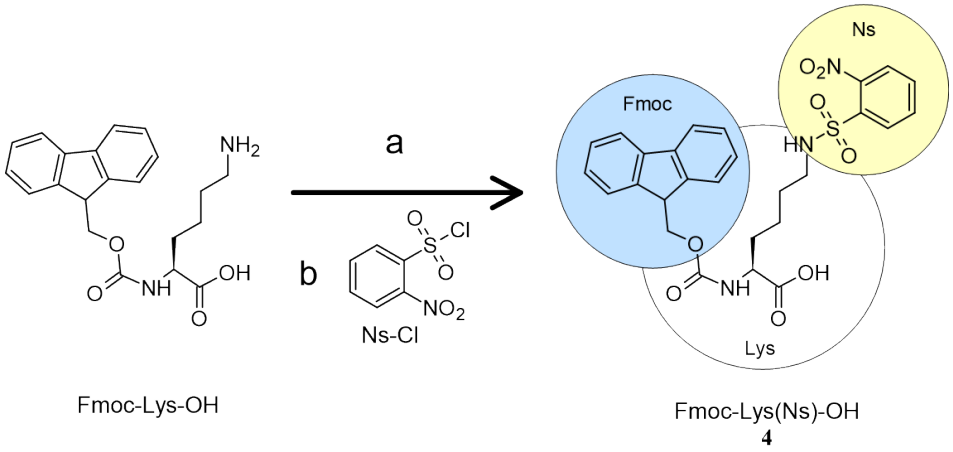
The synthesis of chelator **3** is summarized in **Scheme S1**. Compound **1** was synthesized following a procedure reported by Dadabhoy and coworkers.[1] Cyclen (1,4,7,10−tetraazacyclododecane, 1 eq) and $NaHCO_3$ (3.3 eq) were dissolved in a minimal amount of acetonitrile in a round bottom flask. The air was evacuated and replaced with nitrogen gas. This setup was carried out in an ice bath. Tert−butylbromoacetate (3.3 eq) was introduced to the reaction mixture in a dropwise manner while the mixture was stirred vigorously. This reaction was stirred for 48 h at room temperature (**Scheme S1, step A**). Subsequently, the mixture was filtered, and the filtrate was dried under reduced pressure. The dried product was dissolved in hot toluene, cooled to room temperature, and refrigerated to precipitate the desired compound. This cold mixture was filtered again, washed with cold toluene, and then with cold ether. The precipitate was scraped from the filter paper, identified as compound **1**.

Compound **2** was synthesized following a procedure reported by Faulkner and Burton-Pye[2] and by Moore and coworkers[3] with modifications. In a round bottom flask, Compound **1** (1 eq) was dissolved in a minimal amount of acetonitrile. DIPEA (1.5 eq) was added to the reaction mixture to deprotonate the secondary amine for a few minutes. Methyl 4-(bromomethyl)benzoate (1.1 eq) was dissolved in a minimal amount of acetonitrile and transferred to the reaction mixture. The reaction mixture was stirred for 3 h under nitrogen and at room temperature (**Scheme S1, step B**). After the reaction, the mixture was dried in vacuo. The compound was separated by normal phase flash chromatography with silica and

a linear gradient of DCM to 9:1 DCM:Methanol mobile phase. The target fraction was dried in vacuo again. The yellow oily liquid was triturated in n-hexane to produce compound **2**.

The removal of methyl ester from compound 2 was adapted from Corey and coworkers[4], and Steer and coworkers.[5] In a round bottom flask, compound **2** (1 eq) was dissolved in 3 parts methanol. In a separate solution, lithium hydroxide (LiOH, 5 eq) was prepared in one part of water. These solutions were cooled to around 5 °C before mixing. The reaction mixture was stirred under this cold environment for 24 h. To quench the reaction, pH 4 acetate buffer was mixed with the reaction mixture until the pH of the solution reached 4 (**Scheme S1, step C**). This was done to prevent the deprotection of the tert-butyl ester. The quenched solution was extracted with DCM. The DCM layer was dried in vacuo. The resulting oil was dissolved with minimal THF and precipitated with tert-butyl methyl ether (MTBE). The mixture was triturated and washed with fresh ether until the precipitate was a dry solid powder. The chelator 3 was purified in reversed-phase HPLC using 50 mM acetate buffer pH 5.0 and methanol solvent system.

1.1.3 Fmoc-Lys(Ns)-OH Synthesis

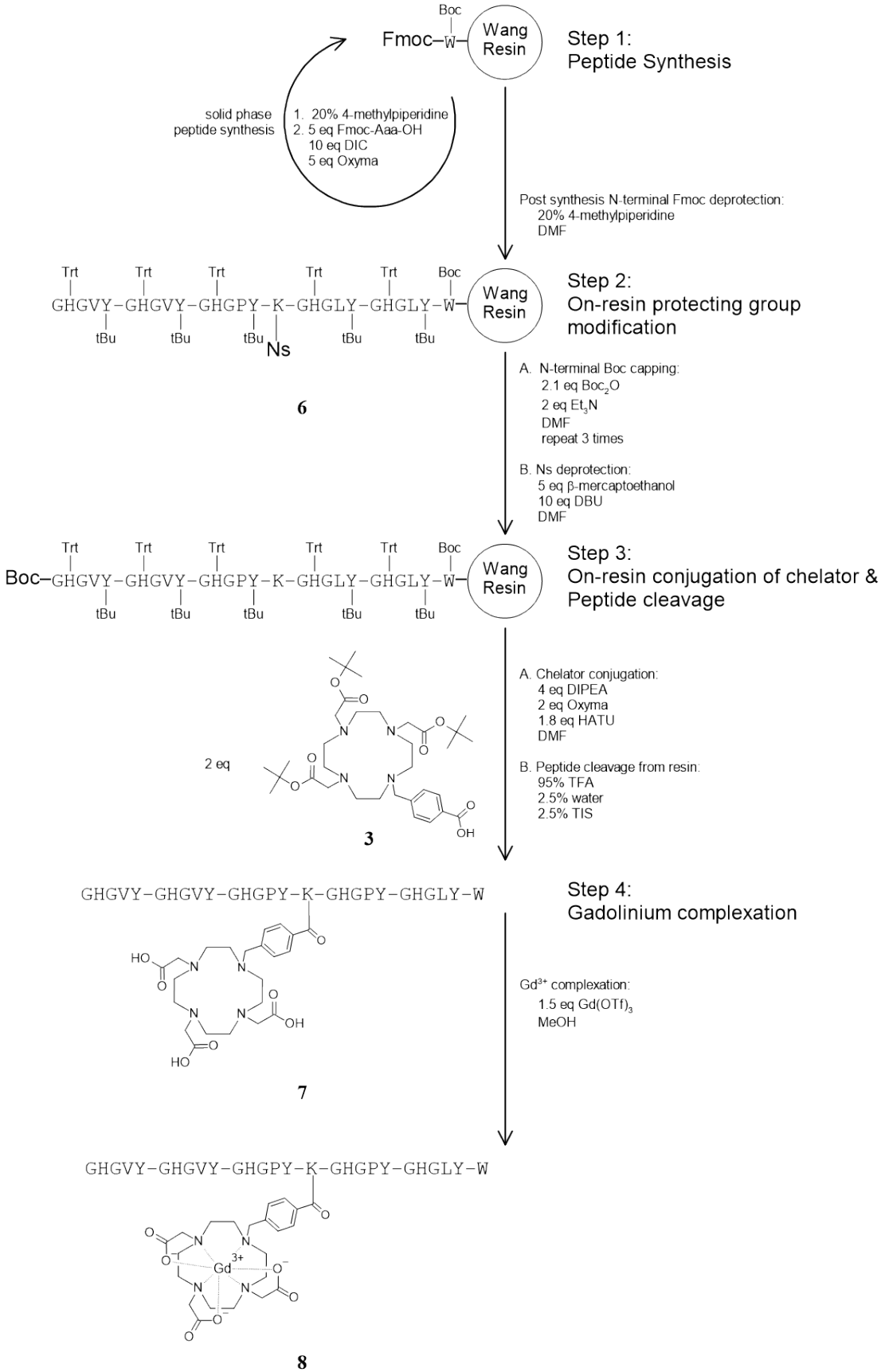


Scheme S2: General synthetic scheme

The synthesis of Fmoc-Lys(Ns)-OH (compound **4**), was previously reported by De Luca and coworkers[6] (**Scheme S2**). Fmoc-Lys-OH (1 eq) was dissolved in 2 parts of 10% w/v K_2CO_3 in water and 5 parts dioxane. This was stirred in an ice bath. Ortho-nitrobenzenesulfonyl chloride (Ns-Cl , 0.9 eq) was dissolved in a small amount of dioxane and slowly introduced to the ice-cold mixture. The reaction mixture was stirred at room temperature for 4 h. Subsequently, 100 mL of water was added to the mixture and transferred to a separatory funnel. Two portions of diethyl ether, 40 mL each, were added to the funnel to extract unreacted Ns-Cl. The water layer was collected. The ether layer was washed with basic water to extract all the lysine compounds. All water extracts were pooled and cooled in an ice bath. To precipitate compound 4, 1 M HCl was introduced into the cold-water extract. This was centrifuged and the supernatant was decanted. The precipitates were washed with 1 M HCl. The precipitates were freeze-dried to remove residual water. The reaction was monitored by normal phase TLC (9:1 DCM:MeOH solvent system). Purity was checked by reversed-phase HPLC.

1.1.4 Peptide synthesis

The synthesis of HBpep (**5**) and [Lys(Ns)]-HBpep (**6**) was performed on a Liberty Blue microwave-assisted automated peptide synthesizer. This was based on the literature procedures by Amblard[7], Collins[8], and Palasek[9]. Before synthesis, Wang resin (solid-support) was loaded with Fmoc-Trp(Boc)-OH, and then the remaining reactive groups of the resin were blocked with acetic anhydride. The loading



Scheme S3: General synthetic scheme

of the first amino acid (Trp, W) was measured by the amount of Fmoc released in the resin. The following amino acids were synthesized using DIC-Oxyma (5 eq excess) as coupling reagents and 20% v/v 4-methylpiperidine as the Fmoc deprotection reagent in the automated microwave-assisted peptide synthesizer (**Scheme S3, step 1**). For peptide **6**, Fmoc-Lys(Ns)-OH (compound **4**) was used for lysine, since Ns protects the ε-amino from acidic and basic reagents. HBpep (**5**) was cleaved in a cocktail of 95/2.5/2.5 TFA:TIS:water for 3 h at room temperature (**Scheme S3, step 3B**). The mixture was filtered, and the peptide was precipitated with MTBE. HBpep (**5**) were purified by reversed phase HPLC. [Lys(Ns)]-HBpep (**6**) was conjugated with chelator **3** before being cleaved from the resin.

1.1.5 Synthesis of peptide-chelator conjugate

While [Lys(Ns)]-HBpep (**6**) was still attached to the resin, the N-terminal was capped with Boc protecting group since Fmoc is labile upon exposure to DBU. Boc_2O (di-tert-butyl decarbonate, 2.1 eq) and Et_3N (triethylamine, 2 eq) were added to [Lys(Ns)]-HBpep (**6**) for 10 min with sonication. (**Scheme S3, step 2A**). Subsequently, the resin was reacted with a mixture of β-mercaptoethanol (5 eq) and DBU (1,8-diazabicyclo[5.4.0]undec-7-ene, 10 eq) in DMF for 3 times with wash in between to remove Ns protection for 30 min at room temperature[6] (**Scheme S3, step 2B**). The resin was washed 3 times with DMF. On a separate reaction vessel, chelator **3** (5 eq), was reacted with the sequential addition of DIPEA (10 eq), Oxyma (5 eq), and HATU (4.5 eq) in 5-minute intervals. The resulting activated ester was introduced to the resin. The resin was irradiated at 40 °C in a microwave reaction. The resin was washed 3 times with DMF and then with DCM (Scheme 1, step 3A). The peptide-chelator conjugate (**7**) was cleaved in a cocktail of 95/2.5/2.5 TFA:TIS:water for 3 hours at room temperature. The mixture was filtered, and the peptide was precipitated with MTBE (**Scheme S3, step 3B**). The peptide-chelator conjugate (**7**) was purified by reversed-phase HPLC.

1.1.6 Gd complexation

Gd^{3+} complexation was done according to Faulkner and Burton-Pye[2] and Do and coworkers[10] with some modifications. Gadolinium triflate, ($Gd(OTf)_3$, 1.5 eq) and peptide-chelator conjugate (compound **7**, 1 eq) were dissolved in methanol and sonicated for 15 min. The reaction mixture was left to stand for 24 h (**Scheme S3, step 4**). Afterward, the solution was dried in vacuo. The Gd-labeled HBpep (compound **8**) was purified by reversed-phase chromatography in pH 5 acetate buffer and methanol solvent system.

1.2 Characterisations

1.2.1 Peptide Quantification

The peptides were quantified by UV-Vis spectroscopy according to the method by Anthis and Clore.[11] Molar absorptivity of HBpep at 205 nm was calculated to be $\varepsilon = 146,300\,M^{-1}cm^{-1}$ based on its sequence. The molar absorptivity of Gd-HBpep was calcutated to be $\varepsilon = 149,080\,M^{-1}cm^{-1}$.

1.2.2 Turbidimetry

The relative turbidity was analysed using a UV-Vis spectrophotometer as reported by Lim and coworkers,[12] and Sun and coworkers.[13] The absorbance at 600 nm (A600) was used to compute the relative turbidity as follows:

$$Relative\,turbidity = 100 - [100 \times (10^{-A_{600nm}})]$$

The Gd-labeled peptide was dissolved in 10 mM acetate buffer pH 4.0 to make a 10 mg/mL stock. One part of the stock solution was mixed with 9 parts of buffer. This was analysed in a range of buffers from pH 3.5 to 9 with 100 mM ionic strength each to assess the response of coacervation as a function of

pH. The absorbance at 600 nm was measured and the relative turbidity was calculated and plotted as a function of pH.

1.2.3 Microscopy

Optical microscopy was used to assess the behaviour of the peptide coacervates at different pH. The peptide mixtures in 1 part stock and 9 part buffer were pipetted into 96-well plate. A range of pH from 5.5 to 7.5 was used according to the relative turbidimetry data to observe the self-assembly behaviour as the turbidity drastically increased. These were viewed under a microscope of a Biotek Cytation 5 cell imaging reader at 4x and 20x magnification range in bright field mode.

1.2.4 CD spectroscopy

CD spectroscopy was used to study the secondary structure features of the Gd-labeled peptide. The method was adapted from Cai and coworkers[14], Le Ferrand and coworkers[15], and Greenfield[16]. This was performed on a JASCO J-1500 CD spectrophotometer. A wavelength scan from 190 to 270 nm was measured for 3 accumulations. Two samples of 1 mg/ml peptide were analysed at (1) pH 4.0 (acetate buffer) and (2) pH 7.5 (10 mM phosphate buffer, 100 mM ionic strength) were analysed. The resulting spectra were smoothed, and DichroWeb was used to calculate the secondary structure estimates of the peptides.[17]

1.2.5 Relaxivity

Inversion recovery NMR experiment is adapted from Helm[10]. This is used to measure the longitudinal relaxation times (T_1). This is based on the pulse sequence: 180°–τ_i–90°–acquisition. τ_i is the delay time and varied at i repetitions to calculate T_1.

$$M_z(\tau) = M_z(\infty)[1 - Ae^{(\frac{-\tau}{T_1})}]$$

$M_z(\tau)$ is the z-magnetisation at a delay τ; it is proportional to the integral or the height of the resonance signal in the NMR spectrum. Equilibrium magnetisation, $M_z(\infty)$, is the magnetisation measured at $\tau \geq 5T_1$. A is equal to 2 for a perfect setting of pulse lengths. T_1 was computed by fitting experimental data using τ and $M_z(\tau)$ in a three-parameter exponential fit ($Y' = B + Fe^{-\tau G}$), such that $T_1 = 1/G$. Relaxivity (r_1) was computed by plotting the reciprocal T_1,obs vs respective concentrations of Gd, c_{CA} in the equation:

$$\frac{1}{(T_1)_{obs}} = \frac{1}{(T_1)_d} + (r_{1,})_{CA} c_{CA}$$

The samples were dissolved in 5% H_2O in 95% D_2O[18] and filled into a capillary tube and then placed inside the 3 mm NMR tube. This method is used to measure T_1 effectively using a small amount of H_2O[18] and minimize the radiation damping of modern high-field NMR spectrometers.[10] Increasing concentrations of free peptides and coacervates (based on the amount of Gd) were dissolved in 10 mM acetate buffer pH 4.0, and 10 mM MES buffer pH 6.5 100 mM ionic strength, respectively. These were also compared with the increasing concentration of Dotarem® (Gadoterate meglumine, Gd-DOTA) as a reference. The [Gd] concentrations were: 0.1 mM, 0.05 mM, 0.025 mM, 0.0125 mM and blank.

NMR experiments were performed on a Varian 500 MHz (11.4T) NMR spectrometer with OpenVnmrJ software. Before the T_1 inversion recovery experiment, the 90 °pulse width was measured to ensure that the signal aligned with the XY plane, thus maximizing the signal for the subsequent experiment. A proton spectrum was acquired and the spectral width was adjusted in the range that covers the water resonance (at 4.6 ppm) for the T_1 inversion recovery. The measured 90 °pulse width was entered for the 90 °pulse, and twice as much for the 180 °pulse of the T_1 inversion recovery. The parameters for minimum T_1 were around 0.01% of the supposed T_1 of the sample, maximum was three times the supposed T_1, and interscan delay was five times the supposed T_1. Preliminary T_1 inversion recovery was acquired, and

the computed T_1 for the respective concentration was used to fine-tune the parameter range of min and max T_1, and interscan delay for the subsequent acquisition. T_1 analysis was performed using Mestrenova software. Once the T_1 was computed, its reciprocal was plotted with respect to the concentration to compute the relaxivity (r_1) of each sample.

2 Supplemental results

2.1 Synthesis

2.1.1 Chelator synthesis (3)

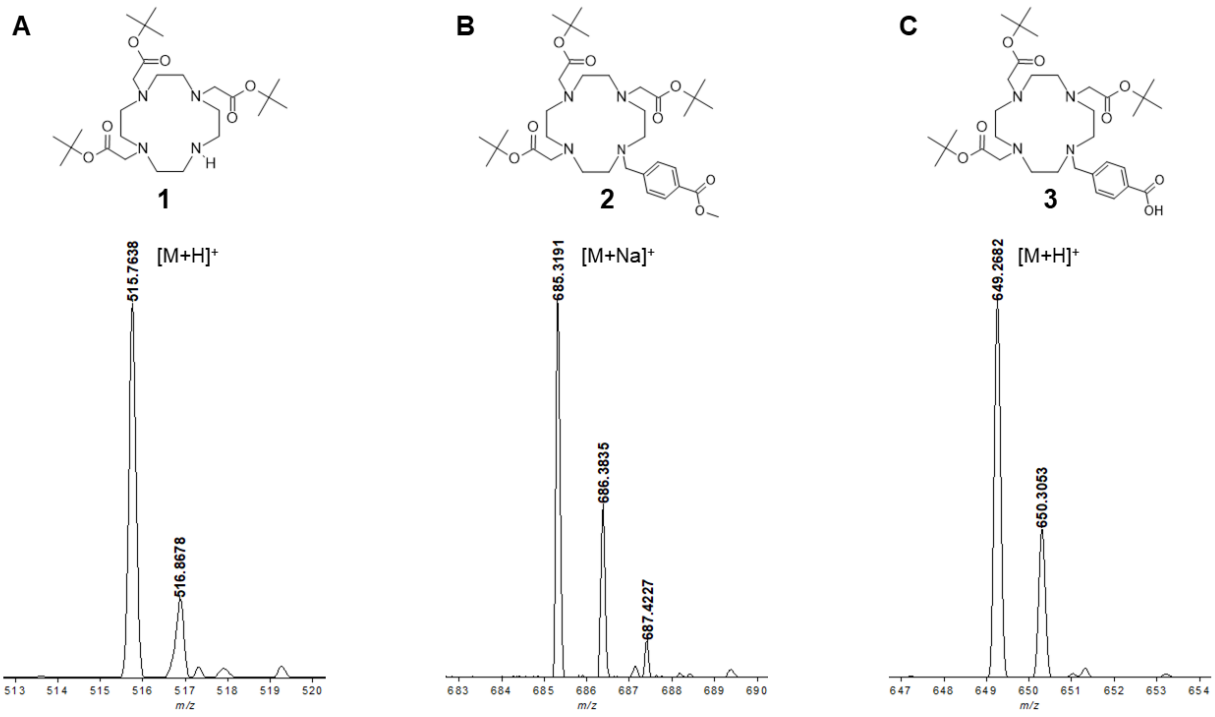


Figure S1: MS spectra of chelators 1(A), 2(B), and 3(C)

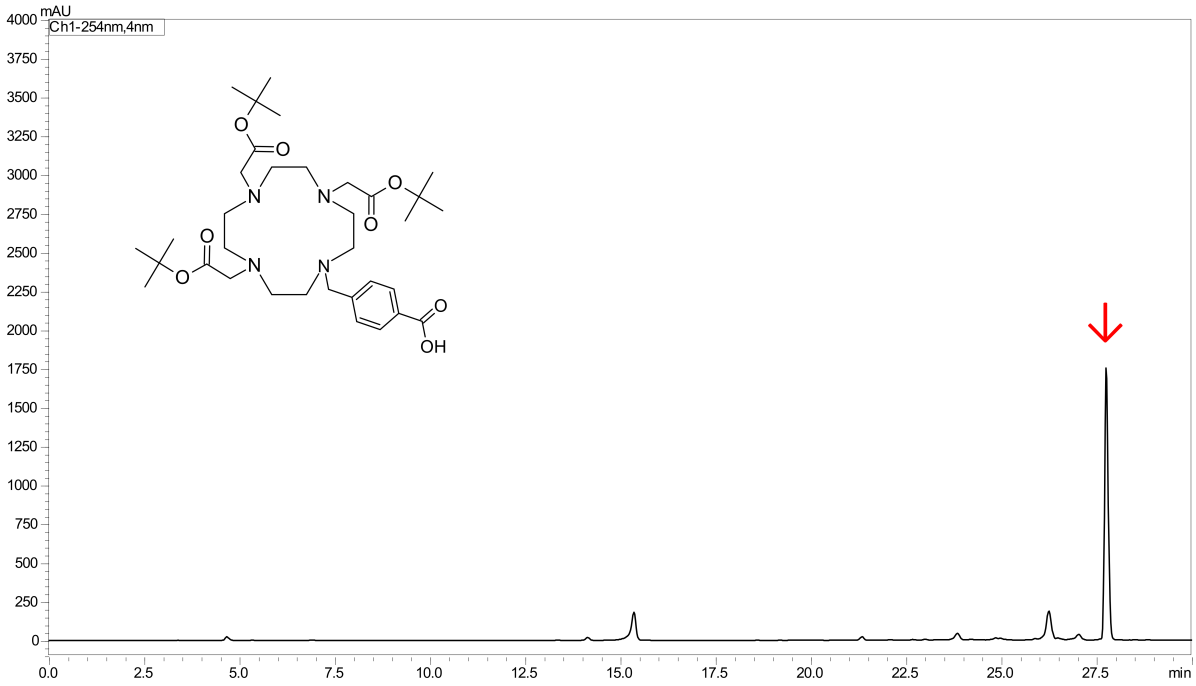


Figure S2: HPLC profile of chelator 3, t_R = 27.762 min, 73.4% purity. Linear gradient of 5% –100% methanol in pH 5.0 acetate buffer was used as the gradient program on an Inertsil ODS-3 (C18) reversed-phase columnn

2.1.2 Synthesis of Fmoc-Lys(Ns)-OH (4)

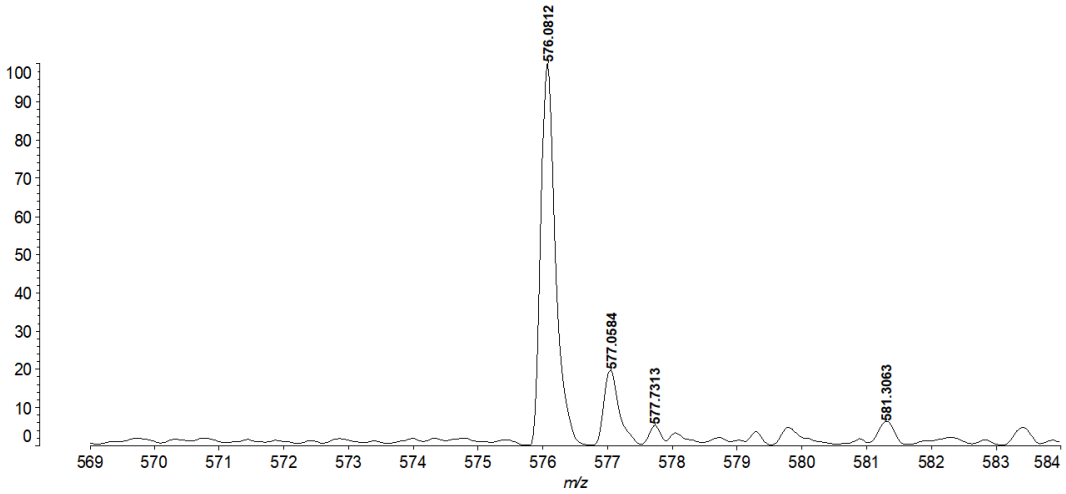


Figure S3: MALDI-TOF MS spectrum of Fmoc-Lys(Ns)-OH (**4**)

2.1.3 Synthesis HBpep (5)

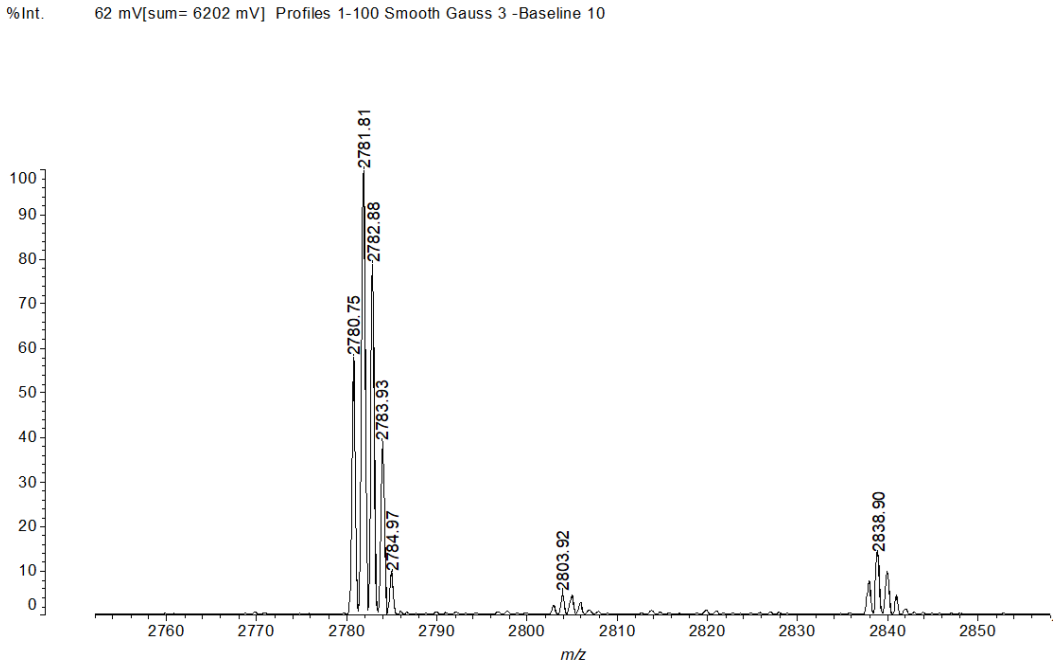


Figure S4: MS spectrum HBpep (**5**)

2.1.4 Reaction monitoring of chelator-peptide conjugate (7)

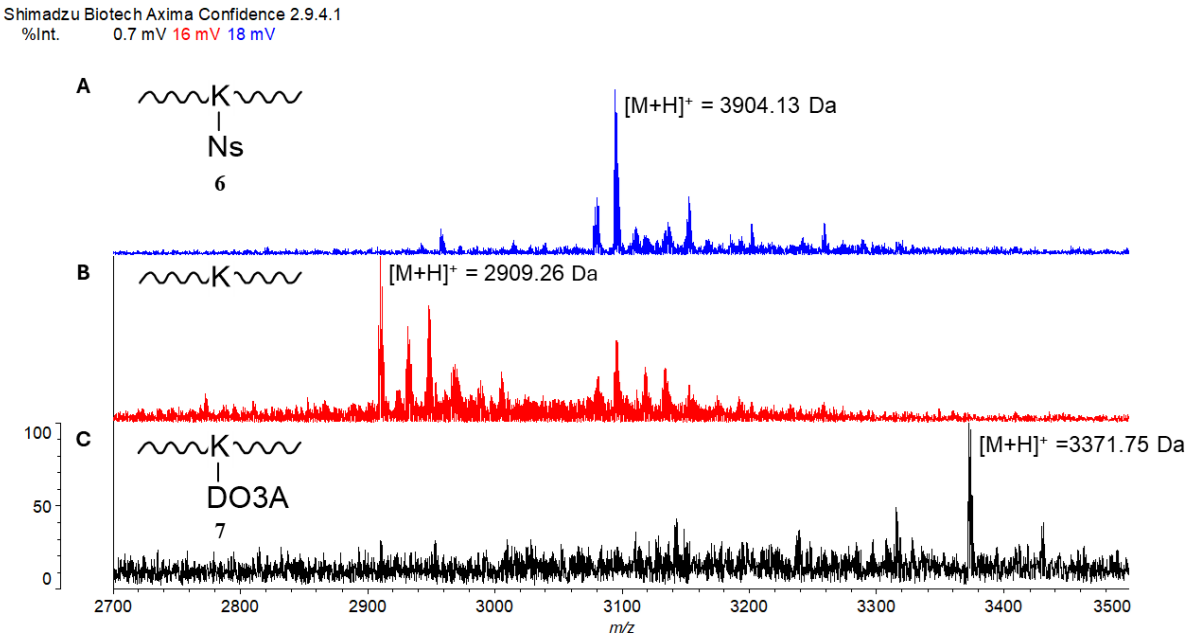


Figure S5: Reaction monitoring of the on-resin conjugation of the chelator to the peptide via MALDI-TOF MS.

2.1.5 Gd complexation of chelator-peptide conjugate (8)

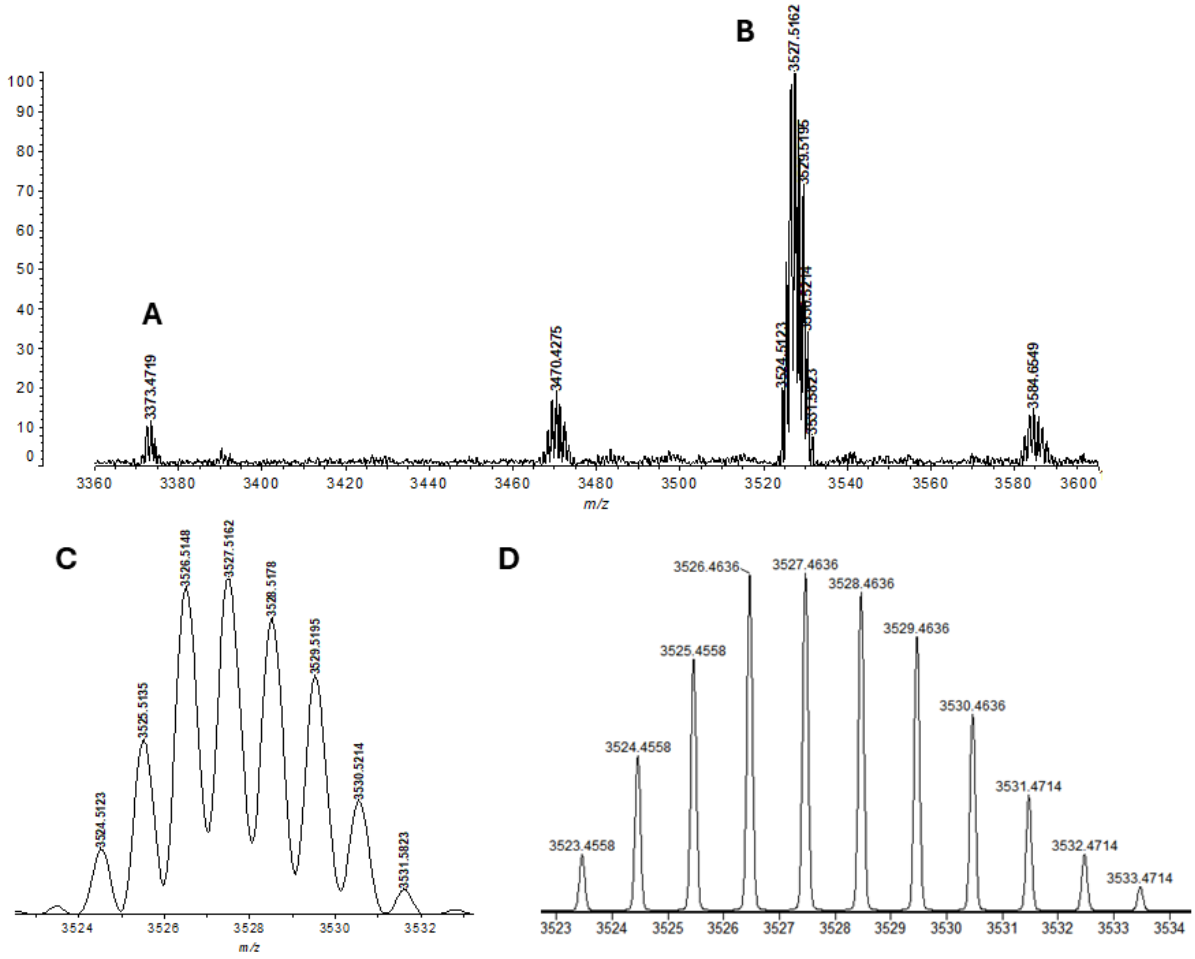


Figure S6: MALDI-TOF MS of (**A**) chelator-peptide conjugate **7**, (**B**) Gd-HBpep, **8**, (**C**) isotopic pattern distriubution of Gd-HBpep, **8**, and (**D**) theoretical mass spectrum of Gd-HBpep, **8**, generated by the MassLynx°isotope model

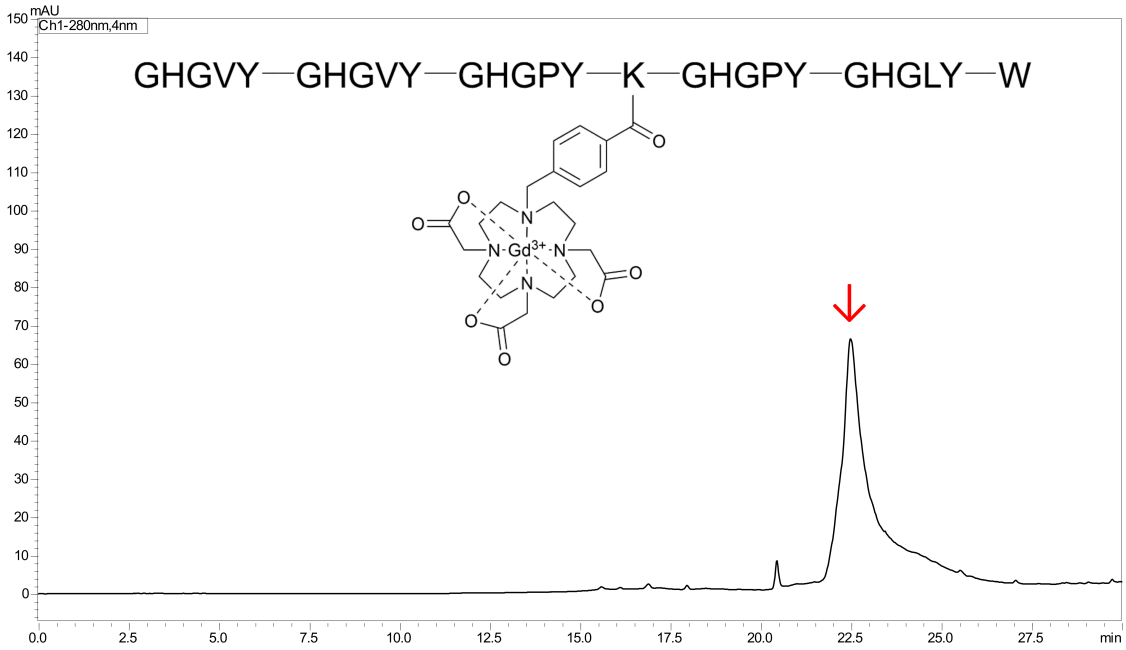


Figure S7: HPLC profile of Gd-HBpep, t_R = 22.494 min, 97.8% purity. Linear gradient of 5% –100% methanol in pH 5.0 acetate buffer was used as the gradient program on an Inertsil ODS-3 (C18) reversed-phase column

2.2 Characterization

2.3 CD spectroscopy

Table 1: Secondary structure calculations from DichroWeb[17] using and SP180t data set.[19]

Algorithm	Helix 1	Helix 2	Strand 1	Strand 2	Turns	Unordered	Total	NMRSD
5, pH 4.0								
CDSSTR	-0.010	0.030	0.340	0.130	0.100	0.390	0.980	0.036
CONTIN 1	0.000	0.068	0.251	0.132	0.123	0.426	1.000	0.077
CONTIN 2	0.000	0.054	0.264	0.133	0.122	0.427	1.000	0.077
K2D	0.090		0.470		n/a	0.440	1.000	3.602
5, pH 7.5 (0.1 M IS)								
CDSSTR	-0.020	-0.020	0.490	0.150	0.120	0.240	0.960	0.059
CONTIN 1	0.000	0.024	0.318	0.129	0.114	0.415	1.000	0.555
CONTIN 2	0.000	0.017	0.359	0.128	0.116	0.381	1.001	0.555
K2D	0.080		0.490		n/a	0.430	1.000	1.797
8, pH 4.0								
CDSSTR	0.010	0.030	0.290	0.130	0.110	0.420	0.970	0.026
CONTIN 1	0.000	0.067	0.237	0.134	0.126	0.436	1.000	0.089
CONTIN 2	0.000	0.056	0.249	0.135	0.127	0.434	1.001	0.089
K2D	0.090		0.480		n/a	0.440	1.010	2.626
8, pH 7.5 (0.1 IS)								
CDSSTR	-0.020	-0.030	0.320	0.230	0.220	0.190	0.910	0.055
CONTIN 1	0.000	0.009	0.376	0.138	0.095	0.382	1.000	0.800
CONTIN 2	0.000	0.003	0.454	0.139	0.086	0.318	1.000	0.800
K2D	0.040		0.480		n/a	0.480	1.000	4.201

References

[1] A. Dadabhoy, S. Faulkner and P. G. Sammes, *Journal of the Chemical Society, Perkin Transactions 2*, 2002, 348–357.

[2] S. Faulkner and B. P. Burton-Pye, *Chemical Communications*, 2005, 259–261.

[3] J. L. Moore, S. M. Taylor and V. A. Soloshonok, *Arkivoc*, 2005, **2005**, 287–292.

[4] E. Corey, I. Székely and C. S. Shiner, *Tetrahedron Letters*, 1977, **18**, 3529–3532.

[5] A. M. Steer, H. L. Bolt, W. D. G. Brittain and S. L. Cobb, *Tetrahedron Letters*, 2018, **59**, 2644–2646.

[6] S. De Luca, R. D. Moglie, A. De Capua and G. Morelli, *Tetrahedron Letters*, 2005, **46**, 6637–6640.

[7] M. Amblard, J.-A. Fehrentz, J. Martinez and G. Subra, *Molecular Biotechnology*, 2006, **33**, 239–254.

[8] J. M. Collins, K. A. Porter, S. K. Singh and G. S. Vanier, *Organic Letters*, 2014, **16**, 940–943.

[9] S. A. Palasek, Z. J. Cox and J. M. Collins, *Journal of Peptide Science*, 2007, **13**, 143–148.

[10] *Contrast Agents for MRI: Experimental Methods*, ed. V. C. Pierre and M. J. Allen, Royal Society of Chemistry, Cambridge, 2017.

[11] N. J. Anthis and G. M. Clore, *Protein Science*, 2013, **22**, 851–858.

[12] Z. W. Lim, Y. Ping and A. Miserez, *Bioconjugate Chemistry*, 2018, **29**, 2176–2180.

[13] Y. Sun, Z. W. Lim, Q. Guo, J. Yu and A. Miserez, *MRS Bulletin*, 2020, **45**, 1039–1047.

[14] H. Cai, B. Gabryelczyk, M. S. S. Manimekalai, G. Grüber, S. Salentinig and A. Miserez, *Soft Matter*, 2017, **13**, 7740–7752.

[15] H. Le Ferrand, M. Duchamp, B. Gabryelczyk, H. Cai and A. Miserez, *Journal of the American Chemical Society*, 2019, **141**, 7202–7210.

[16] N. J. Greenfield, *Nature Protocols*, 2006, **1**, 2876–2890.

[17] A. J. Miles, S. G. Ramalli and B. A. Wallace, *Protein Science*, 2021, **31**, 37–46.

[18] J. V. Hines, G. M. Ammar, J. Buss and P. Schmalbrock, *Bioconjugate Chemistry*, 1999, **10**, 155–158.

[19] A. Abdul-Gader, A. J. Miles and B. A. Wallace, *Bioinformatics*, 2011, **27**, 1630–1636.