Supporting information for

Solid-Phase-Supported Approach for the Preparation of Bioresponsive and Multifunctional MRI Probes

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General remarks

Commercially available reagents and solvents were used without further purification. Rink amide resin 100-200 mesh was purchased from Merck Millipore. Purification of synthesised compounds was performed using silica gel 60 (0.03-0.2 mm) from Carl Roth (Germany). LC-MS spectra were recorded on an Agilent 1100 series LC/MS system with a Polaris 5 C18-Ether column (250 x 4.6 mm). The LC-MS elution conditions are given in Table S1. High resolution mass spectra were recorded on a Bruker Daltonics APEX II (FT-ICR-MS) with an electrospray ionization source. $^1$H and $^{13}$C NMR spectra and relaxometric experiments were performed on a Bruker Avance III 300 MHz spectrometer at 25 °C. Processing was performed using TopSpin 2.1 (Bruker GmbH) and ACD/SpecManager 9.0 (Advanced Chemistry Development, Inc.). The NMR spectra were obtained either in CDCl$_3$ or D$_2$O, using the deuterium lock frequency. The concentration of Gd$^{3+}$ in analysed solutions was determined using the bulk magnetic susceptibility shift (BMS).$^1$ Manual solid phase synthesis was performed with the synthesis 1 apparatus from Heidolph. Reversed-phase HPLC purification was performed on a Varian PrepStar Instrument (Austrailia) with PrepStar SD-1 pump heads. Analytical reversed-phase HPLC was performed with an Atlantis C18 column (4.6 x 150 mm, 5 µm particle size). Semi-preparative reversed-phase HPLC was conducted with a Polaris 5 C18-A column (250 x 21.2 mm). Elution conditions are described in Table S2.

Synthetic procedures

Synthesis of BB1

2-(4,10-Bis-tert-butoxycarbonylmethyl-1,4,7,10-tetraaza-cyclododec-1-yl)-4-(4-nitro-phenyl)-butyric acid tert-butyl ester (1)

![Chemical structure of BB1](image)

The synthesis of 1 was carried out using a previously published procedure.$^2$
2-{4,10-Bis-tert-butoxycarbonylmethyl-7-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propyl]-1,4,7,10tetraaza-cyclododec-1-yl}-4-(4-nitro-phenyl)-butyric acid tert-butyl ester (2)

To a mixture of amine 1 (1.00 g, 1.50 mmol) and K₂CO₃ (0.46 g, 3.31 mmol) in anhydrous acetonitrile (15 ml), N-(3-bromopropyl)phthalimide (0.48 g, 1.80 mmol) was added and the reaction mixture was stirred at 70 °C for 16 h. It was then cooled to room temperature and volatiles were removed under reduced pressure. Water (70 mL) was added and the resultant mixture was extracted with dichloromethane (3 × 100 mL). The organic layer was dried over anhydrous sodium sulphate, filtered and evaporated to obtain yellow oil. The crude product was purified by alumina column chromatography (4% MeOH/CH₂Cl₂) to obtain 2 as a white flocculent powder (1.80 g, 66%).

¹H NMR (300 MHz, CDCl₃), δ (ppm): 1.43 (br., 27H), 1.66-2.06 (m, 4H), 2.25-3.91 (m, 27H), 7.31 (d, J=6 Hz, 2H), 7.54-7.87 (m, 4H), 8.03 (d, J= 6 Hz, 2H). ¹³C NMR (75 MHz, CDCl₃), δ (ppm): 26.3, 28.2, 28.3 (C(CH₃)₃), 31.5, 32.6, 36.6, 49.9, 52.5, 52.8, 53.1, 56.1, 64.3 (-CH₂-), 80.6, 80.9 (C(CH₃)₃), 123.0, 123.5, 129.2, 132.1, 133.7, 146.1, 150.3 (ArC), 168.2, 170.8, 172.3 (C=O). ESI-HRMS: (m/z) [M+H]^+ calcd. for C₄₅H₆₇N₆O₁₀^+, 851.4913, found: 851.4918.

4-(4-Amino-phenyl)-2-{4,10-bis-tert-butoxycarbonylmethyl-7-[3-(1,3-dioxo-1,3-dihydro-isoindol-2-yl)-propyl]-1,4,7,10tetraaza-cyclododec-1-yl}-butyric acid tert-butyl ester (BB1)

A mixture of nitro 2 (0.55 g, 0.65 mmol) and 10% Pd-C (0.10 g, 20% w/w) in absolute ethanol (25 mL) was shaken under a hydrogen atmosphere (35 psi) in a Parr hydrogenator. After completion of the reaction, the catalyst was removed by filtration through celite and the solvent was removed under reduced pressure to obtain a pure light yellow oil of BB1 (0.50 g, 78%).
$^1$H NMR (300 MHz, CDCl$_3$) δ (ppm): 1.26-1.56 (m, 27H), 1.64-1.93 (m, 4H), 2.35-3.77 (br, 27H), 6.52 (d, $J=7.5$ Hz, 2H), 6.91 (d, $J=7.5$ Hz, 2H), 7.60-7.81 (m, 4H). $^{13}$C NMR (75 MHz, CDCl$_3$) δ (ppm): 26.1, 28.1 (C(CH$_3$)$_3$), 31.7, 32.2, 36.5, 49.7, 52.1, 52.3, 52.8, 53.0, 56.1, 64.4 (-CH$_2$-) 80.3, 80.4, (C(CH$_3$)$_3$), 115.1, 122.9, 129.0, 131.9, 132.0, 133.6, 144.1 (ArC), 168.1, 171.0, 172.8 (C=O). ESI-HRMS: (m/z) [M+H]$^+$ calcd. for C$_{45}$H$_{69}$N$_6$O$_8$, 821.5171, found: 821.5164.

Synthesis of BB2

(2-{2-[(tert-Butoxycarbonylmethyl-amino)-ethoxy]-ethoxy}-ethylamino)-acetic acid tert-butyl ester (3)

The synthesis of 3 was carried out using a previously published procedure.$^3$

2-phenylpropan-2-yl 2-bromoacetate

The synthesis of 2-phenylpropan-2-yl 2-bromoacetate was performed in accordance with a previously published procedure.$^4$

[2-(2-[(tert-Butoxycarbonylmethyl-(1-methyl-1-phenyl-ethoxycarbonylmethyl)-amino]-ethoxy]-ethoxy)-ethylamino]-acetic acid tert-butyl ester (4)

Compound 3 (0.8 g, 2.125 mmol) was dissolved in acetonitrile (50 mL) and potassium carbonate (0.294 g, 2.125 mmol) was added. After stirring for 30 min, 2-phenylpropan-2-yl 2-bromoacetate (0.546 g, 2.125 mmol) was dissolved in acetonitrile (5 mL) and slowly added to the mixture by dropwise addition. The reaction mixture was then stirred at room temperature for 2 days. The
resulting mixture was then filtered and the solvent evaporated under reduced pressure to yield a crude oil which was purified by column chromatography (silica gel, MeOH:CH₂Cl₂, 3:97 v/v) giving amine 4 (0.52 g, 44 %) as a yellow oil.

**¹H NMR** (300 MHz, CDCl₃): δ (ppm) 1.46 (m, 18H), 1.77 (s, 6H), 2.78 (t, J=5.19 Hz, 2H), 2.91 (t, J=5.67, 2H), 3.24 – 3.35 (m, 2H), 3.46 (s, 2H), 3.50 – 3.65 (m, 10H), 7.14 – 7.39 (m, 5H).

**¹³C NMR** (75 MHz, CDCl₃): δ (ppm) 28.0, 28.1 (C(CH₃)₃), 28.6 (C(CH₃)₂), 48.7, 51.5, 53.6, 56.6, 70.0, 70.4 (-CH₂-), 80.9, 80.9 (C(CH₃)₃), 82.0 (C(CH₃)₂), 124.2, 126.9, 128.2, 145.6 (ArC), 170.2, 170.8, 171.6 (C=O).  **ESI-HRMS**: (m/z) [M+H]+ calcd. for C₂⁹H₄₈N₂O₈, 553.3483, found: 553.3481.

[[2-(2-[[tert-Butoxycarbonylmethyl-(1-methyl-1-phenylethoxycarbonylmethyl)-amino]-ethoxy]-ethoxy)-ethyl]-[9H-fluoren-9-ylmethoxycarbonyl]-amino]-acetic acid tert-butyl ester (5).]

Compound 5 was synthesized following a slightly modified acylation procedure with FmocCl.⁵ Amine 4 (1.735 g, 3.14 mmol) was dissolved in dioxane (24 mL) and a solution of Na₂CO₃ (0.998 g, 9.42 mmol) in H₂O (24 mL) was added. The reaction mixture was cooled to 0 °C and a solution of 9-fluorenylchloroformate (0.975 g, 3.77 mmol) in dioxane (15 mL) was added dropwise to the mixture. The reaction mixture was then allowed to warm to room temperature and stirred overnight. The solvents were evaporated under reduced pressure and the residue was purified by column chromatography (silica gel, CH₂Cl₂/EtOAc, 80:20 v/v) to yield 5 (1.7 g, 70 %).  **¹H NMR** (300 MHz, CDCl₃): δ (ppm) 1.45 (br. s., 18H), 1.77 (br. s., 6H), 2.86 – 3.01 (m, 2H), 3.28 – 3.72 (m, 14H), 3.93 – 4.09 (m, 2H), 4.17 – 4.30 (m, 1H), 4.36 (d, J=7.18 Hz, 1H), 4.48 (d, J=6.23 Hz, 1H) 7.20 – 7.44 (m, 9H), 7.58 (d, J=7.37 Hz, 2H), 7.75 (d, J=7.55 Hz, 2H).  **¹³C NMR** (75 MHz, CDCl₃): δ (ppm) 28.0, 28.1 (C(CH₃)₃), 28.6 (C(CH₃)₂), 47.3 (-CH-), 47.8, 48.4, 50.7, 53.3, 56.4, 67.3, 67.8, 69.8, 70.1, 70.4 (-CH₂-), 80.8, 81.5, 81.6 (C(CH₃)₃), 82.0 (C(CH₃)₂), 119.9, 124.2, 124.8, 125.1, 126.9, 127.0, 127.6, 128.2, 141.2, 141.3, 143.9, 145.6
(ArC), 156.0, 156.1, 168.9, 169.0, 170.0, 170.7 (C=O). **ESI-HRMS:** (m/z) \([M+H]^+\) calcd. for C\(_{44}H\(_{58}\)N\(_2\)O\(_{10}\)\(^+\), 775.4164, found: 775.4172.

\(\text{tBoc-}\quad [2-(2-[tBoc)-(9H-fluoren-9ylmethoxycarbonyl)-amino]-ethoxy}-ethoxy)-ethyl]-amino]-acetic acid (BB2).

![BB2 structure](image)

Compound 5 (300 mg, 0.387 mmol) was dissolved in a solution of 3 % TFA/CH\(_2\)Cl\(_2\) and stirred for 1 h. The solvent was evaporated under reduced pressure giving a yellow oil which was used in subsequent reactions without further purification. **\(^1\text{H NMR}\)** (300 MHz, CDCl\(_3\)) \(\delta\) (ppm): 1.46 (s, 18H), 3.01-3.19 (m, 2H), 3.36 (s, 2H), 3.41 – 3.70 (m, 12H), 4.00 (d, 2H), 4.15 – 4.30 (m, 1H), 4.37 (d, 1H), 4.49 (d, 1H), 7.30 (t, \(J = 7.18\) Hz, 2H), 7.39 (t, \(J = 7.37\) Hz, 2H), 7.58 (d, \(J = 7.37\) Hz, 2H), 7.75 (d, \(J = 7.37\) Hz, 2H). **\(^{13}\text{C NMR}\)** (75 MHz, CDCl\(_3\)) \(\delta\) (ppm): 27.9, 27.9, 28.0 (C(CH\(_3\))\(_3\)), 47.1, 47.2 (-CH-), 47.8, 48.3, 50.5, 50.7, 53.3, 53.4, 54.7, 56.6, 57.1, 57.2, 67.9, 68.0, 69.7, 70.1, 70.2 (-CH\(_2\)-), 81.5, 81.7, 82.7, 82.8 (C(CH\(_3\))\(_3\)), 119.8, 119.9, 124.7, 124.8, 125.0, 125.0, 127.0, 127.6, 141.1, 141.2, 143.8, 143.9 (ArC), 156.1, 168.9, 169.0, 171.4, 171.4 (C=O). **ESI-HRMS:** (m/z) \([M+H]^+\) calcd. for C\(_{35}H_{49}N_{2}O_{10}\)\(^+\), 657.3382, found: 657.3383.

**BB3** was synthesized according to previously reported procedure. \(^3\)

**Solid phase peptide synthesis**

The synthesis of the initial peptide sequence was carried out using the standard Fmoc chemistry strategy on a manual peptide synthesizer. A Rink Amide MBHA resin (0.1 g, substitution 0.78 mmol g\(^{-1}\)) was used as the solid support. Before the first amino acid was coupled, the resin was allowed to swell in DMF for 1 h and Fmoc deprotection of the resin was carried out using a solution of 20 % piperidine in DMF (3 x 15 min). Prior to each reaction, the resin was allowed to swell in DMF for 1 h. After the coupling of the first amino acid, a capping procedure using an
acetic anhydride/pyridine solution (3:2, 4 mL) was performed for 30 min. The resin was then washed with DMF (5 x 3 mL). Each relevant coupling and deprotection procedure was checked by the Kaiser test for completeness. All reactions on solid phase were performed at room temperature. All LC-MS data was provided for the given compounds after removal from the resin and the deprotection of tBu esters and where applicable, the Mtt protecting group.

**General amino acid coupling procedure**

Fmoc-protected amino acids (3 equiv. relative to the resin substitution) were dissolved in DMF (4 mL) and activated in situ with HBTU (2.9 equiv) and DIPEA (6 equiv). After 10 min of pre-activation, the mixture was added to the pre-swelled resin and agitated for 2 h. After, the solution was removed and the procedure was repeated with half the initial amount of amino acid, HBTU and DIPEA for 1 h. After coupling, the resin was washed with DMF (5 x 3 mL) and CH₂Cl₂ (3 x 3 mL) to remove excess reagents.

**General Fmoc deprotection procedure**

Fmoc deprotections of the resin and amino acids were carried out with 3 treatments (15 min each) of a 20 % piperidine in DMF solution. After deprotection, the resin was washed with DMF (5 x 3 mL) and prepared for the next procedure.

**General procedure for producing analytical data**

After each reaction for compounds 6 - 11, a micro cleavage of the resin was performed to assess the reaction success by means of LC-MS. Below; the synthetic protocols for the reactions described in the main text are reported. The chemical structures and LC-MS data shown represent that of the same compound after micro cleavage from the resin and removal of protecting groups. For compound L, the procedure for removal of the final ligand from the resin is described.
**Compound 6-x (Compound 6 after micro cleavage from the resin)**

The peptide sequence (Lys(Mtt)-Gly-Gly-NH₂) was synthesised following the general procedures previously described. The resin was then treated with a solution of succinic anhydride (78 mg, 0.78 mmol, 10 equiv) and DIPEA (0.272 mL, 1.56 mmol, 20 equiv) in DMF (4 mL) for 5 h. The peptidyl resin was then washed with DMF (5 x 3 mL) and CH₂Cl₂ (3 x 3 mL).

**LC-MS (7 without Mtt protecting group):** (m/z) [M+H]⁺ calcd. for C₁₄H₂₆N₅O₆⁺, 360.2, found: 360.0.

**Compound 7-x (Compound 7 after micro cleavage from the resin)**

**BB1** (128 mg, 0.156 mmol, 2 equiv), HATU (56 mg, 0.148 mmol, 1.9 equiv) and DIPEA (68 µL, 0.390 mmol, 5 equiv) were dissolved in DMF (4 mL) and added to the pre-swollen resin with compound 7. This was allowed to agitate for 18 h. Excess reagents were removed by extensive washing with DMF (5 x 3 mL) and CH₂Cl₂ (3 x 3 mL).

**LC-MS (7 without Mtt and 3 × tBu protecting groups):** (m/z) [M-H]⁻ calcd. for C₄₇H₆₆N₁₁O₁₃⁻, 992.5, found: 992.5.

**Compound 8-x (Compound 8 after micro cleavage from the resin)**
Resin with pthalimide 7 was suspended in isopropanol (4 mL) and ethylene diamine (47 mg, 0.78 mmol, 10 equiv) was added. The mixture was agitated for 24 h. The resin was then washed with isopropanol (3 x 3 mL), DMF (3 x 3 mL) and CH₂Cl₂ (3 x 3 mL) and the reaction progress checked by the Kaiser test.

**LC-MS (8 without Mtt and 3 × tBu protecting groups): (m/z) [M-H]^- calcd. for C₃₉H₆₄N₁₁O₁₁⁻, 862.5, found: 862.6.**

**Compound 9-x (Compound 9 after micro cleavage from the resin)**

Resin with amine 8 was treated with a solution of BB2 (102 mg, 0.156 mmol, 2 equiv), HATU (56 mg, 0.148 mmol, 1.9 equiv) and DIPEA (68 µL, 0.390 mmol, 5 equiv) in DMF (3 mL). The mixture was agitated for 24 h. The resin was washed with DMF (5 x 3 mL) and CH₂Cl₂ (3 x 3 mL) to give 9.

**LC-MS (9 without Mtt and 5 × tBu protecting groups): (m/z) [M-H]^- calcd. for C₆₆H₉₄N₁₃O₂₀⁻, 1388.7, found: 1388.6.**

**Compound 10-x (Compound 10 after micro cleavage from the resin)**

Resin with compound 9 was exposed to a solution of 40 % piperidine in DMF (3 mL) for 15 min. This was repeated a further two times to remove the Fmoc protecting group. The resin was then washed with DMF (5 x 3 mL). A mixture of BB3 (130 mg, 0.187 mmol, 2.4 equiv) and DIPEA (68 µL, 0.390 mmol, 5 equiv) in DMF (3 mL) was then added and agitated for 24 h. The solution was then removed and a further portion of BB3 (2.4 equiv) and DIPEA (5 equiv) in DMF (3 mL)
was added and allowed to shake for a further 24 h. The resin was then washed with DMF (5 x 3 mL) and CH₂Cl₂ (3 x 3 mL).

**LC-MS (10 without Mtt and 8 x tBu protecting groups):** (m/z) [M+H]⁺ calcd. for C₇₀H₁₁₉N₁₈O₂₅⁺, 1611.9, found 1611.8. (m/z) [M+2H]²⁺ calcd. for C₇₀H₁₂₀N₁₈O₂₅²⁺, 806.4, found 806.5. (m/z) [M+3H]³⁺ calcd. for C₇₀H₁₂₁N₁₈O₂₅³⁺, 538.0, found 538.1.

**Compound 11-x (Compound 11 after micro cleavage from the resin)**

Resin with compound 10 was washed with DCM (5 x 3 mL). A solution of TFA/triisopropylsilane (TIS)/CH₂Cl₂ (3:3:94, 3 mL) was then added to the resin and agitated for 2 min. The solution was then removed and the repeated a further 4 times. After 5 treatments, the resin was washed with CH₂Cl₂ (5 x 3 mL) and used further. The resin was then allowed to swell in DMF for 1 h. Biotin (95 mg, 0.390 mmol, 5 equiv), HATU (145 mg, 0.382 mmol, 4.9 equiv), HOBt (52 mg, 0.382 mmol, 4.9 equiv) and DIPEA (136 µL, 0.780 mmol, 10 equiv) were dissolved in DMF (3 mL) and added to the resin. The mixture was shaken for 24 h. The solution was then removed and the resin treated again with a second portion of biotin (5 equiv), HATU (4.9 equiv), HOBt (4.9 equiv) and DIPEA (10 equiv) in DMF (3 mL) for a further 24 h. The solution was then removed and the resin washed with DMF (5 x 3 mL) and CH₂Cl₂ (3 x 3 mL).

**Compound L**
Resin with compound 11 was treated with a deprotection solution of TFA/triisopropylsilane/CH₂Cl₂ (95:2.5:2.5, 3 mL) for 4 h. The solution was collected and the resin was washed twice with the deprotection solution (3 mL x 2) which was also collected. The deprotection solutions were combined and the majority of the solvent was evaporated. The crude product was precipitated from the remaining deprotection solution with cold diethyl ether and stored in the freezer overnight. The mixture was centrifuged at 3000g for 10 min and the solution was removed. The remaining solid was then subject to two further rounds of washing with cold diethyl ether. The crude solid was dried, purified by reverse phase HPLC and lyophilized to yield biotinylated L (35 mg, 24 % (overall yield across all steps)) as a white solid.

**1H NMR:** (300 MHz, D₂O): δ (ppm): 1.17 – 4.38 (br, 109H), 7.29 (dd, 4H)  
**ESI-HRMS:** (m/z) [M-2H]²⁻ calcd. for C₈₀H₁₃₀N₂₀O₂₇S²⁻, 917.4573, found: 917.4577.

**Complex Gd₂L**

Compound L (35 mg, 0.019 mmol) was dissolved in H₂O (5 mL) and the pH adjusted to 7. A solution of GdCl₃.6H₂O (15.57 mg, 0.042 mmol) in H₂O (1 mL) was added and left to stir at room temperature for 24 h while maintaining the pH at 7. Excess Gd³⁺ was removed by treating with Chelex for 24 h. The mixture was filtered and lyophilized to obtain Gd₂L as a white solid.  
**ESI-HRMS:** (m/z) [M-2H]²⁻ calcd. for C₈₀H₁₂₄Gd₂N₂₀O₂₇S²⁻, 1072.3579, found: 1072.3590.
Tables

Table S1. Elution conditions for LC-MS analysis. A flow rate of 1 mL/min was used. Solvent A = 0.1 % CH₃COOH/H₂O. Solvent B = 0.1 % CH₃COOH/MeCN.

| Time (min) | A % | B % |
|------------|-----|-----|
| 0          | 95  | 5   |
| 10         | 80  | 20  |
| 20         | 20  | 80  |
| 25         | 0   | 100 |
| 30         | 0   | 100 |
| 31         | 95  | 5   |
| 33         | 95  | 5   |

Table S2. Elution conditions for analytical and semi-preparative HPLC. Flow rates of 1 and 10 mL/min were used respectively. Solvent A = H₂O. Solvent B = MeCN.

| Time (min) | A % | B % |
|------------|-----|-----|
| 0          | 95  | 5   |
| 5          | 95  | 5   |
| 20         | 0   | 100 |
| 39         | 0   | 100 |
| 40         | 95  | 5   |
Figures

**Figure S1.** HABA assay: corrected UV/Vis absorbance at 500 nm against the equivalents of Gd$_2$L added to avidin (HEPES 25 mM, pH 7.4, 25 °C).

**Figure S2.** Relaxometric titration curves for Gd$_2$L + Avidin with Ca$^{2+}$ ([Gd$^{3+}$] = 1 mM, pH = 7.4, 50 mM HEPES, 25 °C, 7 T).
MRI phantom experiments

MRI measurements were performed on a Bruker BioSpec 70/30 USR magnet (software version Paravision 5.1) using a Bruker volume coil (RF RES 300 1H 075/040 QSN TR) and a balanced steady state free precession (bSSFP) pulse sequence. MRI phantoms consisted of two pairs of 400 µl vials containing either 1 mM of Gd$_2$L-Av in HEPES buffer or HEPES buffer alone, both with and without Ca$^{2+}$. Imaging parameters were field-of-view (FOV)= 25 x 25 mm, matrix size (MTX) 125 x 125, slice thickness 1 mm, flip angle (FA) = 65 °, repetition time (TR) = 2.2 ms, echo time (TE) = 1.1 ms, number of excitations (NEX) = 20, total acquisition time (TA)=23 s 222 ms.
NMR spectra of new compounds

Compound 2
Compound BB1
Compound 4
Compound 5
Compound BB2
Compound L
HRMS spectra of new compounds

Compound 2

Compound BB1
LC-MS data of intermediate solid-phase products

Compound 6-x

Compound 7-x
HPLC data

Compound L

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