Supporting Information

De Novo Design of Ln(III) Coiled Coils for Imaging Applications

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Materials and Methods, one Supplementary Table and ten Supplementary Figures.
1. Materials and Methods

Peptide synthesis and purification.

All peptide synthesis reagents were purchased from AGTC bioproducts Ltd. Peptides were synthesized on a CEM Liberty 1 automated peptide synthesizer on Rink amide MBHA resin (0.25 mmol scale), using standard Fmoc-amino acid solid-phase peptide synthesis protocols and purified and characterized as previously reported, see Figure S1. Peptide concentrations were determined based on the tryptophan absorbance at 280 nm ($\varepsilon_{280\text{ nm}} = 5690 \text{ M}^{-1}\text{cm}^{-1}$) in 7 M aqueous solutions of guanidinium hydrochloride.

Circular Dichroism (CD) Spectroscopy.

CD spectra were recorded in 1 mm pathlength quartz cuvettes on a Jasco J-715 spectropolarimeter. The observed ellipticity in millidegrees was converted into molar ellipticity, ($\Theta$), and is reported in units of deg dmol$^{-1}$ cm$^2$ res$^{-1}$. The % folding was calculated based on the theoretical maximum ellipticity value -39054 deg dmol$^{-1}$ cm$^2$ res$^{-1}$ for our coiled coil, based on reports by Scholtz et al. 3

Aliquots of a 1 mM stock solution of GdCl$_3$/TbCl$_3$, and a 1 M stock solution of CaCl$_2$ were titrated into either a 100 or 30 μM solution of MB1 peptide monomer in 5 mM HEPES buffer pH 7.0 and the CD spectra recorded after 20 min equilibration. Sedimentation equilibrium experiments could be best fit for a simple monomer-to-trimer model in the presence of Gd(III) (vide infra) and so the CD titration data was fit to the following model, $\frac{1}{3}\text{Ln(III)} + \text{MB1} \leftrightarrow \frac{1}{3}(\text{Ln(III)}\text{MB1}_3)$, where the dissociation constant ($K_d$), the inverse of the association constant ($K_a$), can be defined by equation (1).

$$K_d = \frac{[\text{MB1}][\text{Ln(III)}]^{1/3}}{[\text{Ln(III)}\text{MB1}_3]^{1/3}}$$

(1)

In which [MB1] is the free peptide concentration, [Ln(III)] is the free metal concentration and [Ln(III)(MB1)$_3$] is the lanthanide coiled coil complex concentration. This can be expressed as the total metal concentration, [Ln(III)]$_T$, and the total peptide concentration, [MB1]$_T$, using the fraction folded, $f$, see equation (2).
Where the fraction folded, \( f \), is related to the observed molar ellipticity, \( \theta_{\text{obs}} \), molar ellipticity of metal free peptide, \( \theta_{\text{free}} \), and molar ellipticity of metal saturated peptide, \( \theta_{\text{saturated}} \), all at 222 nm, see equation (3).

\[
\frac{K_d}{f_{\text{MB1}}} = \frac{([\text{MB1}]_T - f_{\text{MB1}}) ([\text{Iln(III)}]_T - \frac{f_{\text{MB1}}}{3})^{1/3}}{\left(\frac{f_{\text{MB1}}}{3}\right)^{1/3}}
\] (2)

Equation (2) can be rearranged to determine the dissociation constant using a non-linear least-squares fitting, solving for total metal concentration, using Kaleidagraph (Synergy Software), see equation (4).

\[
f = \frac{\theta_{\text{obs}} - \theta_{\text{free}}}{\theta_{\text{saturated}} - \theta_{\text{free}}}
\] (3)

\[
[\text{Iln(III)}]_T = f \left( \frac{K_d^3}{3 ([\text{MB1}]_T)^2 (1-f)^3} \right) + \frac{[\text{MB1}]_T}{3}
\] (4)

Thermal unfolding data was recorded by monitoring the ellipticity at 222 nm of a 30 μM solution of peptide monomer in 5 mM HEPES buffer pH 7.0 in the absence and presence of 10 μM GdCl₃, using a Jasco Peltier Type PTC-4235 temperature controller ramping from 20 °C to 90 °C at a rate of 0.2 °C min⁻¹.

Analytical Ultracentrifugation.

**Methods:** Equilibrium sedimentation experiments were performed using a Beckman Coulter Proteome Lab XL-I analytical ultracentrifuge with an An-50 Ti rotor and equilibrium cells. Samples of monomer MB1 were run with absorbances of 0.75 (130 μM), 0.50 (87.9 μM) and 0.25 (43.9 μM) in the absence and presence of ⅓ equivalence of GdCl₃ in 10 mM HEPES buffer pH 7.0 at 20 °C, against a reference cell containing buffer. The samples were recorded at 3 rotor speeds (34000, 36000 and 38000 rpm) each with a 20 hour equilibration followed by 4 scans at 280 nm taken at 1 hour intervals. For each speed the 4 scans were then analyzed to ensure that the run had reached equilibrium. Values for the molecular mass and
equilibrium constants were then determined using SEDPHAT.\textsuperscript{4} The specific partial volume was taken to be 0.7571 and the buffer density was approximated to 1.00 using SEDNTERP software (Biomolecular Interaction Technologies Centre, New Hampshire, USA).

Results: In the absence of Gd(III) the mass obtained from a simple fit assuming single species is slightly higher than would be expected (4733 as compared to monomer mass of 4004 amu). This is indicates a small amount of self-association in the system even in the absence of the Gd(III). To assess this further the data was fit to a monomer-to-trimer model. This provides a fit that is at least as good as that for the single species fit, however, close examination of the $K_a$ for these fits shows the interaction to be very weak (10\textsuperscript{−1.5} M\textsuperscript{−1}), consistent with the requirement for Gd(III) to drive oligomerisation.

In the presence of Gd(III) (Figure S3) an equivalent fit to a single species model produces a mass which is significantly above that expected for the dimer (9291 amu). These data suggest that the ligand bound complex is likely to contain a significant quantity of trimer. However the observation that the mass is not that of a trimer indicates that the solution contains an equilibrium between a number of species. To assess this, these data were best fit to a monomer-to-trimer equilibrium model with the major species now the trimer. This model fits well to the data and provides an association constant of 67608297 M\textsuperscript{−2}. To assess whether other oligomerisation states could explain the behavior of the peptide in the experiment, other models were tested including those describing monomer-to-dimer and monomer-to-tetramer equilibriums. Neither of these models fit these data as well as that for the monomer-to-trimer equilibrium (see Table S1).

We are aware that the simple monomer-to-trimer model does not perfectly reflect the proposed assembly mechanism as it does not include the Gd(III). To address this, a more complex stepwise addition model was used to fit these data:

\[\text{Ln(III)} + 3\text{MB1} \leftrightarrow \text{Ln(III)(MB1)} + 2\text{MB1} \leftrightarrow \text{Ln(III)(MB1)}_2 + \text{MB1} \leftrightarrow \text{Ln(III)(MB1)}_3\]
This model also fits to these data, but with a chi$^2$ value that is slightly worse than that for the simple monomer-to-trimer model (9.4 for the stepwise model compared to 8.3 for the simple monomer-to-trimer). This stepwise model suggests an $K_{a1}$ association constant for Gd(III) to the first MB1 of 47863 M$^{-3}$ (see Table S1).

Overall these data confirm that the presence of Gd(III) mediates the formation of the Gd(MB1)$_3$ trimer. The data best fits to a single step trimerisation, although within the limits of the data the more complex sequential assembly cannot be ruled out.

**Mass Spectrometry.**

Peptides were diluted in 10 mM ammonium acetate to a concentration of 7.38 pmol/µl monomer (2.46 pmol/µl trimer) and 10 pmol/µl GdCl$_3$ or TbCl$_3$. The pH of these solutions were identified to be between 6.5 and 7.0. 10 µl of the sample was collected and sprayed by the use of an Advion Biosciences TriVersa NanoMate electrospray source (Ithaca, NY, USA) into a Thermo Fisher LTQ Orbitrap Velos mass spectrometer (Bremen, Germany). Mass spectra were acquired in the orbitrap mass analyser at a resolution of 100,000 at $m/z$ 400 and comprised of 30 scans each comprising of 4 co-added microscans.

**Luminescence.**

Emission spectra were recorded in a 1 cm pathlength quartz cuvette using an Edinburgh Instruments Fluorescence FLS920 system with a 450 W Xenon arc lamp and a Hamamatsu R928 photomultiplier tube. The emission monochromator was fitted with two interchangeable gratings blazed at 500 nm and 1200 nm and the data was collected using F900 spectrometer analysis software. Aliquots of a 1 mM stock solution of TbCl$_3$ were titrated into a 27.6 µM solution of peptide monomer in 10 mM HEPES buffer pH 7.0, and the emission profile recorded after 20 min equilibration. Solutions were excited at 280 nm and the emission was scanned from 305-450 nm (Trp region) or 475-750 nm (Tb(III) region) using 305 and 455 nm long pass filters, respectively. Spectra were corrected for instrument response (grating/PMT) in all cases. Tb(III) luminescence titration data was fit using the same methodology as described for the analogous CD titration (vide supra), where $f$ now
corresponds to the fraction bound and is related to the observed emission intensity, $I$, emission intensity of metal free peptide, $I_0$, and emission intensity of metal saturated peptide, $I_{saturated}$, integrated between 530-560 nm, see equation (5).

$$ f = \frac{I - I_0}{I_{saturated} - I_0} $$

Tb(III) lifetimes in D$_2$O and H$_2$O were determined for Tb(MB1)$_3$ by monitoring solutions containing 10 μM Tb(III) and 100 μM MB1 (to ensure ≥99% of the Tb(III) is bound) in 10 mM HEPES buffer pH 7.0 using a μF Flashlamp light source (50 Hz) on the Edinburgh Instruments spectrofluorimeter, collecting over a 10 ms time range, with a lamp trigger delay of 0.1 ms. Data was fitted to mono-exponential decay kinetics in Kaleidagaph using the Marquardt-Levenburg linear least squares algorithm and from the observed lifetime the number of coordinated water molecules was determined using the Horrocks-Sudnik and Parker-Beeby equations.$^5,6$

**NMR Spectroscopy and Imaging.**

Data was collected on a Bruker DMX 300 spectrometer equipped with a 7 T vertical wide-bore superconducting magnet operating at a proton resonance frequency of 300.13 MHz with a 25 mm RF bird cage coil. The 90° (ca. 45 μs) and 180° (ca. 90 μs) radiofrequency pulses were calibrated for each sample. The spectrometer was controlled by a Silicon Graphics workstation operating XWIN-NMR version 3.5 and ParaVision version 2.1 software. All experiments were recorded at 293 K. The $T_1$ relaxation time was measured using an inversion recovery pulse sequence, $[180°-\tau-90°-\text{acq}]_n$, performing $n = 20$ experiments with logarithmically spaced variable delay times between $5 \times 10^{-6}$ and 16 s. $T_2$ relaxation times were collected using CPMG pulse program, $[90°-(\tau-180°-\tau)-\text{acq}]_m$, $n = 17$ experiments with echoes $m$ ranging between 0 – 4096 with a delay time of 2 ms. A repetition time of 20 s was used for all experiments. The signal attenuation was acquired as a function of time. All NMR experiments were performed in triplicate and were analyzed using Prospa software (Magritek, Wellington, New Zealand). The decay curves were fit with logarithmically spaced points up
to five times the relaxation time, avoiding inclusion of excessive zero values. Solutions containing 10, 25, 50, 75 and 100 µM Dotarem® (from Guerbet UK) were prepared in 10 mM HEPES buffer pH 7.0 from a 0.5 M stock solution. The relaxation rates of Gd(MB1)$_3$ was determined from solutions containing 50, 75, 100, 125 and 150 µM GdCl$_3$ and 6 equivalences of MB1, prepared in 10 mM HEPES pH 7.0. The higher concentrations and excess of peptide (6 monomers per Gd(III)) was used to ensure that >99% of Gd(III) was bound at the lowest concentration (50 µM), see Figure S4.

$T_1$ and $T_2$ maps of water protons in phantom samples prepared in 5 mm NMR tubes and shown in Figures 4 and S10, were acquired using a RARE (Rapid Acquisition with Relaxation Enhancement)$^7$ spin-echo imaging sequence. Horizontal images were acquired with a 1 mm slice thickness, using a $25 \times 25$ mm field-of-view and a $64 \times 64$ ($T_1$ map) or $128 \times 128$ ($T_2$ map) pixel matrix. A repetition time of $T_R = 5$ s was used. $T_1$ relaxation maps were produced from a series of 9 spin echo images with varying $T_1$ inversion recovery delays from 6 - 3500 ms and RARE factor of 1. $T_2$ relaxation maps were produced from 8 echo images with echo times from 103 – 1516 ms and a RARE factor of 64.

**Molecular Dynamics Simulations.**

The Gd(III)-coiled coil model was built using Insight II (Biosym/MSI, San Diego, California), modifying amino acids from the previously published three-stranded coiled-coil structure 3H5F$^8$ and solvated with a water box at least 15 Å larger than the peptide in all dimensions. As the Gd(III)-coiled coil is neutral, no counterions were required. Minimization and molecular dynamic simulations were carried out using AMBER v8.0$^9$ with the ff03$^{10}$ forcefield, with additional parameters for Gd$^{3+}$. Gd$^{3+}$ ions were treated as simple spheres with a charge of +3 and Van der Waals parameters (MOD4 RE format in AMBER) $r = 1.7131$ (radius) and $e = 0.459789$ (well depth). Prior to data-gathering dynamics, the peptide was subjected to 100000 cycles of conjugate gradient minimization and 500 ps equilibration dynamics at 300 K, with constant volume and periodic boundary conditions. Data gathering ran for 10.0 ns also at 300K, with constant volume and periodic boundary conditions, taking snapshots every 10 ps.
2. Figure S1:

Figure S1. A) Analytical reverse phase C18-HPLC chromatograph of pure MB1 (retention time = 19.4 minutes) using H$_2$O/MeCN gradient (0 – 100% MeCN over 40 minutes) in the presence of 0.1% TFA. B) Electrospray mass spectrum showing charge envelope of purified MB1 (mass = 4004 amu).
3. Figure S2:

Figure S2. Electrospray mass spectra of 7.38 pmol/µl MB1 monomer in the presence of 10 pmol/µl (a) GdCl₃ or (b) TbCl₃, in 10 mM ammonium acetate buffer. Though the main species detected corresponds to the monomer, the intact Ln(MB1)₃ trimer species could be detected, whereas no other oligomeric states were observed.
4. Figure S3:

**Figure S3.** Data from the sedimentation equilibrium experiment used to study the assembly of Gd(III) and MB1. *(top)* The distribution of the protein within the AUC cell at each of the 3 speeds used in data analysis (34000, 36000 and 38000 rpm) and at 3 different protein concentrations (130 µM, 87.9 µM and 43.9 µM in the presence of 1/3 equivalence of GdCl$_3$). Shown are the raw data and the fits produced using the simple monomer-to-trimer model in SEDPHAT. The residuals for each fit are shown in the lower figure.
Table S1: The models within SEDPHAT used to fit the two datasets from the sedimentation equilibrium experiments. The minimum Chi^2 produced from each fit is provided alongside the constants produced from that fit.

| Model fitted | Dataset | Chi^2 | Parameters determined from the fit |
|--------------|---------|-------|-----------------------------------|
| M            | No Gd   | 4.8   | Mass of 4733                      |
| M ↔ Trimer   |         | 4.7   | log_{10} K_{a1-3} = -1.50        |
| M ↔ Dimer    |         | 4.72  | log_{10} K_{a1-3} = -0.92        |
| M            | Gd      | 8.9   | Mass of 9291                      |
| M ↔ Dimer    |         | 14.3  | log_{10} K_{a1-2} = 4.36         |
| M ↔ Trimer   |         | 8.3   | log_{10} K_{a1-3} = 7.83         |
| M ↔ Tetramer |         | 649   | log_{10} K_{a1-3} = 5.64         |
| Gd+M ↔ Gd(M)+M ↔ Gd(M)_2+M ↔ Gd(M)_3 | | 9.4 | Log_{10} K_{a1} = 4.68
Log_{10} (K_{a2}/K_{a1}) = -0.84
Log_{10} (K_{a3}/K_{a1}) = -0.15 |
Figure S4: Plot showing the percentage of Gd(III) bound as Gd(MB1)$_3$ based on the experimental association constant (log $K_a$ 5.11) at 10, 50 and 100 µM Gd(III). Percentage bound calculated using ChemEQL.
7. Figure S5:

Figure S5. CD thermal unfolding of 30 μM MB1 in the absence (blue circles) and presence of 10 μM GdCl₃ (red circles) in 5 mM HEPES buffer pH 7.0, monitored by the molar ellipticity at 222 nm.
8. Figure S6:

Figure S6. Absorption (red) and excitation (blue) spectra of a solution containing 10 μM TbCl₃ and 30 μM MB1 in 5 mM HEPES buffer pH 7.0, with λₚₑᵣₚ = 545 nm. Excitation spectrum is corrected for lamp response.
9. Figure S7:

**Figure S7.** CD spectra for the apo peptide and Gd(III)-coiled coil, in 5 mM HEPES buffer pH 7.0, in the absence and presence of 10 mM Ca(II). A 30 μM solution of MB1 (red) remains largely unchanged on addition of 10 mM CaCl₂ (blue). Similarly no significant change if observed to the CD profile of a solution containing 10 μM GdCl₃ and 30 μM MB1 (green) on addition of 10 mM CaCl₂ (purple).
10. Figure S8:

Figure S8. Emission spectra of Tb(MB1)₃ in 5 mM HEPES buffer pH 7.0, in the absence and presence of Ca(II). A solution of 10 μM TbCl₃ and 30 μM MB1 (red) remains largely unchanged on addition of 10 mM CaCl₂ (blue) after a 20 minute equilibration mixing time.
**11. Figure S9:**

*Figure S9.* Relaxivity plot showing reciprocal of relaxation time as a function of Gd(III) concentration, for $T_1$ relaxation rates Gd(MB1)$_3$ (blue diamonds) and Dotarem® (green triangles), and $T_2$ relaxation rates Gd(MB1)$_3$ (red squares) and Dotarem® (purple circles). Gd(MB1)$_3$ solutions are at higher concentrations and contain excess peptide (6 equivalents MB1 per Gd(III)), so as to ensure that >99% of Gd(III) is present as Gd(MB1)$_3$. Data recorded at 293 K in 10 mM HEPES buffer pH 7.0 at 300 MHz, and relaxivity values calculated from gradient.
**12. Figure S10:**

![Figure S10](image)

**Figure S10.** $T_1$ map of phantom samples containing 100 µM Gd(III) in 10 mM HEPES buffer pH 7.0. Samples a-h contain increasing concentrations of MB1; 0.0 (a), 0.1 (b), 0.2 (c), 0.3 (d), 0.4 (e), 0.6 (f), 0.8 (g) and 1.0 equivalence of peptide trimer (h).
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