The metal hydration state within a designed coiled coil can be progressively tuned across the full integer range (3 → 0 aqua ligands), by careful choice of a second sphere terminal residue, including the lesser used Trp. Potential implications include a four-fold change in MRI relaxivity when applied to lanthanide coiled coils.

There is much interest in engineering metal sites within designed miniature protein scaffolds, as they allow us to mimic biologically relevant metal sites, recreate their function, and most excitingly, achieve new function. To fully realize this potential one needs to predictably program in any desired coordination features truly de novo. Nature achieves this with highly evolved sophisticated ligands, proteins, which exploit preorganization and steric in both first and second coordination spheres, to realize this goal.

Promising designs often feature coiled coils, which offer the advantages of protein-like ligands but a simple robust scaffold. Second coordination sphere residues have been used to preorganize first coordination sphere ligands through favourable hydrogen bond formation. Of direct relevance to this work, the steric bulk of second coordination sphere residues has been used to modulate active site access and coordination chemistry, including hydration state. Furthermore, second coordination sphere effects have been found to be important in molecular inorganic chemistry, including, but not limited to, controlling catalysis, interactions with biological targets (which surprisingly receives little attention), and of relevance to the work presented herein, altering the stability, luminescent, or magnetic properties of lanthanide complexes. Despite the importance of second coordination sphere residues in molecular inorganic chemistry and biology, there has been no systematic study of second coordination sphere effects to control designed metal site hydration states beyond one water molecule within coiled coils. This is despite the control of hydration state being a key challenge in metalloprotein design, given how crucial it is for function (e.g. substrate binding, catalysis, MRI relaxivity). Herein we undertake the first such systematic study, and report for the first time that designed metal site hydration states (herein demonstrated for lanthanides) can be programmed to contain three, two, one or no bound water molecules, by selection of a terminal second coordination sphere residue.

We recently reported the first example of a gadolinium coiled coil and its promising MRI relaxivity. The hydration state of the bound lanthanide, which strongly correlates with MRI relaxivity, and as such represents a good model with which to investigate hydration state control, was sensitive to its linear location along the coiled coil. Terminal sites were highly hydrated, whereas sites buried centrally within the lipophilic core were coordinatively saturated. This study starts with the highly hydrated Ln(OH2)3 site generated with MB1-1(2I), as a model with which we can explore hydration state tuning (as a general design principle), over a broad range. The MB1-1(2I) nomenclature refers to the MB1 binding site which features AsnAsp in adjacent layers, towards the N-terminus (1), with Ile (2I) in the non-coordinating second sphere terminal a layer above the binding site (position 2). The terminal second sphere a residue identity was systematically altered (Table 1, Fig. 1 and Fig. S1–S5, ESI†), and its impact on metal hydration state assessed.

It was important to establish whether altering the nature of this terminal second sphere a residue impacts its ability to bind the metal. Circular dichroism (CD) showed that the five apo MB1-1(2X) peptides were all well-folded, and that titration with Gd3+ led to a further signal enhancement at 222 nm, an indication of z-helicity, with binding curves in agreement with data previously reported for MB1-1(2I) (Fig. 2 and Fig. S7, ESI†). Furthermore, intact Tb(Pep) complexes were observed by mass spectrometry (Fig. S8, ESI†). Taken together these data are consistent with Ln3+ binding being retained on altering the second sphere layer.

Whether this terminal second sphere a residue can be used to tune the coordination chemistry of the bound Ln3+ was investigated. The hydration state of the Tb3+ complexes was

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† Electronic supplementary information (ESI) available. See DOI: 10.1039/c9cc08189e
determined by luminescence lifetime decay experiments in H$_2$O/D$_2$O (Fig. S9, ESI†). Our original design, which featured an Ile as the second sphere residue generates a highly hydrated site with three waters bound to the Tb$^{3+}$. When replacing the relatively bulky Ile with the sterically undemanding Ala, did not increase the hydration state, as may have been expected.8,9 This suggests when the double AsnAsp binding site is presented, that the maximum Tb$^{3+}$ hydration state is three. Though we previously found evidence of 3.6 waters bound when only the Asp layer is available.15

When the second sphere residue is Phe, the Tb$^{3+}$ hydration state is reduced to two bound waters, which could be due to greater steric of the benzyl side chain. On incorporation of Tyr, the data is consistent with only one bound water. However, care needs to be taken when interpreting this result, as Tyr sensitization induces a weaker Tb$^{3+}$ emission signal and the Tyr side chain could: (1) be coordinating directly to the Tb$^{3+}$; or (2) the OH group could be contributing to quenching of the Tb$^{3+}$ emission. Finally, the introduction of Trp (its close proximity efficiently sensitizes Tb$^{3+}$ emission), yields a coordinatively saturated Tb$^{3+}$ with no evidence of bound exogenous water molecules (Table 2 and Table S1, ESI†).

This would be consistent with Tb$^{3+}$ bound through both the Asn and Asp side chains (see Fig. 1).

Intriguingly detection of the Tb(Pep)$_3$ +6 ions in the mass spectra appear to be indirectly correlated with hydration state, with the most intense peak observed for Tb(MB1-1(2W))$_3$, which could be indicative of the formation of a more stable site (Fig. S8, ESI†).

### Table 1 Peptide sequences

| Peptide   | Sequence (N→C terminus) |
|-----------|-------------------------|
| MB1-1(2A) | AC-G AANAEWK DAAIEQK IAAIEQK IAAIEQK G-NH$_2$ |
| MB1-1(2I) | AC-G IAANAEWK DAAIEQK IAAIEQK IAAIEQK G-NH$_2$ |
| MB1-1(2F) | AC-G FAANAEWK DAAIEQK IAAIEQK IAAIEQK G-NH$_2$ |
| MB1-1(2Y) | AC-G YAANAEQK DAAIEQK IAAIEQK IAAIEQK G-NH$_2$ |
| MB1-1(2W) | AC-G WAANAEQK DAAIEQK IAAIEQK IAAIEQK G-NH$_2$ |
| MB1-2     | AC-G IAAIEQK IAANEWK DAAIEQK IAAIEQK G-NH$_2$ |
| MB1-4     | AC-G IAAIEQK IAAIEQK IAAIEQK DAAIEQK G-NH$_2$ |
| MB1-4(37W)| AC-G IAAIEQK IAAIEQK IAAIEQK IAAIEQK DAAIEQK WG-NH$_2$ |

Relevant residues are bold and underlined. Sequences contain a single Trp/Tyr residue (see footnote ‡).
Replacing the terminal second sphere Ile with Trp converts a highly hydrated Ln(OH)₃ site into a coordinatively saturated site, which better resembles buried sites located centrally in the coiled coil (e.g. MB1-2). This profound change in coordination chemistry should be mirrored by the MRI properties of the Gd³⁺ complex. The longitudinal (r₁) and transverse (r₂) relaxivity of the Gd(MB1-1(2W))₃ complex at 7 T (Fig. S10, ESI†) and Table 2: r₁ = 3.9 ± 0.4 mM⁻¹ s⁻¹ and r₂ = 24.2 ± 2.4 mM⁻¹ s⁻¹ are the same within error to those reported previously for Gd(MB1-2)₃ (Table 2), consistent with no bound water molecules (Table 2). Merely changing the non-coordinating second sphere residue directly above the Gd³⁺ binding site, Ile → Trp, leads to an almost four-fold reduction in transverse relaxivity.

One can account for this dramatic change in Ln³⁺ hydration state by considering the crystal structure of a comparable coiled coil with a similar Trp layer (state by considering the crystal structure of a comparable coiled coil (Fig. S11, ESI†)). These results suggest that a terminal second sphere residue directly above the Gd³⁺ binding site, Ile → Trp, leads to an almost four-fold reduction in transverse relaxivity.

To establish if this strategy could be applied more widely, we considered the crystal structure of a comparable coiled coil and protein designers more widely, for the truly de novo design of any desired metal site, the ultimate goal of metalloprotein design. This study has for the first time demonstrated that it is possible to truly control the hydration state of metal binding sites (from three, two, one and zero waters), by changing a terminal second sphere residue. With metal hydration often dictating the resulting chemistry, herein demonstrated with MRI, but also key for substrate access, “vacant” coordination sites, and catalysis more widely, this is a crucial goal that we have now realized. These findings will be of use to metallo-coiled coil and protein designers more widely, for the truly de novo design of any desired metal site, the ultimate goal of metalloprotein design.

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Conflicts of interest

There are no conflicts to declare.

Notes and references

† MB1-1(2Y), MB1-1(2F) and MB1-4(37W) feature Gln in place of the external Trp residue (f position), so as to retain a single UV absorbing residue in the sequence.

‡ Tb³⁺ and Gd³⁺ are routinely used interchangeably given their almost identical coordination chemistry. Herein we take advantage of the visible emission afforded by Tb, but not Gd, to interrogate the binding site.
One Trp has been solved as being in two equal occupancy conformations, but only one conformation is shown here. Coiled coils are based on the heptad repeat HxxHxxc, where H corresponds to a hydrophobic amino acid, and the relative position within the heptad is referred to as abcd ef g. Both a and d residues are located within the hydrophobic core of a three stranded coiled coil, but their side chains are directed differently relative to the helix backbone.

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