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PAPER

Luminescent lanthanide-binding peptides: sensitising the excited states of Eu(III) and Tb(III) with a 1,8-naphthalimide-based antenna†

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The investigation into the luminescence properties of a lanthanide-binding peptide, derived from the Ca-binding loop of the parvalbumin, and modified by incorporating a 1,8-naphthalimide (**Naph**) chromophore at the N-terminus is described. Here, the **Naph** is used as a sensitising antenna, which can be excited at lower energy than classical aromatic amino acids, such as tryptophan (the dodecapeptide of which was also synthesised and studied herein). The syntheses of the **Naph** antenna, its solid phase incorporation into the dodecapeptide, and the NMR investigation into the formation of the corresponding lanthanide complexes in solution is presented. We also show that this **Naph** antenna can be successfully employed to sensitize the excited states of both europium and terbium ions, the results of which was used to determined the stability constants of their formation complexes, and we demonstrated that our peptide 'loop' can selectively bind these lanthanide ions over Ca(II).

Introduction

The use of lanthanide(III) complexes in structural analysis,[1–3] for probing supramolecular interactions,[4,5] or in various biomedical applications[5–9] is of great current interest in chemistry. This is in particularly due to the unique magnetic and photophysical properties that these ions possess.[10] Gd(III) complexes and conjugates have been employed as MRI relaxation contrast agents, while, with the exception of La(III) and Lu(III), most of the remaining lanthanides have unique luminescent properties, which have been explored in the development of various luminescent supramolecular devices. This includes the development of luminescent switches, sensors, logic gate mimics, imaging agents, *etc*. using ions such as Eu(III) and Tb(III), which emit in the visible ranges,[11–13] whereas ions such as Nd(III), Yb(III) emit in the near infra-red; an area of particular interest for biological, and telecommunication applications.[14–16] The main advantages of using these ions in the development of luminescent probes and devices is attributed to their long-lived excited states and line-like emission bands which occurs at long wavelengths. These features overcome interferences from short-lived background fluorescence and light scattering from biological matter.[17] As these are Lapporte forbidden transitions, these excited states are most effectively populated by using sensitizing antennae, often embedded, or conjugated, *via* short spacers into macrocyclic structures.[18] In particular, the use of polyaminocarboxylate based ligands, such as cyclen, has been widely investigated for such applications.[19–27] In contrast, the use of peptides that can be synthetically modified with such sensitizing antennae, and possess donor moieties that can enable the binding of lanthanide ions, has been much less explored. Such systems could be of significant value, particularly for use in biological media, for instance, with the view of generating novel luminescent protein mimics, employed to investigate protein-protein or protein-substrate interactions.

Due to the similar ionic radii of Ca(II), the lanthanides have been used as substitutes for Ca(II) ions in various Ca-binding peptides and proteins,[28–31] or with the aim of developing luminescent peptides.[32,33] For the latter, such structures usually possess amino acids with aromatic residues, such as tryptophan, tyrosine and phenylalanine, which can be used to sensitize the excited state of Tb(III).[34] Recently, this area of research has attracted significant attention, particularly through the work of Imperiali *et al.*, who has developed lanthanide-binding tags appended to various proteins (LBTs).[34,35] These have been used to elucidate protein structure,[3,36] detect protein-protein interactions and to phase X-ray crystallographic data.[37] Some other examples within this area of research includes that of Vázquez *et al.*,[38] who developed novel sensors for investigating protein-substrate interactions, Franklin *et al.*[39–40] who investigated the DNA binding affinity and hydrolysis of such lanthanide based peptides. Recently, Ito *et al.*[41] have also developed lanthanide complexes containing the cyclic peptide c(RGDfK) to visualize the α(v)β(3)-integrin-expressing tumor cells, while Delangle *et al.*[42] have developed Gd(III)-based complexes of cyclic decapeptide with two independent faces as "regioselectively addressable functionalised templates" (RAFTs).

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All the above examples have been formed by using natural occurring amino acids, and while these complexes are clearly useful for *in vitro* studies, there are intrinsic limitations for their use *in vivo*. Firstly, the stability of the lanthanide-peptide complexes formed are limited, as such peptides bear only simple binding groups such as carboxylates, phenolates, or amides. Secondly, only few of these amino acids can be employed as antennae. Consequently, modified amino acids have to be used to introduce strong donor groups for reinforcing metal complexation[43,44] or to improve the luminescence properties.[45,46]

We have recently reported preliminary results for the use of 1,8-naphthalimide (**Naph**) structures to sensitize the excited states of both Eu(III) and Tb(III).[47,48] This sensitizing antenna is particularly attractive as it can be excited at lower energy (345 nm), and by simple synthetic modifications, the photophysical properties can be easily tuned. Hence, we have employed this structure in our laboratory in various supramolecular systems and devices.[49,50] Herein, we give full account of the use of this antenna within peptide structures designed to form stable complexes with lanthanide ions for use in biological applications.

The peptide sequence chosen for this investigation is shown in Fig. 1 as the dodecapeptide **P1**; designed on the Ca(II) binding loop of the parvalbumin protein, an amino acid sequence which includes three Glu and three Asp amino acids; each one able to provide a chelating carboxylate residues for binding to Ln(III). In **P1**, the **Naph** antenna, the synthesis of which is shown in Fig. 1, was incorporated at the N-terminus of the peptide. Furthermore, we also developed **P2** for use in comparison studies. This system lacks the **Naph** antenna, but has instead, a Trp moiety that can be employed as such an antenna.

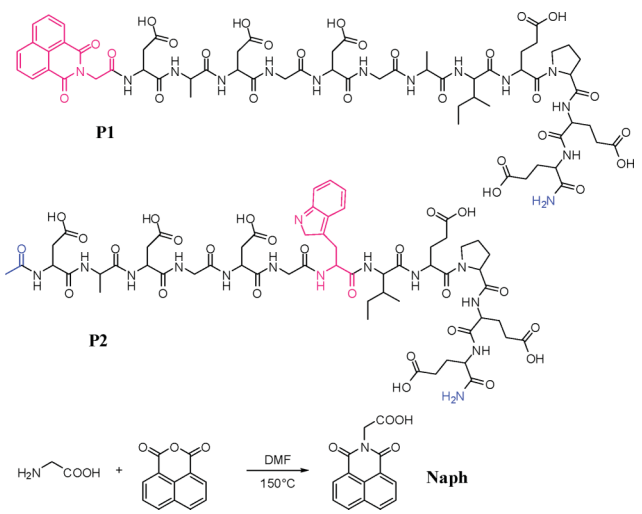

Fig. 1 The two dodecapeptides **P1** and **P2** developed in this study and the synthesis of the **Naph** antenna that was incorporated into **P1**.

Results and discussion

Syntheses of the dodecapeptides P1 and P2

The dodecapeptide sequence **DADGDGWIEPEE** of the model peptide **P2** was synthesised using standard manual solid-phase synthesis based on Fmoc/^{t}Bu protection strategy.[51] The peptide was cleaved off the resin and the side chains were deprotected using a mixture of TFA/TIS/H_2O 95/2.5/2.5, and purified by reverse-phase HPLC, and characterised by Maldi-Tof MS (See ESI).

The synthesis of the **Naph** derivative is shown in Fig. 1, and was formed in one step by reacting glycine (1.2 eq.) with naphthalic anhydride (1 eq.) in DMF at 150 °C over 24 h. The resulting brown oil was purified by precipitation from methanol to give a white solid in 56% yield (see Experimental section).

The peptide sequence **DADGDGAIEPEE** was synthesised using standard automated solid phase peptide synthesis according to the Fmoc/^{t}Bu strategy and HBTU-HOBt-DIEA coupling chemistry in NMP. The **Naph** antenna was then coupled manually at the N-terminus of the peptide sequence using a double coupling procedure. The resulting modified polymer-bound peptide **Naph**-Asp(^{t}Bu)-Ala-Asp(^{t}Bu)-Gly-Asp(^{t}Bu)-Gly-Ala-Ile-Glu(^{t}Bu)-Pro-Glu(^{t}Bu)-Glu(^{t}Bu) was then cleaved from the resin and the side chains were deprotected using TFA/thioanisole/TIS/EDT/H_2O (v/v/v/v/v = 77/5.75/5.75/5.75/5.75), and **P1** was finally purified by reverse-phase HPLC, and characterised by their ESMS (see ESI†).

Photophysical evaluation of P1 and P2

The photophysical properties of **P1** and **P2** were investigated in 10 mM HEPES buffer solution in the presence of 0.1 M NaCl, to maintain a constant ionic strength. The UV-Vis absorption, the fluorescence emission and the excitation spectra, were recorded both in the absence, as well as in the presence of *one* equivalent of either Eu(III) or Tb(III).

The UV-vis absorption spectrum of **P1** was dominated by a broad band centred at 344 nm ($\log \varepsilon = 4.04$) which was assigned to the n–π^* transition of the **Naph** antenna (see Fig. S3, ESI†). Excitation into this band gave rise to a fluorescence emission typical of such **Naph** moieties, with λ_{max} = 393 nm. In analogous manner, the Trp-antenna-based peptide **P2** was analysed in buffered solution, and the UV-vis absorption spectrum showed a broad band with λ_{max} at 280 nm, which had the typical Trp structure. Excitation of this band gave rise to the characteristic Trp emission centred at 334 nm.

Upon addition of one equivalent of $TbCl_3$ or $EuCl_3$ to **P1**, no significant changes were seen in the UV-vis absorption spectra of **P1**, but the characteristic emission spectra of Eu(III) or Tb(III) ions were observed as shown in Fig. 2a and 2c respectively. This emission is most likely due to an energy transfer mechanism occurring from the **Naph**, to the Eu(III) and Tb(III) excited states. This was confirmed by recording the fluorescence excitation spectra of both systems in the presence of Eu(III) and Tb(III), by fixing the emission at 616 nm ($^5D_4 \rightarrow {}^7F_2$ transition) and 544 nm ($^5D_0 \rightarrow {}^7F_5$ transition), respectively (see ESI, Fig. S4–S5†). The excitation spectra were found to be structurally similar to the absorption spectra of these systems, confirming the successful energy transfer from these antennae to the Eu(III) and Tb(III) excited states. Additionally, in the case of Tb(III), a new band centred at 270 nm was observed and attributed to the formation of a 4f–5d transition (which could be MLCT in nature).[52,53] In a similar manner, **P2** was analysed and similar results were observed to those described above (see ESI, Fig. S7–S9†). As expected, **P2** was also able to sensitise the Tb(III) excited state, and the relative intensity of the emission at 544 nm was significantly greater than

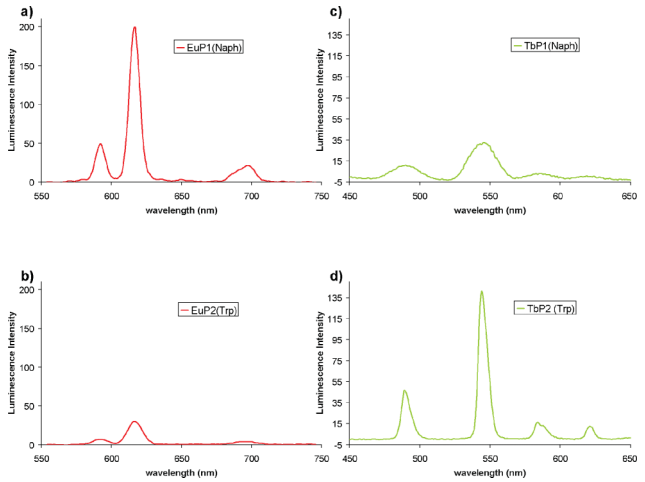

Fig. 2 (a) Emission spectra of **P1** (30 μM) in the presence of Eu(III) (17 μM) with excitation of **Naph** at 345 nm (slit width 5 nm for excitation and emission). (b) Emission spectra of **P2** (30 μM) in the presence of Eu(III) (17 μM) with excitation of Trp at 280 nm (slit width 20 nm for excitation and 10 nm for emission). (c) Emission spectra of **P1** (30 μM) in the presence of Tb(III) (17 μM) with excitation of **Naph** at 345 nm (slit width 20 nm for excitation and emission). (d) Emission spectra of **P2** (30 μM) in the presence of Tb(III) (17 μM) with excitation of Trp at 280 nm (slit width 5 nm for excitation and 2.5 nm for emission).

that seen for **P1**. It is possible that this is partially due to closer proximity of this antenna to the lanthanide in comparison to **P1**, where the **Naph** antenna is located at the N-terminus. But there is also certainly a difference in the mechanism of the energy transfer to the Tb(III) excited state ($^5D_4$ = 20 500 cm^{-1}) which is thought to occur *via* the triplet state of the Trp (24 830 cm^{-1}),[55] and which might occur through the singlet state of the **Naph** (27 980 cm^{-1}), because the triplet state is too low in energy (18 540 cm^{-1}).[54] On the contrary, the relative intensity of Eu(III) luminescence at 616 nm was found to be significantly greater in the case of **P1** than that seen for **P2**, Fig. 2a and 2b, despite the difference in the location of these two antennae within the peptide structures. Even though we did not quantify the efficiency of the energy transfer for these two systems, it is clear from these results that the **Naph** antenna can be successfully employed for populating the Eu(III) excited state, and that it does so more efficiently than Trp. This can be explained because the T_1 energy of **Naph** is better suited for populating Eu(III) ($^5D_0$ = 17 200 cm^{-1})[9] than the T_1 energy of Trp, and additionally in the case of Trp a non-radiatively deactivated LMCT could also be formed.

Photophysical titrations of P1 and P2 using Eu(III) and Tb(III)

We next investigated the formation of these lanthanide-based peptides by carrying out careful spectroscopic titration in solutions, where the fluorescence emission and the lanthanide emission spectra were monitored. A typical evolution of the time-resolved Eu(III) emission for the titration of **P1**, upon excitation of the antenna at 345 nm, is shown in Fig. 3. Here the characteristic line-like and narrow emission bands of the Eu(III) emission became apparent upon binding of the ion to the peptide; the binding of which was monitored by observing the changes in the Eu(III) ΔJ = 2 transition at 617 nm. The results are shown as an inset in Fig. 3. First, it should be pointed out that **P1** displays the

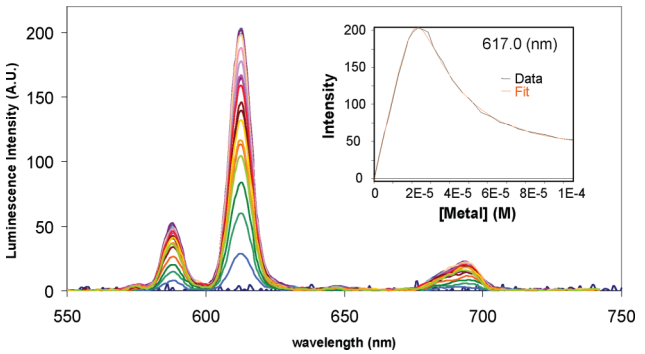

Fig. 3 The overall changes in the Eu(III) emission upon titrating **P1** (21 μM) with $EuCl_3$ at pH 7.0 (10 mM HEPES) and in 0.1 M NaCl. Inset: corresponding titration profile at 617 nm and the best fit obtained using the non-linear regression analysis programme SPECFIT.

same behaviour than **P2** towards Ln(III) complexation, where the metal-centred luminescence increased upon formation of the lanthanide peptide complex, within the use of one equivalent of the lanthanide ions. However, beyond the use of one equivalent, the Eu(III) emission reduced in intensity, see inset in Fig. 3. This was attributed to the formation of mononuclear and binuclear species, the latter resulting in a quenching of the luminescence intensity as previously observed.[56–58] Indeed, upon further analysis of these changes using the non-linear regression analysis program SPECFIT, this hypothesis was confirmed, as these changes were best fitted to two step binding equilibria.

The stability constants determined from the fitting of the changes in the Eu(III) emission of both **P1** and **P2** are summarized in Table 1. They show that both **P1** and **P2** give rise to similar binding constants for these ions. Unfortunately, due to the low solubility of the Tb_2**P1** species, we were unable to perform full titrations on this system, and, hence, unable to determine the Tb(III) affinity for **P1**. From Table 1, we can, however, conclude that the formation of the binuclear complexes is more difficult in terms of binding affinity than the formation of the mononuclear complexes. The stepwise stability constant is *ca.* 5 for all the systems; nearly two orders of magnitude lower than the formation of the mononuclear species. This difference can be explained by unfavourable electrostatic repulsion between the two lanthanide ions. This has previously been observed, even for systems with a large cavity, such as the one of a modified α-cyclodextrin (where the distance between the two Ln(III) ions is 5.74 Å in the solid state), and for which the stepwise formation constant of the binuclear species is nearly four orders of magnitude lower than the one of the mononuclear species.[59] Concerning the mononuclear complex, the stability constants are of the same order of magnitude than the 17-amino acid peptide developed by Imperiali *et al.* ($\log \beta_{Tb} = 7.2$) and optimised by screening methods, where the coordination sphere of

Table 1 Stability constants of Ln-**P** complexes in HEPES buffer 10 mM, pH = 7.0, 0.1 M NaCl at 298 K. $\beta_{mn} = [Ln_mP_n]/[Ln]^m[P]^n$

		Tb(III)	Eu(III)
P1	$\log \beta_{11}$	—	6.8 (0.1)
	$\log \beta_{21}$	—	11.8 (0.1)
P2	$\log \beta_{11}$	6.8 (0.1)	6.75 (0.08)
	$\log \beta_{21}$	11.9 (0.1)	11.9 (0.1)

Tb(III) is fully saturated, without any water molecule coordinated.[35] It is also higher than the stability constants reported for natural Ca(II) binding loops in calmodulin, where the affinity was found to be 5.5 for Tb(III) and 6.2 for Eu(III).[60] Finally, and possibly most importantly, the presence of the new sensitizer, the **Naph** antenna, does not significantly alter the affinity of the peptide sequence for Eu(III); being $\log \beta = 6.8$ for **P1**, compared to $\log \beta = 6.75$ for the unmodified version **P2**.

In addition to explore the binding of the lanthanide ions by **P1** and **P2**, we were also interested in assessing the selectivity of our system for these lanthanide ions with respect to endogenous cations such as Ca(II), Cu(II), and Zn(II). In order to do that, we chose the model compound **P2** and we performed competition experiments starting with the Tb(III) complex Tb_2**P2** (typically, [Tb(III)] = 5 × [**P2**] at the start of the titration), and we monitored the decrease of the Tb(III) luminescence intensity upon addition of the desired cation. A complete quenching of the luminescence intensity was observed upon addition of 5 eq. of Cu^{2+}, as is demonstrated in Fig. 4, whereas the addition of 1000 eq. of Ca(II) resulted in 60% of quenching (Fig. S10, ESI†), and the addition of 2000 eq. of Zn(II) resulted in less than 40% of quenching, making it impossible to determine a stability constant from these changes. For Cu(II), the best model to fit the data using SPECFIT involved the formation of mixed species between M(II)/Ln(III) and **P2**, whereas for Ca(II) only the formation of Ca**P2** was observed. The results are summarized in Table 2. The Ca(II) complex formed with **P2** is clearly less stable than the one formed with native

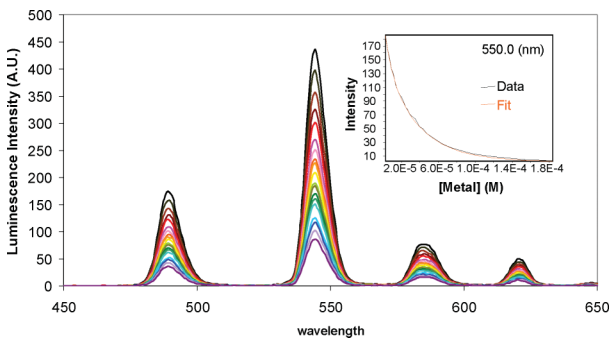

Fig. 4 Evolution of the luminescence spectra of **P2** (18.8 μM)-Tb(III) (75.2 μM) upon titration by $CuCl_2$ and after excitation of Trp at 280 nm at pH 7.0 (10 mM HEPES) and in 0.1 M NaCl. Inset: The corresponding titration profile at 550 nm and the best fit obtained using SPECFIT.

Table 2 Stability constants of M-**P2** complexes in HEPES buffer 10 mM, pH = 7.0, 0.1 M NaCl at 298 K. $\beta_{pmn} = [M_pLn_mP2_n]/[M]^p[Ln]^m[P]^n$

	Ca(II)	Cu(II)
$\log \beta_{101}$	4.61 (0.05)	7.53 (0.05)
$\log \beta_{111}$		11.7 (0.1)

parvalbumin ($\log \beta_{CaP} \sim 8$).[61] This could be attributed to the modification made from the native Parvalbumin binding loop, and also from the fact that other residues, not strictly from the binding loop could contribute to the stability of the complex in the naturally occurring protein. We achieved a good selectivity for Ln(III) over Ca(II) as the stability of Ln(III) complexes is two order of magnitude higher than seen for the corresponding Ca(II) complex. Previous work in this field has shown that the Ln(III) complexes formed with the first calmodulin domain are one order of magnitude more stable than the corresponding Ca(II) complexes.[60]

Elucidation of the structure of the metallo-peptides in solution

In order to gain a better insight into the structure of the metallo-peptides formed, we used circular dichroism experiments to monitor their formation. The CD spectra of **P1**, **P1** with one and two eq. of Eu(III), **P2**, **P2** with one and two eq. of Tb(III) are shown Fig. S11–S12 in ESI.† The same trend was observed for both systems upon binding to the lanthanides: a stronger negative band at 200 nm was observed for the free peptides, than for the metallo-peptides. This is characteristic of a random coil configuration in the absence of lanthanide ions.[62] Moreover, additional changes were observed upon addition of the lanthanide ions such as the transition centred at 220 nm became more negative which is indicative of some folding interactions. However, we were unable to identify any typical β-sheet or α-helix signatures from these spectral changes, and no significant changes were observed that allowed us to distinguish between the mononuclear and the binuclear species in these spectra. Because of this, we thus decided to carry out NMR experiments on these systems to get more information on the structure of the resulting metallo-peptides.

The NMR titration of both **P1** and **P2** were undertaken using the diamagnetic ion, La(III), and Eu(III) which is paramagnetic, in 90 : 10 H_2O:D_2O solutions. Selected spectra from these investigations are shown as stack-plots in Fig. 5. On all occasions, we only observed changes in the ^{1}H NMR spectra up to the addition

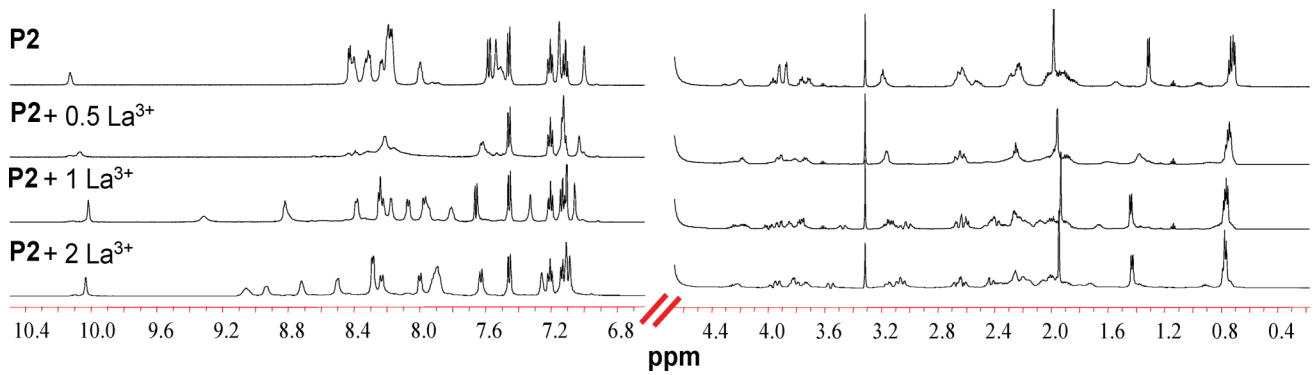

Fig. 5 Partial ^{1}H NMR spectra (600 MHz, 298 K) of the titration of **P2** with La^{3+} in a 9/1 H_2O/D_2O solution.

of 2 equivalents of metal ion added, with no significant changes occurring at higher equivalents. This confirms the formation of 1 : 1 and 2 : 1 complexes (see also Fig. S13, ESI†). In the case of **P1**, we could not determine the structure because in H_2O/D_2O solutions, the NH proton resonances were too broad, and becoming almost invisible when the pH was adjusted to 7. Although in the presence of La(III) the NH signals appear again (see Fig. S14, ESI†), they remained too broad for any further analysis. However, the NH-resonances were better resolved for **P2** and its complexes, which allowed us to analyse those changes in more details. This difference between **P1** and **P2** is possibly due to a difference in dynamics between the two peptides in solution. For the titration of **P2** with La(III) the addition of less than one equivalent of La(III) resulted in a broadening in the NMR spectra, suggesting the formation of a labile 1 : 1 complex, with dynamic equilibrium between the free and the bound form of **P2**. Upon addition of one equivalent of La(III), the spectrum sharpened significantly, making it possible to obtain a complete assignment of the resonances. To achieve this, we carried out t-ROESY experiments. A partial spectrum is shown in Fig. 6a, and demonstrates that while we did not observe enough long-range correlations to allow for a full structure determination, a correlation between the NH of Asp1 and the Hγ of Ile8 was observed. This suggests a folding of the **P2** peptide upon complexation with the La(III) ion, which further confirms the conformation changes observed in the CD spectra above. Further analysis of the chemical shifts of the different protons identified in these experiments, showed that the Hβ protons of the three Asp and the Hα protons of Trp and Ile were all shifted upon interaction of **P2** with La(III). These results are summarised in Table 3, and the changes in the NMR spectra suggest a direct coordination

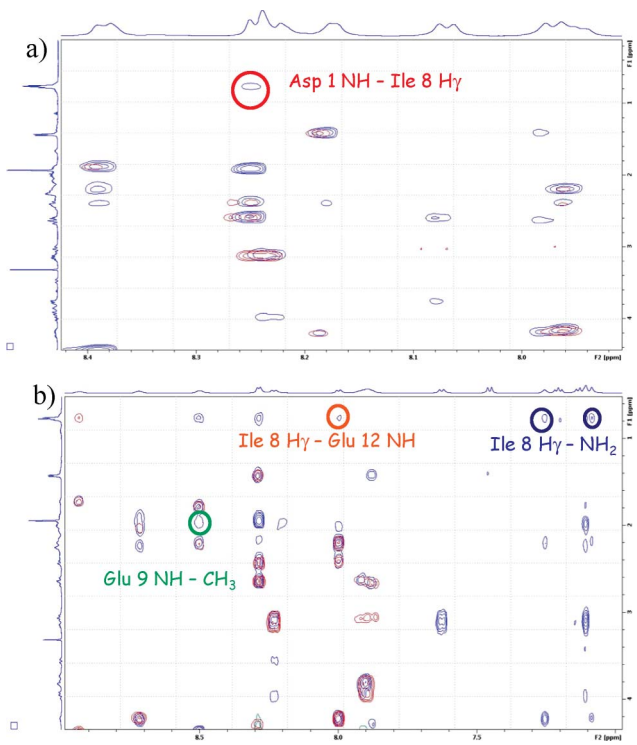

Fig. 6 Partial ^{1}H t-ROESY spectra at 600 MHz, 298 K with (a) [**P2**] = [La] = 0.63 mM and (b) [**P2**] = [La]/2 = 0.63 mM.

Table 3 ^{1}H NMR (600 MHz) chemical shifts (δ ppm) for **P2**, basified **P2** (**P2** + 6 eq. NaOH) and its La(III) complexes in H_2O/D_2O (v/v = 9/1) at 298 K

	P2	**P2** + 6 eq. NaOH	**P2**La	**P2**La$_2$
Asp1 Hβ	2.72–2.80	2.51–2.62	2.39–2.69	2.42–2.63
Asp1 Hβ	2.77–2.83	2.64	2.63–2.99	2.66–3.05
Asp1 Hβ	2.78	2.63	2.60–3.03	2.63–3.07
Glu 9 Hβ	1.93–2.05	1.88–2.25	1.89–2.18	1.77–2.20
Hγ	2.43			
Glu 11 Hβ	1.84–2.00	1.95–2.22	2.00–2.25	2.03–2.24
Hγ	2.42			4.72
Glu 12 Hβ	1.93–2.09	1.92–2.24	2.20–2.41	2.20–2.41
Hγ	2.41			
Trp Hα	4.60	4.58	4.90	4.95
Ile Hα	3.99	9.96	4.46	4.37

of the three Asp in **P2** to the La(III) ion, as well as the amide backbone of the Trp. Importantly, the coordination of the amide of the Trp in the position 7 of the Ca-binding loop is consistent with what has been observed for typical coordination of metal ions in EF hand motif for other peptide-Ln complexes both by X-ray in the solid state[35] and by NMR in solution.[40] We had previously determined the number of water molecules directly coordinated to the Ln(III) ion in the mononuclear complex by luminescence lifetime measurements. We found a q value of ~3 for both **P1** and **P2** complexes,[47] showing the coordinatively unsaturated nature of the lanthanide ions within **P1** and **P2**. These results, together with the stability constant values confirm that the introduction of Naph does not affect the coordination sphere of the Ln(III). Moreover, these results are consistent with the direct coordination of three Asp, and suggest that possibly one or two of the Asp residues are coordinating to the ion in a bidentate manner in **P2**. Upon addition of a second equivalent of La(III) to **P2** further shifts were observed for the side chains of two of the three Glu residues, suggesting that the second metal ion is coordinated within the Glu pocket of **P2**. This was further confirmed by the t-ROESY correlations, Fig. 6b, observed between the Hγ of Ile8 and the NH of Glu12, and between the Hγ of Ile8 and the C-terminal NH_2. This is also an indication of the folding of the peptide at the C-terminus. It should be noted that a correlation is also observed between the NH of Glu9 and the CH_3 at the N-terminus, confirming that we still have a folding of the N-terminus part of the peptide.

Conclusions

In summary, we have developed a peptide, based on the Ca-binding loop of the parvalbumin, bearing a sensitising naphthalimide chromophore that can used to populate both the excited states Eu(III) and Tb(III). The **Naph** has been coupled to a glycine and then easily introduced at the N-terminus of the peptide using standard peptide solid-phase synthesis. The binding loop used and studied through the use of a similar peptide bearing a Trp chromophore does show a good selectivity for Ln(III) over Cu(II), Zn(II), and more importantly Ca(II). The presence of two coordination pockets composed of Asp and Glu, and corresponding to the formation of 1/1 and 2/1 Ln/P complexes respectively was evidenced. The introduction of the **Naph** antenna does not affect the stability of the lanthanide complexes formed with the peptide, and does not change the number of water molecules directly coordinated to the Ln(III) ions. This is a first

step towards the optimisation of peptide- lanthanide complexes as the use of lower excitation energies than the natural amino acids can provide is compulsory for further possible *in vivo* applications.

Experimental section

General

All chemicals were purchased at the purest grade commercially available and were used without further purification unless otherwise mentioned. Solvents used were HPLC grade unless otherwise mentioned. Dichloromethane for peptide synthesis was distilled prior to use. Water was purified with a Waters Milli-Q system to give a specific resistance <15 MΩ cm.

Chromatographic analysis and purification were performed on a BioCAD SPRINT Perfusion Chromatography Workstation (PerSeptive Biosystems) using Phenomenex Gemini columns (5 Å, C18, 4.6mmd/250mmL (analytic) 100mmd:250mmL (semi-preparative), solvent A = H_2O/TFA (v/v = 99.9/0.1), solvent B = CH_3CN/TFA (v/v = 99.9/0.1), flow rate: 1 mL min^{-1} (analytical) 4.5 mL min^{-1} (semi-preparative). The gradient employed was 2 to 60% B in 18 column volumes with UV monitoring at 214 and 350 nm (for **P1**) or only 214 nm for **P2**.

The peptides were characterized by Matrix Assisted Laser Desorption Ionisation- Time Of Flight- Mass Spectroscopy (α-cyano-4-hydroxy-cinnamic acid matrix).

Mass spectrum of **Naph** was determined using electrospray on a Micromass LCT spectrometer. High-resolution mass spectra were determined relative to a standard of leucine enkephaline.

Synthesis

1,3-Dioxo-1*H*-benz[d,e]isoquinoline-2(3H)-acetic acid (Naph). Glycine (3.7 mmol) and 1,8-naphthalic anhydride (3 mmol) were mixed together with 20 mL of DMSO in a pressure tube. The mixture was heated at 150 °C to allow the dissolution, and was kept under continuous stirring at this temperature during 24h. It was then evaporated to give a brown solid. The crude product was washed with methanol and filtrated to give a white solid in 56% yield.^{1}H NMR (DMSO-d_6, 40 MHz), δ (ppm): 8.54 (m, 4H), 7.92 (t, J = 7.76 Hz, 2H), 7.76 (s, 2H).

^{13}C NMR (DMSO-d6, 150 MHz), δ (ppm): 169.2 (COOH), 163.0 (CO), 134.9, 131.4, 131.1, 127.36, 127.33, 121.5 (C_{ar}), 41.1 (CH_2). ESI-MS m/z: [L + Na]$^+$ calculated for $C_{14}H_9NO_4Na$: 278.04; Found, 278.05.

Peptide P1: Naph-DADGDGAIEPEE. The polymer-bound precursor peptide Asp(*t*Bu)-Ala-Asp(*t*Bu)-Gly-Asp(*t*Bu)-Gly-Ala-Ile-Glu(*t*Bu)-Pro-Glu(*t*Bu)-Glu(*t*Bu) was prepared by standard solid phase peptide synthesis according to the Fmoc-*t*Bu strategy with HBTU/HOBt/DIEA coupling chemistry, in NMP solvent. Single coupling cycles using a 10-fold excess of Fmoc-amino acid derivatives to resin-bound peptide were employed. Assembly of the amino acid sequence, starting from a Rink Amide MBHA resin was carried out on an automated peptide synthesizer (Applied Biosystems 433A). The Naphthalimide (2 eq.) was then coupled manually using PyBOP (2 eq.). Coupling reaction was monitored using the qualitative Kaiser Test. The peptide was cleaved from the resin and deprotected by treatment with TFA/thioanisole/TIS/EDT/H_2O (v/v/v/v/v = 77/5.75/5.75/5.75/5.75) for 2 h. After concentration the residue was precipitated in cold diethylether to yield a white powder. The solid residue was dissolved in MeOH/H_2O (v/v = 60/40) and purified by RP-HPLC to yield **Naph**-DADGDGAIEPEE as a white powder. Maldi-Tof -MS: m/z = 1475.4 [M+K$^+$].

Peptide P2: DADGDGWIEPEE. The peptide with protected side chains Ac-Asp(*t*Bu)-Ala-Asp(*t*Bu)-Gly-Asp(*t*Bu)-Gly-Trp(Boc)-Ile-Glu(*t*Bu)-Pro-Glu(*t*Bu)-Glu(*t*Bu)-NH_2 was assembled manually by solid-phase peptide synthesis on Rink-amide resin (substitution 0.6 mmol g^{-1}) using Fmoc chemistry.[51] Couplings were performed with *N*-α-Fmoc-protected amino acids (2 eq.), PyBOP (2 eq.) and DIEA (pH ≈ 8–9) in DMF. Coupling reaction was monitored by TNBS test. For non complete reactions, a second coupling was achieved followed by DMF/pyridine/Ac_2O (v/v/v = 7/2/1) treatment. Fmoc deprotection was achieved with DMF/piperidine (v/v = 4/1). After the last coupling, the acetylation was performed with a DMF/pyridine/Ac_2O 7/2/1 mixture. The peptide was cleaved from the resin and deprotected by treatment with TFA/TIS/H_2O (v/v/v = 95/2.5/2.5) for 2 h. After concentration the residue was precipitated in cold diethylether to yield a white powder. The solid residue was dissolved in water and purified by RP-HPLC to yield Ac-DADGDGWIEPEE-NH_2 as a white powder. Maldi-Tof -MS: m/z = 1394.8 [M+Na$^+$].

Preparation of liquid samples

The peptide concentration was systematically determined by measuring the UV-absorption of the tryptophan residue for **P2** (λ_{max} = 280 nm, ε_{max} = 5690 cm^{-1} M^{-1}), and the **Naph** residue for **P1** (λ_{max} = 345 nm, ε_{max} = 11 500 cm^{-1} M^{-1}). Metal stock solutions were prepared by dissolving $LnCl_3 \cdot 6H_2O$ or $LnOTf_3$ in H_2O or D_2O. Their exact Ln^{3+} concentrations were systematically determined by colorimetric titration in acetate buffer (pH = 4.5) using standardized H_2Na_2edta solution and xylenol orange as an indicator,[63] except for Ca(II) solutions, which were titrated at pH 12 using Calcon as an indicator.

Luminescence titrations

Solutions were all made at pH = 7.0 in 10 mM HEPES buffer, 0.1 M NaCl. UV-Vis spectra were recorded on a Varian UV-Vis spectrophotometer from 250 to 500 nm using excitation and emission slit width of 1 nm with a medium scan speed. Luminescence and lifetimes were measured on a Varian Cary Eclipse Fluorescence spectrophotometer following an excitation at 345 nm for **P1**, and at 280 nm for **P2**. The following settings were as follow: for fluorescence mode: PMT detector: 600 mV, scan speed: 600 nm min^{-1}, averaging time: 0.1 s, data interval: 1 nm. For phosphorescence mode: delay time: 0.1 ms, flash count: 1, PMT detector: 1000 mV, data interval: 1 nm, total decay time: 0.02 s, gate time: 10 ms.

Spectra were analysed by using the program SPECFIT, which employs a singular value decomposition algorithm and refines the data according to a global least-squares analysis procedure.[64,65] The values obtained are the average of at least three titrations.

Terbium and europium luminescence lifetimes were measured by recording the decay of the emission intensity at 545 nm and 616 nm, respectively, after excitation at 345 nm for **P1** and 280 nm for **P2**. It was also checked that direct excitation at 368 nm and

396 nm give the same results. The lifetimes were measured in such conditions that only the 1/1 complex is present in solution. The signals were analyzed as single-exponential decays. As the peptide is highly hydrated, the lifetimes were measured for different H_2O/D_2O ratio ranging from 0.2/0.8 to 1/0, and the lifetime in D_2O was extrapolated from the measured values.

NMR experiments

All the NMR experiments were performed on a Bruker Avance II 600 MHz spectrometer equipped with a 5 mm TCI cryoprobe. The spectra were recorded at 298 K in H_2O/D_2O (v/v, 95/5) using watergate W5 when necessary[66] with a bandwidth of 7200 Hz and a power of 4.05 dB for the Watergate pulse. 2D NMR spectra were acquired in phase-sensitive mode with TPPI for quadrature detection in the indirect dimension using 2048×128 matrices. TOCSY experiments were performed by using a MLEV-17 spin-lock sequence with a mixing time of 70 ms. Off-resonance ROESY experiments were recorded with a mixing time of 300 ms (5000 Hz spin-lock). The spectra of **P2** were recorded at 0.63 mM. A titrated solution of NaOH was added and spectra were recorded up to 6 equivalents of NaOH added with respect to **P2**. A titrated solution of Ln(III) (La or Eu) was then added and spectra were recorded up to 4 equivalents of metal ion with respect to **P2**. The same procedure was followed with **P1** and the corresponding spectra were recorded with a concentration of **P1** of 0.20 mM.

Circular dichroism

CD spectra were recorded at 25 °C on a JASCO's J-810 dichrograph in a cell with a 0.1 cm path length. The peptide concentration was 100 μM in 10 mM HEPES, NaCl 0.1 M at pH 7.4. All spectra were obtained from 260 to 180 nm at intervals of 1 nm, a band width of 2 nm. The contribution of the buffer was always subtracted and three spectra were averaged for each sample. CD spectra are reported in molar ellipticity per α-amino acid residue.

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