Structural studies in aqueous solution of new binuclear lanthanide luminescent peptide conjugates†

Célia S. Bonnet,[a] Marc Devocelle[b] and Thorfinnur Gunnlaugsson*[a]

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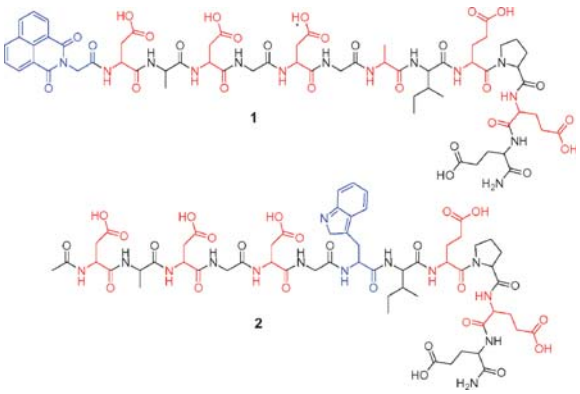
The synthesis, NMR and luminescent analysis of a novel polypeptide possessing a sensitising naphthalimide antenna at the amino terminus, and a model compound with a Trp moiety, and their Eu(III) and Tb(III) complexes are described.

The design and synthesis of novel targeting molecules for biological imaging, structural analysis, dynamics, and for investigating bio-supramolecular interactions such as protein-substrate communications is a topical area of research.[1,2] The use of paramagnetic luminescent lanthanide ions to achieve such endeavours has recently become an important part of such investigations.[3–8] With their unique photophysical properties and high coordination requirements,[9] they are ideal targeting agents, as they often possess vacant binding sites that can be used to coordinate to biological guests such as anions[10,11] drugs,[12] or enzyme cofactors.[13]

We have been interested in the development of lanthanide complexes for sensing[14] and phosphodiester hydrolysis.[15] With the aim of developing novel targeting and biocompatible luminescent lanthanide probes, we have developed Tb(III) and Eu(III) complexes of **1**, a synthetically modified polypeptide containing the 1,8-naphthalimide (**Naph**) chromophore as a sensitising antenna at the N-terminal. This short peptide sequence, was designed based on the parvalbumin protein Ca(II) binding loop,[16] which consists of six Glu and Asp carboxylate residues, that can be employed to complex lanthanide ions. For comparison purposes, we also made **2**, which lacks the **Naph** antenna, but instead, was synthetically modified to incorporate a Trp moiety at the 7th position. Such modifications have previously been shown by Szabo *et al.* to give rise to efficient sensitised Tb(III) emission upon binding of the ion.[17] In this *communication* we demonstrate, using luminescence and NMR spectroscopy, the formation of mono- and bimetallic lanthanide complexes of **1** and **2**. Furthermore, we show that the incorporation of the **Naph** antenna in **1**, gives rise to efficient Eu(III) and Tb(III) sensitised luminescence.



The peptide sequence **DADGDGAIEPEE** of the naphthalimide based peptide **1** was synthesised using standard automated solid phase peptide synthesis according to the Fmoc-*t*Bu strategy and HBTU (2-(1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate)–HOBt (*N*-hydroxybenzotriazole)–DIEA (*N*,*N*-diisopropylethylamine) coupling chemistry in NMP (*N*-methylpyrrolidone). The 1,8-naphthalimide antenna was then coupled manually at the N-terminus of the peptide sequence using a double coupling procedure with two equivalents of **Naph**, two equivalents of PyBOP (benzotriazol-1-yl-oxytripyrrolidinophosphonium hexafluorophosphate) and six equivalents of DIEA in DMF. The resulting modified peptide was subsequently deprotected and cleaved from the resin using TFA–H_2O–thioanisole–TIS (triisopropylsilane). This was followed by a purification using reverse-phase HPLC. The model compound **2** was synthesised in an analogous manner. Both **1** and **2**, were characterised using MALDI-TOF or ESMS which gave 1475.4 ($M + K^+$) and 1394.8 ($M + Na^+$), respectively (ESI†). The two peptides were also analysed by ^1H, ^{13}C, HSQC, TOCSY and t-ROESY NMR spectroscopy in H_2O,‡ which showed that the two apopeptides adopted a random coil conformation in water. Both were stable in solution for a period of over one month.

The ability of **1** to complex and sensitise Tb(III) and Eu(III) was investigated in buffered solutions at pH 7.0 (0.01 M HEPES, 0.1 M NaCl). The absorption spectrum of the **Naph** moiety in **1** exhibited a broad band centred at 344 nm ($\log \varepsilon = 4.04$) which was assigned to the n–π^* transition, Fig. 1. Excitation of this band gave rise to a fluorescence emission arising from the **Naph** moiety with $\lambda_{max} = 393$ nm. Similarly, peptide **2**, which possesses a Trp antenna, exhibited a band centred at $\lambda = 280$ nm in the absorption spectrum. Excitation of this band gave rise to the characteristic emission of Trp at 334 nm. Upon addition of one equivalent of $TbCl_3$ or $EuCl_3$ to **1**, no significant changes were observed in the absorption spectra. However, the fluorescence was significantly quenched, indicating a potential energy transfer occurring from the

[a] *School of Chemistry, Centre for Synthesis and Chemical Biology, Trinity College Dublin, Dublin 2, Ireland. E-mail: gunnlaut@tcd.ie; Fax: +353 1 671 2826; Tel: +353 1 896 3459*
[b] *Centre for Synthesis and Chemical Biology, Department of Pharmaceutical and Medicinal Chemistry, Royal College of Surgeons in Ireland, 123 St. Stephen's Green, Dublin 2, Ireland*

† Electronic supplementary information (ESI) available: MS; NMR spectra of **1** and **2** and complexes; τ-values. See DOI: 10.1039/b811388b

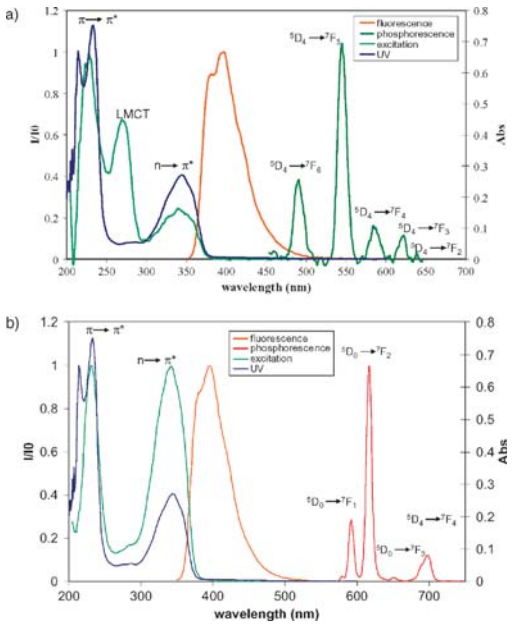


Fig. 1 (a) The absorption and the fluorescence emission spectra of **1** and one equivalent of Tb(III), and the characteristic Tb(III) emission observed upon excitation at 344 nm in pH 7.0 buffered solution. Also shown is the delayed excitation spectrum recorded upon excitation of the **Naph** band and observing the emission of the Tb(III) at 545 nm. (b) The same spectra observed in the presence of Eu(III). The 616 nm Eu(III) transition was used when recording the delayed excitation spectra of this complex.

S_1 state, *via* the T_1 of the antenna, to the Eu(III) and Tb(III) excited states. Indeed, this was found to be the case, as shown in Fig. 1, with the appearance of the characteristic line-like emission bands at 492, 545, 584, 622 and 638 nm, for the deactivation of the $^5D_4 \rightarrow {}^7F_J$ (J = 6, 5, 4, 3, 2) of Tb(III), and at 592, 617, 651 and 698 nm for the deactivation of the $^5D_4 \rightarrow {}^7F_J$ (J = 1, 2, 3, 4) of Eu(III). These luminescence emissions clearly show that the **Naph** moiety was capable of sensitising these excited states efficiently, which was further confirmed by recording the delayed excitation spectra of **1** in the presence of these ions, Fig. 1. Moreover, for Tb(III), a new band centred at 270 nm was observed in the excitation spectrum, attributed to the formation of a LMCT transition.[6a]

In order to gain a better insight into the species present in solution and of the structural environment of the metal, luminescent titrations were performed on **1** and on the model compound **2**, at pH 7.0. Unfortunately, in the case of **1**, after the addition of one equivalent, precipitation occurred, thus preventing further titrations. However, for **2**, full titrations were successfully performed, where, in the case of $TbCl_3$ (Fig. 2), excitation at 280 nm gave rise to a sharp enhancement in the Tb(III) emission up to the addition of one equivalent of Tb(III). This was followed by a subsequent decrease in the lanthanide emission at higher concentrations of Tb(III). The resulting data were analysed using the non-linear least squares regression programme SPECFIT, the results of which are shown as an inset in Fig. 2. The best fits were obtained by using both 1 : 1 and 2 : 1 (Tb : **2**) binding models, giving complexes with conditional stability constants of log β_{11} = 6.8 (±0.1) and log β_{21} = 11.9 (±0.1) (ESI†). It is worth noting that the binding affinity found for the mononuclear species is quite significant in comparison to other peptides of comparable size.[3,4] While we did not initially expect to observe the formation of both the 1 : 1 and

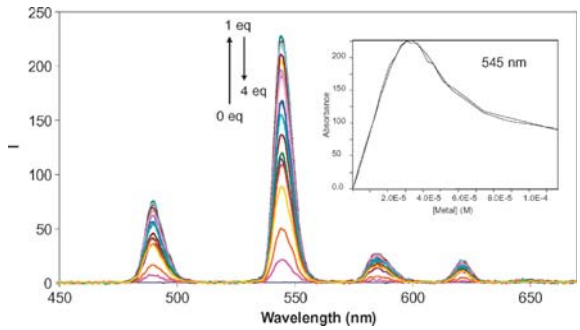


Fig. 2 The overall changes in the Tb(III) emission upon titrating **2** (31 μM) with $TbCl_3$ at pH 7.0 (10 mM HEPES) and in 0.1 M NaCl. Inset: the corresponding titration profile at 545 nm and the observed non-linear regression analysis fit, obtained using the SPECFIT.

2 : 1 stoichiometries in solution, it is possible that the additional non-coordinating acidic residues involved in the coordination of the second metal ion might potentially increase the binding affinity for the first lanthanide ions. The nature of such phenomena has recently been noted by Imperiali *et al.*[18] It is also important to note that the binding affinity of the second metal ion is about two orders of magnitude weaker than that observed for the 1 : 1 binding, which is most likely due to unfavourable electrostatic repulsion between the two metal ions upon formation of the 2 : 1 stoichiometry. This would also explain the quenching observed in the Tb(III) emission, in Fig. 2, after one equivalent addition, which could be due to self-quenching between the two metal ion centres.[19]

These results would also imply that each of the metal centres in **2** was coordinatively unsaturated within the peptide structures. Therefore, we evaluated the number of metal bound water molecules, or the hydration state (the q-value),[20] of the mononuclear complex of **2**·Tb (ESI†) by measuring the excited state lifetimes of Tb(III) in H_2O and D_2O, respectively. The excited state decay for Tb(III) was best fitted to a single exponential, giving lifetimes of 0.82 ms and 1.92 ms for H_2O and D_2O, respectively. From these results, a q value of ~3 was determined. Similarly, lifetime measurements were also carried out on the mononuclear Tb(III) complex of **1**. However, these lifetimes were too short to determine q accurately. We attribute that to a possible back energy transfer occurring from a $^5D_4 \rightarrow T_1$ excited state of the **Naph** moiety. Degassing the sample did not result in any significant enhancement in the lifetimes of the Tb(III) emission. This might suggest that other deactivation pathways are also possibly occurring. Because of this, the lifetime measurements were carried out using the mono Eu(III) complex of **1**, from which lifetimes of 0.270 ms and 0.884 ms were determined for H_2O and D_2O, respectively. Again, a q value of ~3 was determined, which confirmed the coordinatively unsaturated nature of the lanthanide ions within **1** and **2**.

From these measurements we can also conclude that the presence of the **Naph** moiety in **1**, or the Trp in **2**, does not affect the coordination sphere of the metal ions within these peptide structures. Taking into account the speciation distribution discussed above, and a q value of ~3 within the first coordination sphere of the lanthanide ions, we propose that three carboxylate arms might be coordinating the metal ion, some of which might be bound in a bidentate manner. This would leave three further carboxylates free for the coordination of the second metal ion. With the aim of evaluating this, we attempted to resolve the structure in solution of these complexes in H_2O by carrying out a

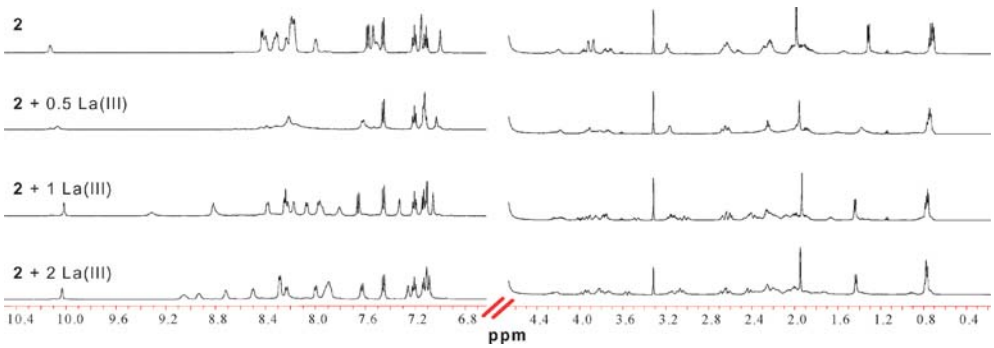


Fig. 3 Partial ^{1}H NMR spectra (600 MHz) of the titration of **2** with $LaCl_3$ in a 9 : 1 H_2O : D_2O mixture.

detailed NMR analysis of **2** using Eu(III) and La(III). The formation of the mono- and the bimetallic species of **2** was monitored by recording the ^{1}H, ^{13}C, HSQC, TOCSY and t-ROESY NMR spectra at different Ln : **2** ratios. For that purpose, the peptide was treated with six equivalents of NaOH and brought to pH $\sim$ 7. The evolution of the complex formation in the ^{1}H NMR spectrum is shown in Fig. 3 for La(III), and clearly demonstrates that substantial changes occur between 0 $\rightarrow$ 2 equivalents of La(III). No significant changes were observed in the spectra thereafter, confirming the stepwise formation of the 1 : 1 and the 2 : 1 complexes. Similar stoichiometry changes were also observed for Eu(III) (ESI†). In the case of La(III), up to one equivalent, the spectra obtained were broad, suggesting a dynamic equilibrium in solution between the bound and unbound form of **2**. At one equivalent of La(III) the spectrum sharpened and a complete assignment of the complex was possible. In particular, significant shifts were observed for the Hβ protons of the three Asp and the Hα protons of Trp and Ile. These suggest a direct coordination of the three Asp, as well as the amide backbone of the Trp moiety to La(III). While the t-ROESY spectrum did not show enough long-range correlations to allow a full structural analysis of the complex, a correlation between NH of Asp1 and the side chain of Ile8 was observed, which proved the folding of the peptide upon complexation. Also, at two equivalents of La(III), further shifts were observed for the side chains of two of the three Glu residues, suggesting that the second metal ion was involved in coordination to these amino acids within the Glu pocket. Moreover correlation between the NH of Glu12 and the side chain of Ile8 further confirmed the folding of the peptide at the C-terminus. Furthermore, the CD spectrum of **2** was significantly induced upon addition of both one and two equivalents of Tb(III) (ESI†).

In summary, we have demonstrated the ability of **1** and **2** to form 1 : 1 and 2 : 1 complexes with lanthanide ions, the peptide possessing two coordination pockets composed of the Asp and the Glu respectively. We are currently evaluating these systems in greater detail.

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Notes and references

‡ The spectra were obtained using a Bruker Avance II 600 MHz spectrometer equipped with a 5 mm TCI cryoprobe. The watergate W5 sequence was implemented when necessary.

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