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DNA Sensing by a Eu-Binding Peptide Containing a Proflavine Unit

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Supporting Information

ABSTRACT: Synthesis of a lanthanide-binding peptide (LBP) for the detection of double-stranded DNA is presented. A proflavine moiety was introduced into a high affinity LBP involving two unnatural chelating amino acids in the Ln ion coordination. The Eu^{3+}−LBP complex is demonstrated to bind to ct-DNA and to sensitize Eu luminescence. The DNA binding process is effectively detected via the Eu-centered luminescence thanks to the intimate coupling between the LBP scaffold and DNA intercalating unit.

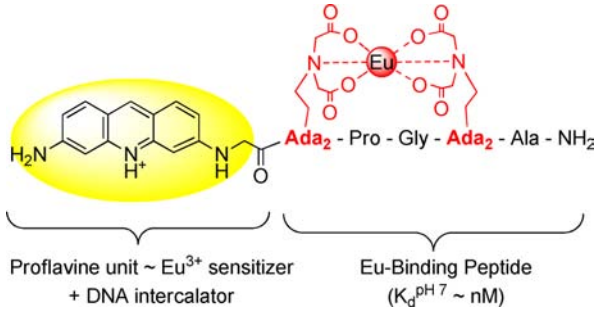
Lanthanide (Ln) complexes are promising luminescent probes for many biological applications,[1] such as sensing of biologically relevant species[2] and in particular DNA.[3] Indeed, photophysical properties of these metal ions present several advantages over fluorescence-based systems, such as sharp line-like emission bands within the visible or near-infrared region and time-resolved emission with long-lived excited states and luminescence lifetimes in the μs−ms time scale. Their luminescence is thus easily detected from the autofluorescence of the biological material (ns time scale) and gives rise to a significant improvement of the signal-to-noise ratio.

Among Ln-based luminescent probes, Ln-binding peptides (LBPs) are promising molecular tools because they combine interesting structural features of the peptide backbone with high hydrophilicity and solubility in water.[4] Another asset of using peptides as Ln ligands is their versatile synthesis and insertion in proteins by solid phase peptide synthesis (SPPS), which gives access to a large set of peptides with easy functionalization or by bioengineering. LBPs of high affinities for Ln ions have been designed either by optimizing Ca^{2+} binding sites[5] or by the rational insertion of unnatural chelating amino acids in short peptide sequences with well-defined structures.[6] Interestingly, peptide sequences of up to 20 encoded amino acids that tightly and selectively complex lanthanide ions have been demonstrated to be valuable tools to probe the structure, function, and dynamics of proteins with biophysical technics such as X-ray diffraction, paramagnetic NMR, or luminescence (Lanthanide Resonance Energy Transfer, LRET).[7]

Compounds that bind DNA, an essential biomolecule that is storing and dispensing genetic data required for life, are extremely useful as biochemical tools for visualization of DNA both in vitro and inside the cell.[8] The exceptional properties of LBPs may also be exploited to probe DNA. For instance, a Gd-binding peptide with a helix-turn-helix structure was demonstrated to bind DNA thanks to its MRI contrast efficiency, which is sensitive to the formation of the DNA−peptide adduct.[9] However, to the best of our knowledge, the time-resolved luminescence of Ln−LBP complexes has never been exploited to probe DNA recognition.

Herein, we present **Pfl-P^{22}**, a Ln-binding peptide conjugate possessing a proflavine moiety, which serves both as a Eu-sensitizer and a DNA binding group (Scheme 1). Proflavine

Scheme 1. EuPfl−P^{22}, a Eu^{3+} Complex with a Ln-Binding Peptide (Pfl−P^{22}) That Intercalates into ds-DNA



(Pfl) or 3,6-diaminoacridine belongs to the widely studied acridine family, which represents an important class of anti-tumor drugs and has been demonstrated to interact with double-stranded DNA via an intercalative process.[10] Pfl binds to ds-DNA with a binding constant $K \approx 10^5\ M^{-1}$ on the basis of the fluorescence intensity change of this aromatic compound upon intercalation.[11] Besides, Pfl bears two amino groups, which are useful for synthetic transformation and further incorporation in peptide sequences. This tricyclic aromatic core shows an absorption in the visible range (445 nm or 22 472 cm^{-1}) with a large molar extinction coefficient (≈ 40 000 $M^{-1}cm^{-1}$) and low energy excited levels (510 nm or 19 608 cm^{-1} for fluorescence and 576 nm or 17 400 cm^{-1} for phosphorescence).[12] Because natural aromatic amino acids such as tryptophan and tyrosine are poor Eu antennas, aromatic moieties with lower triplet states—naphthalimide,[4c,d] acridone, and carbostyril 124[13]—were introduced in LBPs as more efficient Eu sensitizers. Besides, Schiff Bases with triplet states as low as 17 270 cm^{-1} in energy[14] were demonstrated to significantly sensitize the latter metal ion. Therefore, the proflavine DNA intercalating unit is also expected to effectively sensitize the Eu metal ion.

The peptide **Pfl−P^{22}** (Scheme 1) combines a Ln-binding sequence with two unnatural Ada_2 amino acids carrying

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aminodiacetate chelating groups[6a] and a Pfl moiety appended to the N-terminus. The Eu**Pfl**–$\mathbf{P^{22}}$ complex was demonstrated to interact with ct-DNA as efficiently as the Pfl core alone, and the binding process is detected by the evolution of the time-resolved Eu-centered luminescence.

The synthesis of **Pfl**–$\mathbf{P^{22}}$ was achieved by solid phase peptide synthesis on Rink amide MBHA resin using standard SPPS conditions and starting from natural amino acids, Fmoc-$Ada_2(t\mathrm{Bu})_2$–OH[6a], and finally the Pfl derivative **3** in the last coupling step (Scheme S1, Supporting Information (SI)). Compound **3** bears a carboxymethyl function introduced on the amino group in position 6 in order to be directly coupled to the N-terminus of the peptide. It was synthesized from Pfl in three steps with an overall yield of 27%. The pure peptide was obtained after preparative HPLC purification. Its identity and purity were confirmed by electrospray ionization mass spectrometry and ^{1}H NMR (SI). Pfl is highly basic—the pK_a value is 9.65—due to the aromatic nitrogen atom.[15] The functionalization of the amino group in position 6 does not significantly affect the Pfl protonation as demonstrated by the pK_a value of **Pfl**–$\mathbf{P^{22}}$ (pK_a = 9.9) determined by the evolution of the UV spectra as a function of pH (Figure S3, SI). The aromatic amine is thus protonated in the experimental conditions used in this work, i.e., aqueous buffer at pH 7.

The spectroscopic properties of **Pfl**–$\mathbf{P^{22}}$ are characteristic of the Pfl moiety: the absorption spectrum exhibits a broad band centered at 449 nm (ε = 39 200 $M^{-1}cm^{-1}$), and excitation of this band gives rise to fluorescence emission with λ_{max} = 510 nm. The time-resolved emission spectrum of Eu**Pfl**–$\mathbf{P^{22}}$ in HEPES buffer at pH 7 shows the Eu-centered $^5D_0 \rightarrow {}^7F_J$ emission bands (J = 1–4) upon excitation of the Pfl moiety at 450 nm (Figure 1b). The excitation spectrum of Eu**Pfl**–$\mathbf{P^{22}}$ (Figure 1a) recorded for the most intense emission of Eu^{3+} (λ_{em} = 616 nm) shows the same features as the UV spectrum of the complex and demonstrates the sensitization of the metal ion by the Pfl moiety at 450 nm. For comparison, Figure 1b shows the emission spectrum of the Eu^{3+} complex with the parent peptide $\mathbf{P^{22}}$, which contains the natural tryptophan moiety instead of Pfl ($\mathbf{P^{22}}$: Ac-Trp-Ada_2-Pro-Gly-Ada_2-Gly-NH_2).[6a] This spectrum is barely detectable because Trp has higher energy levels that lead mainly to photoinduced electron transfer (PET) at the expense of Eu^{3+} sensitization.[6d] The low energy chromophore Pfl is demonstrated here to significantly sensitize Eu^{3+} in the complex Eu**Pfl**–$\mathbf{P^{22}}$, which is easily detected in the low micromolar range with our experimental equipment.

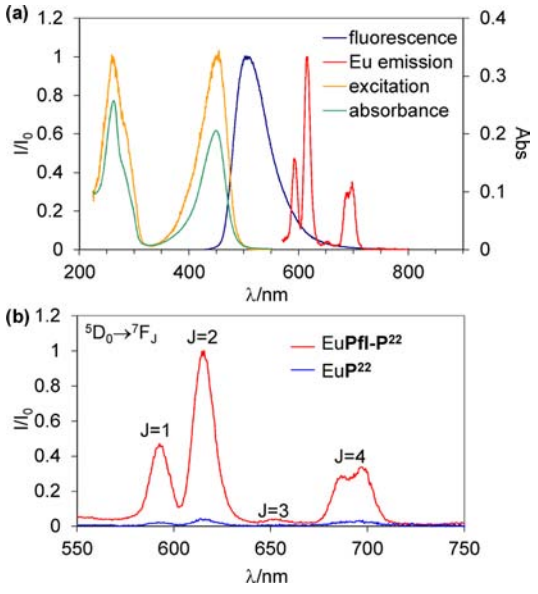

Figure 1. (a) Spectroscopic properties of Eu**Pfl**–$\mathbf{P^{22}}$ (5 μM) in HEPES (10 mM pH 7, KCl 0.1 M) at 298 K. Fluorescence and Eu^{3+} emission observed upon excitation at 450 nm, excitation spectrum upon delayed emission at 616 nm. (b) Comparison of the delayed emission spectra of Eu**Pfl**–$\mathbf{P^{22}}$ (excitation of Pfl at 450 nm) and Eu$\mathbf{P^{22}}$ (excitation of Trp at 280 nm), 5 μM in HEPES (10 mM pH 7, KCl 0.1 M).

The complexation of Eu^{3+} could thus be followed by the evolution of the time-resolved luminescence spectra of the metal ion upon excitation of the Pfl moiety at 450 nm, during titrations of solutions of **Pfl**–$\mathbf{P^{22}}$ by $EuCl_3$ (Figure S4, SI). A sharp end-point is detected for 1 Eu equiv., which indicates the formation of the Eu**Pfl**–$\mathbf{P^{22}}$ complex with a large stability constant, as previously observed for Tb$\mathbf{P^{22}}$. The signal does not evolve in excess of Eu demonstrating the absence of polymetallic species formation. Electrospray ionization mass spectrometry spectra (Figure S5, SI) show the exclusive formation of the Eu**Pfl**–$\mathbf{P^{22}}$ complex whatever the Eu/peptide ratio: $[Eu\mathbf{Pfl}–\mathbf{P^{22}}\text{-}3H]^+$ m/z = 1074.3 and $[Eu\mathbf{Pfl}–\mathbf{P^{22}}\text{-}2H]^{2+}$ m/z = 537.6. The luminescence decay of Eu^{3+} in Eu**Pfl**–$\mathbf{P^{22}}$ is a single exponential function at room temperature, thus indicating the presence of only one emissive Eu^{3+} center (Figure S6, SI). The hydration number $q_{Eu} \approx 3$ (Table S1, SI) is common for Ada_2-chelating peptides such as $\mathbf{P^{22}}$.[6a] We can therefore conclude that the replacement of the Trp residue with a Pfl moiety in the peptide sequence does not modify the peptide Ln-binding properties and increases significantly the Eu-centered emission. Delayed Eu luminescence in the Eu**Pfl**–$\mathbf{P^{22}}$ complex was therefore exploited to monitor its interaction with DNA.

The addition of calf thymus DNA (ct-DNA) to Eu**Pfl**–$\mathbf{P^{22}}$ induces an efficient quenching of the Eu-centered luminescence as shown in Figure 2a. The Eu luminescence lifetime is constant during the whole titration demonstrating that the metal ion is not released from the peptide when the complex interacts with DNA. Other spectroscopic features were used to infer into the interaction mechanism of Eu**PPfl**–$\mathbf{P^{22}}$ with ct-DNA. The UV spectrum of Eu**Pfl**–$\mathbf{P^{22}}$ is significantly modified by addition of ct-DNA and shows the characteristic bathochromic ($\Delta\lambda$ = +5 nm) and hypochromic (27%) effects observed when aromatic compounds intercalate into DNA (Figure S7, SI).[16] The modification of the absorption spectrum of Pfl in Eu**Pfl**–$\mathbf{P^{22}}$ is accompanied by a fluorescence quenching of Pfl of ca. 45% (Figure 2b). These features are similar to those evidenced using Pfl alone (Figure S8, SI), which has been demonstrated to intercalate into ct-DNA.[11,17] DNA melting experiments reinforce an intercalative process. Indeed, the melting temperature of ct-DNA was measured in the absence (67 °C) and in the presence (74 °C) of Eu**Pfl**–$\mathbf{P^{22}}$, which evidence an increase of +7 °C when bound to Eu**Pfl**–$\mathbf{P^{22}}$ (Figure S9, SI). This value is slightly lower than Pfl alone, for which we measured a ΔTm value of +9 °C (Figure S10, SI), similar to literature data.[18] The UV, fluorescence, and Eu time-resolved titrations were analyzed for DNA binding with the classical simple binding model (eq 1),[19]

$$X + DNA = C \qquad (1)$$

where X is Pfl or Eu**Pfl**–$\mathbf{P^{22}}$, DNA is taken to be a DNA base pair, and C is the complex with DNA. The association constant,

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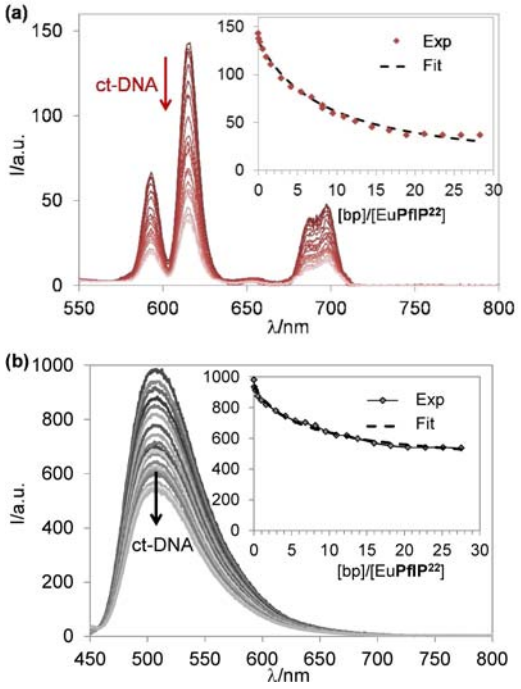

Figure 2. (a) Eu-centered emission and (b) fluorescence during the titration of Eu**Pfl**–$\mathbf{P^{22}}$ (5 μM) with ct-DNA (0–22 μM) in HEPES 10 mM pH 7, NaCl 50 mM at 298 K. Insets: Variation of the maximum intensity; squares are experimental points, and dashed lines represent calculated data.

$K = 10^{4.6(2)}$ M^{-1}, is very similar to the constant obtained for Pfl alone, considering Pfl fluorescence quenching ($K = 10^{4.7(1)}$ M^{-1}).

In conclusion, the introduction of a proflavine DNA intercalating unit into a Ln-binding peptide was successful in enhancing europium sensitization in comparison with the common indole sensitizer found in the natural amino acid tryptophan. Besides, this novel conjugate interacts with ct-DNA as efficiently as the proflavine intercalator itself and benefits from the time-resolved Eu-centered luminescence to detect DNA intercalation thanks to the Eu-binding peptide unit. This first DNA-sensing lanthanide binding peptide is currently modified by varying the nature of the intercalating unit and the peptide sequence in order to optimize both DNA binding and Eu sensitization.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental details and supplementary spectroscopic figures. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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