

COMMUNICATION

Programmed stereoselective assembly of DNA-binding helical metalloptides

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A flexible and versatile synthetic approach for the construction of water-stable DNA-binding chiral peptide helicates and that is based in the solid phase peptide synthesis (SPPS) methodology is reported.

The development of non-natural agents that can reproduce the DNA recognition properties of natural transcription factors remains a major goal in biological chemistry,¹ as they might have a great impact in fundamental and applied biological research, and even lead to the development of gene-targeted therapies.² Most of the efforts in this area have been directed towards the development of small organic binders, but in the past few years there has been a growing interest in the use of coordination and metallo-supramolecular compounds with unique structural and spectroscopic properties.³

Helicates have shown promising and unusual DNA-binding properties,⁴ including major groove binding,⁵ and recognition of DNA three-way junctions.⁶ However, nearly 20 years after the pioneering studies by Prof. Jean-Marie Lehn,⁷ helicates are still not viable alternatives to traditional DNA-binding agents. The slow development in the biological chemistry of metal helicates ultimately derives from the lack of efficient and versatile methodologies for their enantioselective synthesis,⁸ which usually result in mixtures of isomers of difficult separation,⁹ insoluble or unstable species in water,¹⁰ and above all, lack of versatility for structural and functional optimization.¹¹ Prof. Scott recently proposed a new synthetic approach to DNA-binding helicates based in the self-assembly of aldehydes and amines in the presence of M(II) ions that allows the formation of water-stable and optically pure helicates.¹² Although this method represents a great improvement over the traditional routes, it does not guarantee the straightforward access to structural variants to efficiently study the chemical and functional space.

Given our experience in the study of DNA recognition agents,¹³ DNA-binding peptides,¹⁴ metalloptides,¹⁵ and helicates,¹⁰ we decided to investigate into this problem by developing an alternative approach to DNA-binding chiral

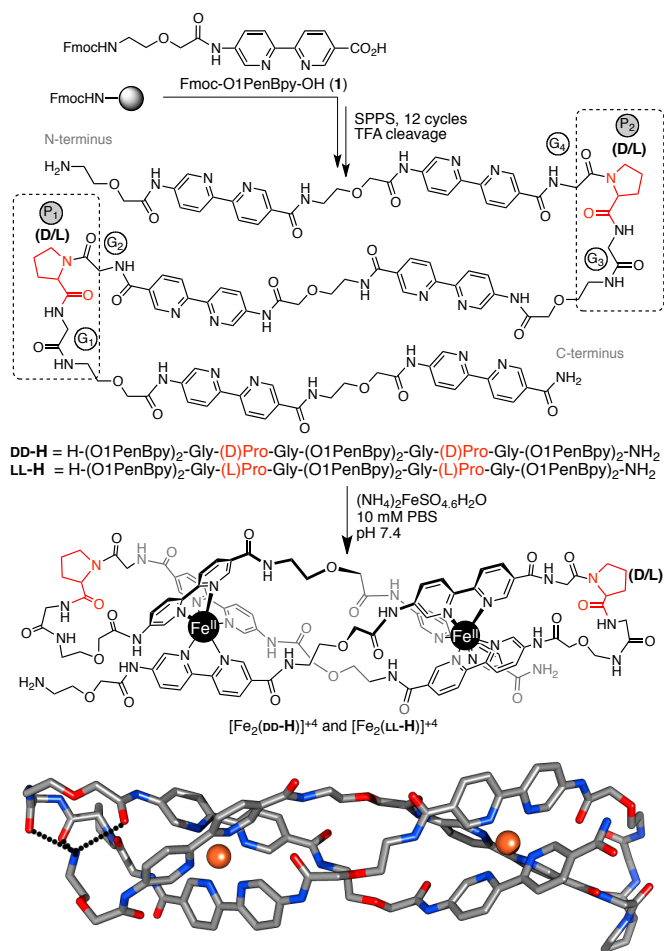
helicates. Herein we report our proposal, which potentially combines the biocompatibility, modularity and structural control of peptides with the synthetic flexibility and versatility of solid-phase peptide synthesis (SPPS) methodology, which allows the straightforward construction and modification of the helicates and, therefore, the easy optimization of their biophysical properties.

2,2'-bipyridine (Bpy) is a privileged chelator that leads to stable complexes with many metal ions that has been extensively used in coordination and supramolecular chemistry.¹⁶ Thus, we decided to synthesize a Bpy analog appropriately modified for its application in SPPS as the basic component of our peptide helicates. Hence, the Bpy unit was derivatized with 5-amino-3-oxapentanoic acid (O1Pen) as a Fmoc-protected achiral amino acid (Fmoc-O1PenBpy-OH, **1**, Scheme 1).¹⁵

Following the synthesis of the amino acid building block, we designed a peptidic ligand capable of folding into a single-stranded dinuclear hairpin helicate; the selected peptide sequence contains six O1PenBpy units, which satisfy the coordination requirements of two octahedral metal centers, arranged in three sets (or substrands) of two consecutive Bpy residues connected through two short loops. The loops, whose structures have been previously optimized,^{15b} are key components of this design, as they include a β -turn promoting -[(D/L)-Pro]-Gly- sequence that preorganize the ligand and induce the folding of the peptidic chain into discrete dinuclear species, thus avoiding the formation of higher order and polymeric complexes in the presence of metal ions (Scheme 1).

In order to gain some information about the structural viability of our design, molecular modeling was performed on the Fe(II) helicate derived from the peptide ligand **LL-H** (Scheme 1). The system was optimized following a hierarchical protocol that consists in: 1) Molecular Dynamics simulation on the loops of the metalloptide; 2) clustering of the resulting MD, and 3) refinement by QM/MM geometry minimization of the most populated cluster representatives (see ESI). The final calculation was performed with solvent and electronic

embedding effects. The lowest energy model presents a helical geometry (Scheme 1) with both Fe(II) ions in octahedral configurations and the six bipyridine rings ordered around the metal centers. The first coordination sphere of the metal ions is barely constrained by the entire peptide framework, indicating that an excellent complementarity between organic and inorganic moieties is achieved in this design.



Scheme 1. Solid phase peptide synthesis of the peptide ligands **LL-H** and **DD-H**. Introducing of L-Pro or D-Pro residues in the loops selects a particular supramolecular chirality in the final helicates ($\Lambda\Lambda$ - or $\Delta\Delta$ -, respectively). Below, molecular representation of the most stable $\Lambda\Lambda$ - $[\text{Fe}_2(\text{LL-H})]^{4+}$ helicate highlighting the interaction of the ammonium group of the N-terminal end with the C-terminal loop.

Supported by the computational results, we synthesized the two enantiomeric peptide ligands, each of them equipped with two L-Pro (**LL-H**) or D-Pro residues (**DD-H**) in their loops (Scheme 1). Both peptidic strands were obtained following standard Fmoc/*t*Bu solid-phase protocols,¹⁷ and purified by reverse-phase HPLC (see ESI). Incubation of 4.0 μM solutions of **LL-H** and **DD-H** in PBS buffer (pH = 5.1, 298 K) with increasing concentrations of Fe(II) resulted in a bathochromic shift of the bipyridine absorption band from 304 to 322 nm, as well as in the appearance of a new band centered at 545 nm, typical of octahedral trisbipyridine Fe(II) complexes (see ESI). The increase in the absorption intensity of the d-d band was

used to calculate the binding constants, which were the same within the experimental error for both **LL-H** and **DD-H**,¹⁸ thus for the first association $\beta_{1,1}(\text{LL-H}) \approx \beta_{1,1}(\text{DD-H}) \approx 12.9 \mu\text{M}$, and the overall formation constants $\beta_{2,1}(\text{LL-H}) \approx \beta_{2,1}(\text{DD-H}) \approx 18.9 \mu\text{M}$.^{19,20} The successful assembly of the helicate, which can be considered as quantitative, was further confirmed by MALDI-TOF mass spectrometry showing a major peak at 2337.9 (see ESI). To our knowledge, SPPS has never been used for the preparation of helicates. Moreover, fully-peptide ligands have not been used before as precursors of metal helicates, and there are only a handful of examples of organic strands equipped with oligopeptides as spacers or functional appendages.²¹

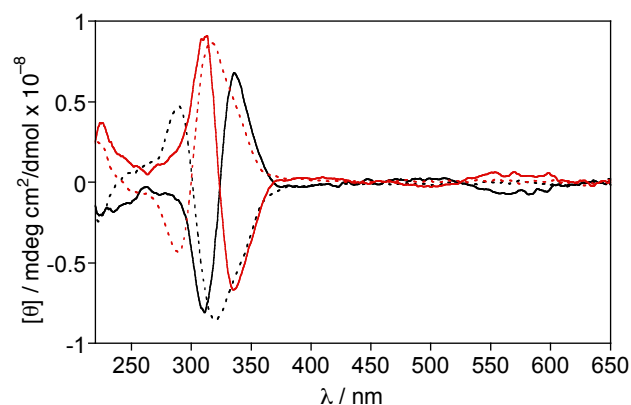


Figure 1. CD spectra of **LL-H** and **DD-H** peptide ligands (50 μM) and their corresponding helicates. **LL-H** before (black solid line, $\Lambda\Lambda$ - $[\text{Fe}_2(\text{LL-H})]^{4+}$) addition of 2 eq. of Fe(II) and **DD-H** before (red dashed line) and after (red solid line, $\Delta\Delta$ - $[\text{Fe}_2(\text{DD-H})]^{4+}$) addition of 2 eq. of Fe(II). All measurements were made in PBS buffer pH = 7.4, 298 K.

As expected, **LL-H** and its enantiomer **DD-H** give rise to mirror image CD spectra with two bands of opposite signs at approximately 290 and 324 nm with a crossover at 303 nm (Figure 1 and ESI). The intensity of these bands decreases upon addition of Fe(II), while at the same time they display a bathochromic shift to 302 and 333 nm, respectively. The sign of the Cotton effect of the helicates is consistent with a Λ -configuration on each metal center for the **LL-H** derivative ($\Lambda\Lambda$ - $[\text{Fe}_2(\text{LL-H})]^{4+}$), and a Δ -configuration for the **DD-H** analog ($\Delta\Delta$ - $[\text{Fe}_2(\text{DD-H})]^{4+}$).²² These data confirm that the enantiomeric peptide ligands give rise to the enantiomeric helicates.²³ This means that the chiral proline residues do not only direct the folding of the peptide chain into a discrete dinuclear helicate, but also encode its chirality, which is selected under thermodynamic control at room temperature in water media upon incubation with Fe(II) ions. Controlling the metal center configuration in helicates usually requires demanding synthetic procedures for obtaining chiral organic ligands,²⁴ which complicates the access to multiple structural variants that are required for the systematic studies involved in the optimization of their biological properties. In our approach, however, the chirality of the peptide helicates arises naturally from two single Pro residues located in the loops connecting the three bisBpy substrands (Scheme 1).

The chiral peptide helicates seem to be very stable in water at room temperature, as no degradation or racemization processes were detected for several weeks. We also performed thermal denaturation experiments in which the changes in absorbance at 525 nm were monitored upon increasing the temperature from 20 to 100 °C. The sigmoidal melting profiles observed for both helicates were consistent with cooperative transitions in the decomplexation of the Fe(II) ions (Figure 2, and ESI). Both melting curves were qualitatively similar; with a T_m temperature of ≈ 80.5 °C. Van't Hoff analysis of the thermal denaturation afforded the enthalpic and entropic changes of the unfolding process. As expected, the unfolding process of the helicate displays a positive entropic variation ($\Delta S \approx 447.1$ J/mol), consistent with the increase in molecular disorder resulting from the disassembly of the complexes; the unfolding process is also highly endothermic ($\Delta H \approx 157.9$ kJ/mol), in agreement with the large association constants obtained in the UV/Vis titrations and reported studies of related supramolecular metal complexes.²⁵ Alternatively, global analysis of the unfolding process using the Gibbs-Helmholtz equation provided qualitatively similar results.²⁶

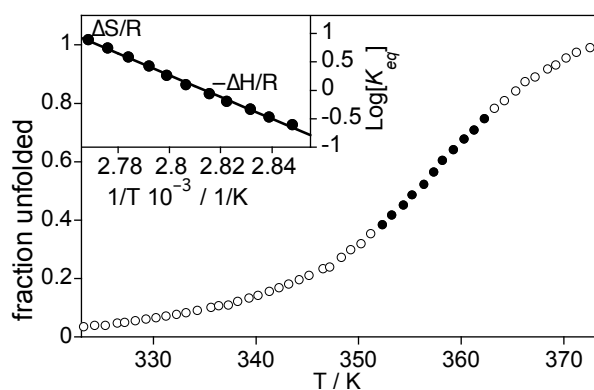
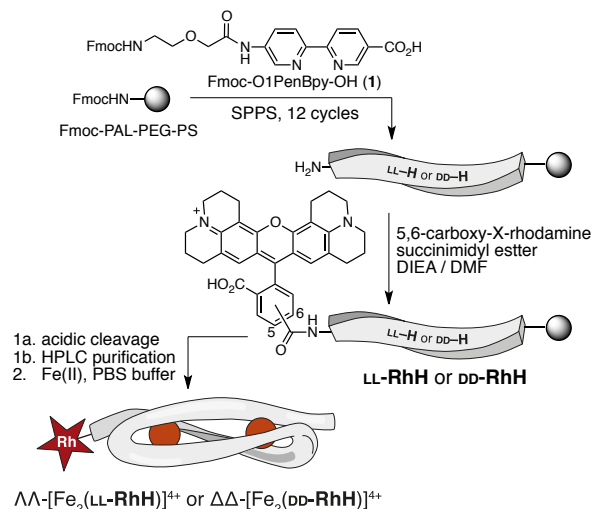


Figure 2. Left: Thermal denaturation curve and Van't Hoff analysis of the thermal denaturation process of a 7 μM solution of $\Lambda\Lambda\text{-}[\text{Fe}_2(\text{LL-H})]^{4+}$ in PBS buffer 10 mM, NaCl 10 mM, pH 7.2. Black points represent the data near the transition used in the Van't Hoff plot shown in the inset.

Having at hand the methodology for the efficient assembly of water-stable and optically pure peptide helicates, we decided to explore the DNA binding properties of these species. In particular, we were interested in studying the affinity and chiral selectivity of their binding to three-way DNA junction,²⁷ data that remain unknown until now.

Towards this end, we synthesized the N-terminal rhodamine labeled derivatives of the previously described **LL-H** and **DD-H** peptide ligands. In short, once the core peptide sequences were fully assembled, and still attached to the solid support, their N-terminal amines were reacted with 5-(and 6)-carboxy-X-rhodamine succinimidyl ester to yield the fluorescent peptidic ligands **LL-RhH** and **DD-RhH**, which were then cleaved from the resin and purified as described before, and incubated with 2 eq. of Fe(II) to form the corresponding fluorescent helicates $\Lambda\Lambda\text{-}[\text{Fe}_2(\text{LL-RhH})]^{4+}$ and $\Delta\Delta\text{-}[\text{Fe}_2(\text{DD-RhH})]^{4+}$ (Scheme 2).



Scheme 2. Solid phase peptide synthesis of the Rhodamine-labeled peptide ligands **LL-RhoH** and **DD-RhoH** by solid phase modification of the core peptides, and their corresponding helicates ($\Lambda\Lambda\text{-}[\text{Fe}_2(\text{LL-RhoH})]^{4+}$ or $\Delta\Delta\text{-}[\text{Fe}_2(\text{DD-RhoH})]^{4+}$ respectively). See Scheme 1 for complete sequences of **LL-H** and **DD-H**.

Incubation of 1 μM solutions of the rhodamine-labeled helicates with increasing concentrations of the DNA three-way junction **Y₁Y₂Y₃** (see ESI) produced a concentration-dependent increase in the fluorescence anisotropy that could be fitted to a modified 1:1 binding mode, including higher order aggregates resulting from non-specific interactions (Figure 3).¹⁴ The $\Lambda\Lambda\text{-}[\text{Fe}_2(\text{LL-RhH})]^{4+}$ peptide helicate displayed significantly higher affinity—almost 150 fold—for the **Y₁Y₂Y₃** DNA junction than the enantiomeric $\Delta\Delta\text{-}[\text{Fe}_2(\text{DD-RhH})]^{4+}$, with apparent dissociation constants for the 1:1 complexes of 0.25 ± 0.04 μM and 37.6 ± 1.0 μM , respectively.²⁸

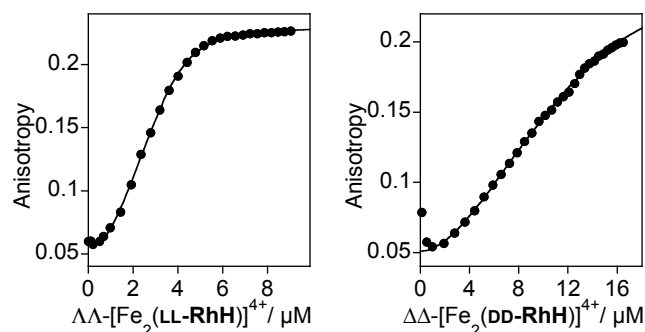


Figure 3. Fluorescence anisotropy titrations of 1 μM solutions of peptidic helicates with a DNA three-way junction (**Y₁Y₂Y₃**) in 10 mM phosphate buffer, NaCl 10 mM; pH: 7.3, 20 °C. $\Lambda\Lambda\text{-}[\text{Fe}_2(\text{LL-RhH})]^{4+}$ (left) and $\Delta\Delta\text{-}[\text{Fe}_2(\text{DD-RhH})]^{4+}$ (right). Lines represent the best fit to 1:1 binding models including the contribution of nonspecific electrostatic complexes. DNA oligo sequences: **Y₁**: 5'-CAC CGC TCT GGT CCT C-3'; **Y₂**: 5'-CAG GCT GTG AGC GGT G-3'; **Y₃**: 5'-GAG GAC CAA CAG CCT G-3'.

In summary, herein we present a simple and versatile methodology for the enantioselective assembly of water-stable and DNA-binding helicates. This novel approach relies in the modularity and synthetic flexibility of solid-phase peptide synthesis and in the preorganization of the peptide ligand provided by two single proline residues, which also encode the

helical chirality of their corresponding helicates formed under thermodynamic control in water media in the presence of Fe(II) ions. We have demonstrated the potential of this approach synthesizing fluorescently-labeled helicates that show chiral discrimination and high selectivity for three-way DNA junctions.

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Electronic Supplementary Information (ESI) available: synthetic procedures and characterization of peptide ligands and helicates, computational details, spectroscopic studies (UV, CD, melting). See DOI: 10.1039/c000000x/

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