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Assembly at the DNA

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Reversible supramolecular assembly at specific DNA sites: Ni-promoted, bivalent DNA binding with designed peptide and bipyridyl-bisbenzamidine components

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(Dedication---optional)

Transcription Factors (TFs) are specialized proteins that regulate gene expression by binding to specific DNA regulatory sequences.^[1] TFs are grouped into families according to the structure of their DNA binding domains, and in many cases their interaction with the DNA occurs as part of multimeric complexes. It is also known that the DNA recognition process by many TFs is coupled to the folding of their DNA binding domains into well-defined secondary structures, typically α -helices, which create a complementary contacting surface with the DNA major groove.^[2] In some cases, such as in the zinc-finger family, the α -helical folding is mediated by coordination of a metal ion (Zn^{+2}) to amino acid side chains, typically Cys and His residues.^[3]

As a result of their central biological role, there has been a great interest in the development of miniaturized models of TFs capable of reproducing the DNA binding properties of the natural proteins.^[4, 5] The more successful approaches have relied either on engineering the DNA binding regions into pre-folded secondary structures,^[6] or in the conjugation of these peptidic modules to other DNA-binding units, such as minor groove binders.^[7] Also, in the context of mimicking nature, several groups have developed synthetic constructs whose DNA recognition properties can be modulated by application of external stimuli, such as light or metal ions.^[8]

Herein we present a new approach for the specific recognition of DNA sequences that combines in a single system many of the attributes of these previous designs: bivalence, conditional folding, responsiveness to external stimuli (metal ions), and even reversibility. The strategy, which relies on a planned, dual role for nickel (II) as both a dimerizing agent and folding promoter, involves a programmed self-assembly of dissimilar components.

Design and synthesis: As starting point for our design we chose the yeast transcription factor GCN4, an archetypical bZIP TF that specifically binds to ATF/CREB (5'-ATGA(c/g)TCAT-3') or AP1 (5'-ATGA(c)TCAT-3') sites as a leucine zipper-mediated dimer of uninterrupted α -helices.^[9] The DNA interaction occurs through the N-terminal basic regions, which undergo a transition from a disordered structure to an alpha helix upon DNA binding.^[10] While an isolated basic region peptide is unable to interact to its DNA consensus site, we have shown that appropriate conjugation to other DNA binders, such as distamycin analogues or bisbenzamidines, results in hybrid systems that can recognize composite target sites.^[7]

Relying on these precedents, we considered an alternative method for connecting the basic region peptide and the minor

groove-binding unit based on their coordination to a transition metal and formation of a heterodimeric complex. Moreover, we envisioned that an appropriate modification of the basic region might allow the use of the metal not only as tethering unit but also as a promoter of the required α -helical folding.^[11] Therefore, we decided to make a GCN4 basic region derivative in which two amino acids located in consecutive helical turns are mutated to histidines (His). In particular, we synthesized the peptide **brHis₂**, featuring the amino acids Asp226-Arg249 of the GCN4 DNA binding domain, but with residues Leu230 and Arg234 replaced by His. The addition of Ni(II) or related metal ions to this peptide might induce the formation of an α -helix by nucleating the N-terminal turns, while simultaneously providing a coordination site for bisbenzamidine derivatives equipped with a bipyridine ligand.^[12] Given that the bisbenzamidine binds the minor groove of A/T rich DNA sites,^[13] the bipyridine derivative might be able to recruit the modified GCN4 basic region (**brHis₂**) into the adjacent major groove featuring a consensus DNA binding sequence (Figure 1).^[14]

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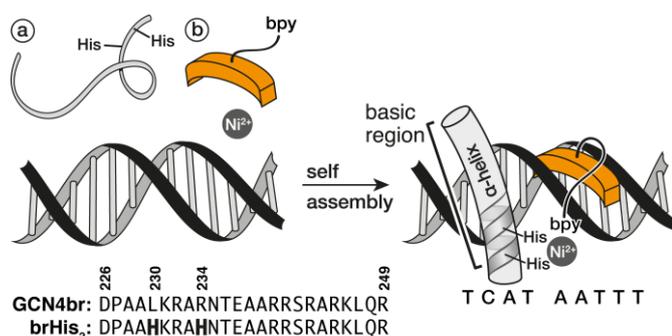
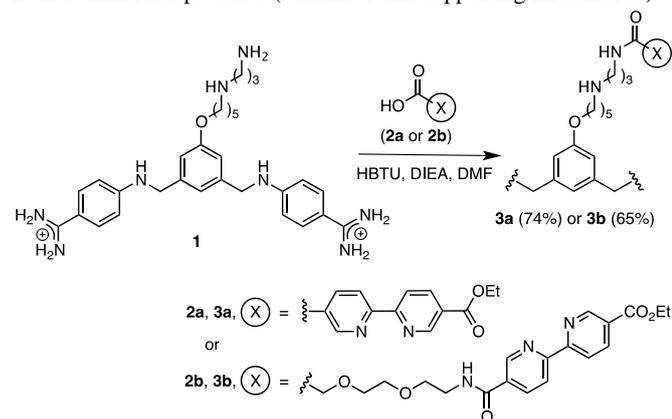


Figure 1. Outline of the cooperative assembly strategy. (a) Unfolded **brHis₂** that will fold into an α -helix in the complex (right); (b) *Bisbenzamide* minor groove binder. **brHis₂**: *Aba*-DPAAHKRAHNTAARRSRARKLQR-*NH₂*; “bpy” : bipyridine chelator tethered to the *bisbenzamide*; and *Aba*: 4-Acetamidobenzoic acid.

The GCN4-based peptide **brHis₂** was synthesized following standard SPPS procedures. On the other hand, we synthesized two *bisbenzamide*-bipyridine derivatives featuring linkers of different lengths between the DNA-binding *bisbenzamide* and the bipyridine ligand (**3a** and **3b**); both constructs were assembled from the aminobisbenzamide **1**, which was synthesized in three steps from commercial products (Scheme 1 and supporting information).



Scheme 1: Synthesis of bipyridine-*bisbenzamide* derivatives for coordination with the GCN4-based peptide.

DNA binding studies: The DNA binding properties of the synthetic constructs were studied by electrophoretic mobility assays (EMSA) in polyacrylamide gel under non-denaturing conditions, and using SybrGold as DNA stain. As expected, incubation of peptide **brHis₂** in the presence of *bisbenzamidines* **3a** or **3b**, with the ds-oligonucleotide **AP1^{hs}•A/T**, which contains the consensus target sequences in adjacent positions, does not induce the formation of retarded bands in the gel (Figure 2a, lane 2). However, addition of increasing concentrations of $\text{Ni}(\text{ClO}_4)_2$ to the previous mixtures generates clear retarded bands (Figure 2a, lanes 3-5). In the case of **3b** these bands are fainter and show some smearing (Figure 2a, lanes 6-8), and therefore the *bisbenzamide* **3a** was chosen for further characterization. In agreement with the proposed interaction model, no new slower-migrating bands were observed when the mixtures do not include the minor groove binding component **3a** (figure 2b, lane 2); but the formation of the retarded band is again restored if **3a** is added to the mixture of the other components (Figure 2b, lanes 3-5). This result confirms the requirement of the bipyridine-equipped minor groove anchor for specific DNA binding of the peptide moiety. It was also interesting to observe that the complex can also

be assembled upon addition of increasing amounts of **brHis₂** onto a mixture of **AP1^{hs}•A/T**, $\text{Ni}(\text{II})$ and **3b** (Figure 2, lanes 7-9).

Taken together, these results indicate that the system dynamically assembles independently of the order in which the components are mixed, and any potential competitive homodimeric [$(\text{brHis}_2)_2\text{Ni}^{2+}$] or [$(\text{3a})_2\text{Ni}^{2+}$] species are kinetically labile and evolve towards the desired [$(\text{brHis}_2)(\text{3a})\text{Ni}^{2+}$] complex in the presence of the target DNA oligo **AP1^{hs}•A/T**.

We next performed a forward titration by adding premixed combinations of equimolar amounts of **brHis₂**, **3a**, and $\text{Ni}(\text{ClO}_4)_2$ (5 equiv), to a ³²P-labeled **AP1^{hs}•A/T** dsDNA oligonucleotide. Considering the [$(\text{brHis}_2)(\text{3a})\text{Ni}^{2+}$] complex as a single species binding to the DNA we could calculate an approximate dissociation constant around 0.45 μM at 4 °C (Figure 3a), albeit we acknowledge that this is an oversimplification of a much more complex equilibrium.^[7e]

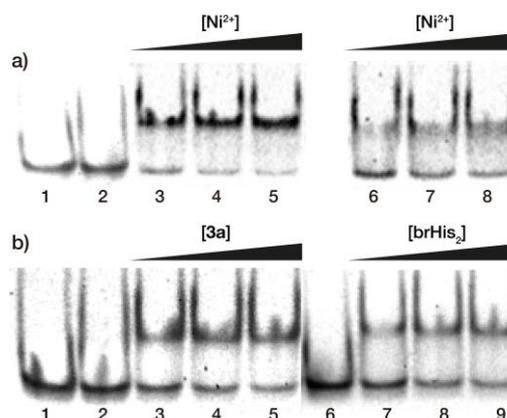


Figure 2. Comparative EMSA analysis of the DNA binding processes.

a) Lanes 1-8: **AP1^{hs}•A/T** (0.1 μM); lane 2-5: 1 μM of **brHis₂** and **3a**, lanes 3-5: 0.6, 1 and 1.6 μM of $\text{Ni}(\text{ClO}_4)_2$; lanes 6-8: 1 μM of **brHis₂** and **3b**, lanes 6-8: 0.6, 1 and 1.6 μM of $\text{Ni}(\text{ClO}_4)_2$; b) Lanes 1-9: **AP1^{hs}•A/T** (0.1 μM); lane 2-5: 0.8 μM of **brHis₂** and 1.6 μM of $\text{Ni}(\text{ClO}_4)_2$; lanes 3-5: 0.8, 1 and 1.6 μM of **3a**; lane 6-9: 0.8 μM of **3a** and 1.6 μM of $\text{Ni}(\text{ClO}_4)_2$; lanes 7-9: 0.8, 1 and 1.6 μM of **brHis₂**. The protocol for fluorescence EMSAs involved addition of peptide solutions in 20 mM Tris-HCl pH 7.5 to 18 mM Tris-HCl pH 7.5, 90 mM KCl, 1.8 mM MgCl_2 , 9% glycerol 0.11 $\text{mg}\cdot\text{mL}^{-1}$ BSA, 2.25% NP-40 and 100 nM of DNA (4 °C, 30 min) and loading into the gel. Products were resolved by PAGE on a 10% nondenaturing polyacrylamide gel and 0.5X TBE buffer over 40 min at 4 °C and by staining with SybrGold (5 μL in 50 mL of TBE) for 10 min, followed by fluorescence visualization. *Note:* we needed to run the EMSA in the absence of EDTA to avoid sequestration of the nickel cation, this is the reason of the smearing of the bands. Oligo sequence **AP1^{hs}•A/T**: 5'-ACGAACG TCAT•AATTT CCTC -3' (peptide binding site in italics and minor groove binding site underlined, only one strand shown).

We next studied the sequence selectivity of the system by using other dsDNAs containing specific mutations. Not surprisingly, with DNAs like **AP1^{hs}•mA/T** (TCAT•AGTTT), which lack the A/T rich tract required for binding of the minor groove agent, we do not observe the formation of retarded bands. Moreover, we neither observe stable DNA complexes with oligonucleotides mutated in the recognition site of the peptide (**mAP1^{hs}•A/T**, TCGT•AATTT). This result contrasts with previously studied covalent derivatives that do bind to these DNAs with a substantial affinity.^[7c,d]

The system is even capable of discerning sequences that feature shorter A/T rich binding regions, such as in **AP1^{hs}•sA/T** (TCAT•AATT) (Figure 3c). This selectivity could be ascribed to the lower affinity of **3a** for AATT versus AATTT, as revealed by

fluorescence titration experiments ($K_D \approx 0.17 \mu\text{M}$ for **AP1^{hs}•sA/T**, versus a $K_D \approx 0.035 \mu\text{M}$ with **AP1^{hs}•A/T**, see the supporting information).^[15]

The above observations highlight the higher specificity attainable through cooperative recognition in noncovalent multicomponent systems.

Not surprisingly, a truncated derivative of the basic region lacking the N-terminal capping motif (Asp-Pro-Ala)^[16] failed to promote the formation of retarded bands in EMSA experiments (see the supporting information). Likewise, a related GCN4 basic region peptide, featuring a cysteine residue instead of the histidine at position 230, also failed to yield the desired DNA-binding assembly, either in the presence of nickel, or of other metal ions (see the supporting information).

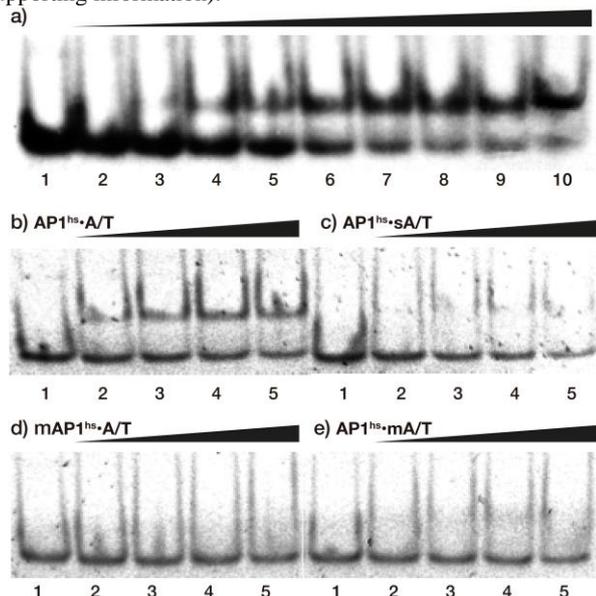


Figure 3. a) Radioactive EMSA titration of with the mixture of the target dsDNA with a mixture of **brHis₂**, **3a** and $\text{Ni}(\text{ClO}_4)_2$. Lanes 1-10: **AP1^{hs}•A/T** (50 nM, ~ 100 pM labeled with P^{32}); Lanes 2-10: 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.7, 0.8 and 1 μM of an equimolecular mixture of **brHis₂** and **3a**, in presence of 5 equiv of $\text{Ni}(\text{ClO}_4)_2$. b-e) EMSA analysis (SybrGold staining) of the DNA selectivity. Lanes 1-5 in gels b-e contain the indicated ds-oligonucleotide (0.1 μM) and 0, 0.2, 0.4, 0.6 and 0.8 μM of an equimolecular mixture of **brHis₂** and **3a**, with 5 equiv of $\text{Ni}(\text{ClO}_4)_2$. b) **AP1^{hs}•A/T**. c) **AP1^{hs}•sA/T**. d) **mAP1^{hs}•A/T**. e) **AP1^{hs}•mA/T**. Oligonucleotide sequences (only one strand is shown) : **AP1^{hs}•sA/T**: 5'-ACGAACG TCAT•AATT CCTC-3', **mAP1^{hs}•A/T**: 5'-ACGAACG TCGT•AATT CCTC-3', **AP1^{hs}•mA/T**: 5'-ACGAACG TCAT•AGTTT CCTC-3'.

We also studied the selectivity of the system with regard to the metal ion (Co^{2+} , Ni^{2+} , Cd^{2+} , Zn^{2+} , Cu^{2+} , Hg^{2+} , and Fe^{2+} salts). Using comparable conditions, we only observed the formation of slower-migrating bands in the presence of Ni^{2+} (see the supporting information). Therefore it seems that nickel has the right coordination properties to promote an effective heterodimeric assembly in the presence of the cognate DNA.^[17]

As expected for a rather unstructured peptide, the circular dichroism spectrum of **brHis₂**, even in the presence of **3a** and **AP1^{hs}•A/T**, presents a relatively weak negative signal at 222 nm (Figure 4 left, curve **b**); addition of $\text{Ni}(\text{ClO}_4)_2$ to the mixture promotes a considerable increase of the negative ellipticity intensity at 222 nm, consistent with the folding of the peptide chain into an α -helical conformation (Figure 4 left, curve **c**).^[18]

The key role of the nickel ion as adaptor component mediating the assembly of the system prompted us to check the possibility of dismantling the DNA complex by using an external nickel chelator. Achieving stimuli-responsive reversibility in DNA binders is a main challenge that cannot be easily accomplished.^[8] Gratifyingly, addition of 10 equiv of EDTA to the supramolecular complex resulting from mixing **brHis₂**, **3a**, Ni^{2+} and **AP1^{hs}•A/T** promoted a drastic decrease in the helicity of the peptide (Figure 4 left, curve **d**), which correlates with the disruption of the DNA complex, as demonstrated by EMSA experiments (inset in the figure). Therefore, while the presence of nickel is critical for the DNA interaction, the resulting multicomponent supramolecular complex can be dismantled by the addition of an external nickel chelator.

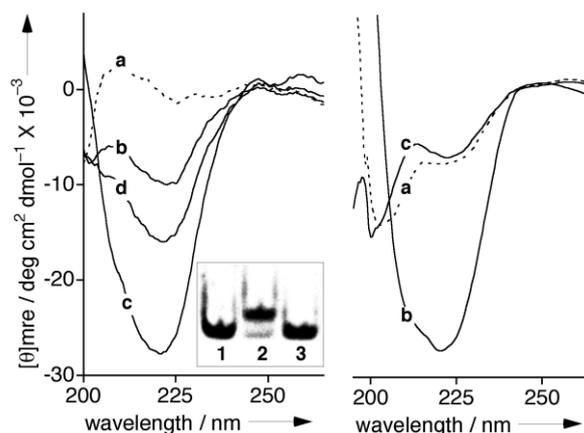


Figure 4. Left: Circular dichroism spectra of different components in presence of **AP1^{hs}•A/T** (5 μM) in Tris-HCl buffer 20 mM, pH 7.5, NaCl 100 mM at 20°C. a) **3a** (5 μM); b) **3a** (5 μM) and **brHis₂** (5 μM); c) **3a** (5 μM), **brHis₂** (5 μM) and $\text{Ni}(\text{ClO}_4)_2$ (10 μM); d) same as in a) after addition of 20 equiv of EDTA; 30 equiv of EDTA leads to a full recovery of the initial helicity (data not shown). Inset: EMSA analysis (SybrGold staining). Lanes 1-3: **AP1^{hs}•A/T** (100 nM); lane 2: 1000 nM of **brHis₂** and **3a**, and 5 equiv of $\text{Ni}(\text{ClO}_4)_2$; lane 3: 1 μM of **brHis₂** and **3a**, and 5 equiv of $\text{Ni}(\text{ClO}_4)_2$ after addition of 1.8 mM of EDTA. Right: Circular dichroism spectra of **3a** (5 μM) and **brHis₂** (5 μM): a) in presence of 2 equiv of $\text{Ni}(\text{ClO}_4)_2$; b) same as in a) after addition of the consensus DNA **AP1^{hs}•A/T**; c) same as in a) after addition of the mutated **mAP1^{hs}•A/T** oligonucleotide. The contribution of the **AP1^{hs}•A/T** oligo has been subtracted from the spectra.

Importantly, control experiments showed that mixing $\text{Ni}(\text{ClO}_4)_2$ with the peptide **brHis₂** does not promote an α -helical folding, even in the presence of the bispyridine partner **3a** (Figure 4 right, curve **a**). However, addition of the DNA **AP1^{hs}•A/T** to this mixture induces the formation of the α -helix (Figure 4 right, curve **b**). As expected, a mutated ds oligonucleotide (**mAP1^{hs}•A/T**) does not induce such folding (Figure 4 right, curve **c**). Therefore, the presence of $\text{Ni}(\text{II})$ is not sufficient for inducing the folding transition of the peptide into an α -helix, which also requires the template effect of the target DNA.

In summary, we have introduced a new approach for achieving a highly selective, bivalent recognition of designed nine DNA base pairs. The strategy involves the nickel-promoted assembly of instructed components consisting of a bishistidine-modified peptide derived from a bZIP transcription factor, and a bisbenzimidazole equipped with a bipyridine unit. Key for the success of the approach is the dual role of the metal as an α -helix-nucleating factor and as heterodimerizing staple. The multicomponent nature of the system,

and the kinetic lability of the metal coordination, facilitates the disassembly of the supramolecular structure upon addition of external agents that sequester the nickel cation. Overall, we have devised a supramolecular system with emergent properties that reproduces some key characteristics of naturally occurring DNA binding proteins, such as bivalence, selectivity, responsiveness to external agents and reversibility. The system represents an infrequent case of self-assembly, as it involves four different components: a metal, a peptide, a small molecule and a nucleic acid.

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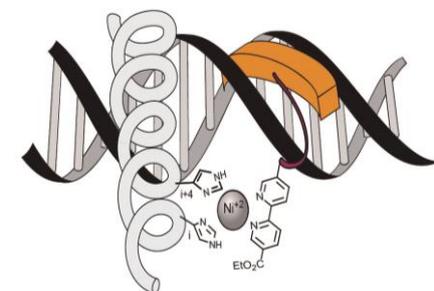
Keywords: DNA recognition · metallosupramolecular chemistry · peptides · self assembly · minor groove binder

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Assembly at the DNA

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**Reversible supramolecular assembly
at specific DNA sites: Ni-promoted,
bivalent DNA binding with designed
peptide and bipyridyl-*bis*benzamidine
components**



Nickel (II) salts promote the assembly, at specific DNA sites, of instructed components consisting of a bis-histidine-modified peptide derived from a bZIP transcription factor, and a *bis*benzamidine unit equipped with a bipyridine. This programmed supramolecular system with emergent properties reproduces some key characteristics of naturally occurring DNA binding proteins, such as bivalence, selectivity, responsiveness to external agents and reversibility.