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COMMUNICATION

Ruthenium Bipyridyl Complexes as Photocleavable Dimerizers: Deactivation of DNA-Binding Peptides Using Visible Light

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We report a photolabile biselectrophilic Ru(II) complex that can be used for homo- or heterodimerization of cysteine-containing peptides. The resulting dimers can be efficiently disassembled by long-wavelength light. As proof-of-concept, we describe the preparation of homo- and heterodimeric bZIP peptides whose DNA-binding properties can be turned off using visible light.

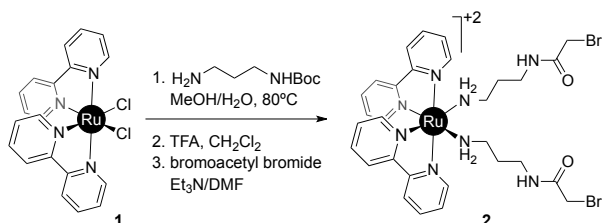
Cells respond to external signals that modulate a wide variety of processes through the production and/or activation of biomolecular agents.¹ Likewise, scientists have long pursued to mimic this type of natural control mechanisms by developing molecules that become functional only after receiving an external stimulus.² This has been usually approached through the synthesis of bioactive compounds masked with photosensitive protecting groups—caged compounds—so that irradiation with UV light releases the parent molecules, thus triggering a biological response.³ In this context, we have developed several light-activated DNA-binding agents,⁴ and peptide derivatives,⁵ which rely in the use of *o*-nitrobenzyl derivatives as UV-photolabile groups.⁶ Curiously, while the photoactivation of biological functions has been widely applied, the inverse approach involving light-induced deactivation has been scarcely studied.⁷ Photocontrolled DNA binding of peptides and proteins has also been attained through the incorporation of azobenzene chromophores into their DNA recognition domains.⁸ Although some of these derivatives have even been applied in cellular contexts,⁹ the *azo*-photoswitches typically suffer from small differences in DNA affinities of their ON and OFF states, as well as difficulties for obtaining well-shifted equilibria.¹⁰

Herein we describe a light-sensitive bis-electrophilic dimerizing linker based on a Ru(II) complex, and its application for the development of DNA binding agents whose interaction with the DNA can be suppressed by irradiation. Importantly, and in contrast with common photolabile groups that require the use of UV light typically below or around 360 nm,¹¹ the deactivation process is promoted by long-wavelength visible light,¹² which is much less harmful in biological environments.¹³ Key elements behind our design are the need of a dimeric state for an efficient DNA binding

by bZIP peptides, and the ability of Ru(II) bipyridyl complexes to act both as dimerizers and as photo-cleavable units.

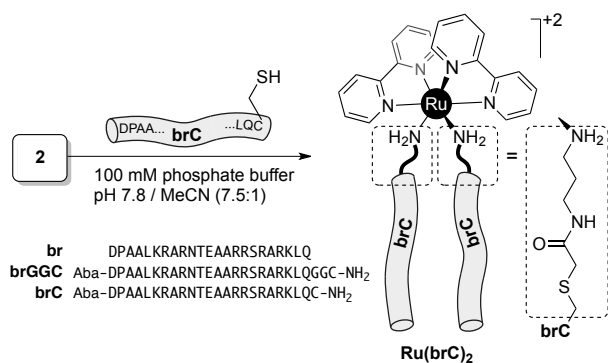
The bZIP (basic region leucine zipper) proteins are a family of eukaryotic transcription factors (TF) that bind to specific DNA sequences as homo or heterodimers.¹⁴ bZIP TFs contain a highly charged N-terminal basic region (**br**) that makes specific contacts with the DNA major groove, and a C-terminal coiled-coil leucine zipper domain that mediates dimerization.¹⁵ It is known that monomeric basic regions do not interact with significant affinity with their DNA targets, unless they are linked to other DNA binders,¹⁶ or engineered into prefolded constructs.¹⁷ Alternatively, it is also possible to obtain efficient DNA binders by replacing the leucine zipper dimerization domain with artificial connectors.¹⁸ In this context, we envisioned that an artificial photocleavable linker connecting two bZIP basic regions might allow for a light-triggered transition between DNA-binding dimeric peptides and inactive monomeric species. In this way we could disable a DNA interaction through the application of light, and hence set the basis for the conditional photo-deactivation of biological functions.

In order to avoid the use of high-energy UV light, which is typically required for the cleavage of standard *o*-nitrobenzyl cages,^{6,11} we decided to design other dimerizing platforms that could be cleaved using long wavelength light. Towards this aim we were attracted by the work of R. Etchenique and coworkers on the use of ruthenium(II) bipyridyl complexes as photolabile amine caging groups.¹⁹ Although these complexes have never been used in peptides or as temporary connectors, they were attractive for our purposes owing to their sensitivity to visible light. On the other hand, considering the versatility and wide applicability of cysteine alkylations for the selective bioconjugation of peptides and proteins, we decided to synthesize a photoactive ruthenium complex in the form of a bis-electrophilic moiety containing two thiol-reactive bromoacetyl units.²⁰ Thus, the reagent **2** was readily obtained by treatment of the commercially available precursor *cis*-dichlorobis(2,2'-bipyridine)ruthenium(II) (Ru(bpy)₂Cl₂, **1**) with excess of *N*-Boc-1,3-propanediamine, followed by removal of the Boc protecting groups and acylation of the resulting amines with 2-bromoacetic acid (Scheme 1). The whole process was accomplished with an overall yield of 34%.



Scheme 1. Synthesis of the electrophilic linker **2**.

As reference system for implementing the strategy we chose the GCN4 transcription factor, an archetypical bZIP protein that specifically binds to the ATF/CREB (5'-ATGAcgTCAT-3') or AP1 (5'-ATGA(c/g)TCAT-3') sites.¹⁴ The DNA-binding peptide sequence (**br**) was based on earlier studies that identified the minimum-length of the GCN4 basic region that retains the DNA binding properties of the full domain when engineered as a disulfide dimer, such as in (**brGGC**)₂SS (Scheme 2).¹⁸ The photolabile dimer **Ru(brC)**₂ was assembled by treatment of dibromide **2** with 2.2 equiv of the core GCN4 basic region containing a C-terminal nucleophilic Cys (**brC**),²¹ in 100 mM aqueous phosphate buffer (pH 7.8) containing 15% of acetonitrile. HPLC analysis of the reaction showed the complete disappearance of the dibromide after 1 h at rt, and the formation of a new major product identified as the expected hybrid by HPLC-MS (isolated in yields over 30%).



Scheme 2. Synthesis of the GCN4 basic region dimer **Ru(brC)**₂ by bis-alkylation with the reactive linker **2**. **br**: Sequence of the minimum GCN4 basic region capable of high affinity DNA binding as a disulfide dimer. Aba = acetamido benzoyl (included as a chromophore to quantify the peptide).

The DNA binding of **Ru(brC)**₂ was first studied by electrophoretic mobility shift assays (EMSA) under non-denaturing conditions,²² and using SYBR gold for DNA staining.²³ Thus, when a double stranded oligonucleotide containing the target ATF/CREB site (**ATF/CREB**) is incubated with increasing concentrations of **Ru(brC)**₂ in Tris-HCl buffer at 25 °C, we observe new retarded bands consistent with the formation of a specific DNA/**Ru(brC)**₂ complex (Fig. 1, lanes 2–8, band b). The shift of these bands is in line with that observed when the same oligonucleotide is incubated with the reference disulfide dimer (**brGGC**)₂SS (Fig. 1, lanes 9–10, band c).¹⁸ Control EMSA experiments with oligos containing random sequences do not show retarded bands, thus confirming that **Ru(brC)**₂ is a highly selective DNA binder (see the ESI). Moreover, in agreement with the EMSA, circular dichroism experiments reveal that addition of 1 equiv of the dsDNA **ATF/CREB** to a 5 μM solution of **Ru(brC)**₂ promotes a significant increase in the ellipticity of the negative bands at 208 and 222 nm, consistent with the folding of the basic regions into α-helices (see the ESI).^{15,18} Fluorescence anisotropy titrations using a TAMRA-labeled

ATF/CREB confirmed the high affinity interaction of the peptide dimer with the DNA, with an apparent K_D of 12 nM at 20 °C (see the ESI), which is over three times stronger than that of (**brGGC**)₂SS.^{5,24} The DNA interaction in presence of excess of competing calf thymus DNA displays a decreased—but still significant— K_D of 190 nM.

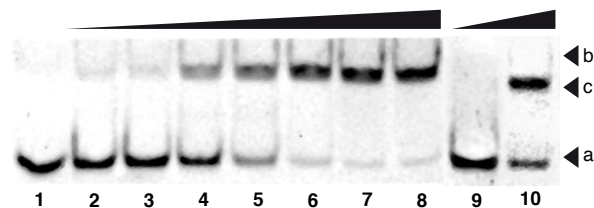


Fig. 1 DNA binding of **Ru(brC)**₂ studied by EMSA. Lanes 1–10: 100 nM target **ATF/CREB**; lanes 2–8: 50, 100, 200, 300, 450, 750, 1000 nM **Ru(brC)**₂; lane 10: 450 nM (**brGGC**)₂SS. Band **a** corresponds to the free oligonucleotide; slow-migrating bands **b** and **c** are consistent with the complexes between the DNA and the dimeric peptides **Ru(brC)**₂ and (**brGGC**)₂SS, respectively. Peptides and dsDNA were mixed in 18 mM Tris-HCl pH 7.5, 90 mM KCl, 1.8 mM MgCl₂, 1.8 mM EDTA, 9% glycerol, 0.11 mg/mL BSA, 2.25% NP-40 for 30 min at 20°C, and loaded into gel. Gels were run on 10% nondenaturing polyacrylamide and 0.5X TBE buffer over 40 min, and stained with SyBrGold (5 μL in 50 mL of 1X TBE) for 10 min. **ATF/CREB** (binding site in italics, only one strand is shown): 5'-CGG *ATGACGTCAT* TTTTTC-3'.

We next studied the photolysis of **Ru(brC)**₂ in the absence of DNA. Interestingly, irradiation of a ≈ 20 μM solution of the complex **Ru(brC)**₂ in milliQ water for 20 min with a long-wavelength light source (530–550 nm) or for 2 min with a 455 nm LED source, led to the almost complete photolysis of the starting complex.²⁵ HPLC analysis of the irradiated mixture showed the formation of two new products with UV and MS spectra consistent with the asymmetric cleavage of the linker and the release of one of the **brC** arms as a terminal amine, while the metal complex remains attached to the other **brC** peptide (Fig. 2).

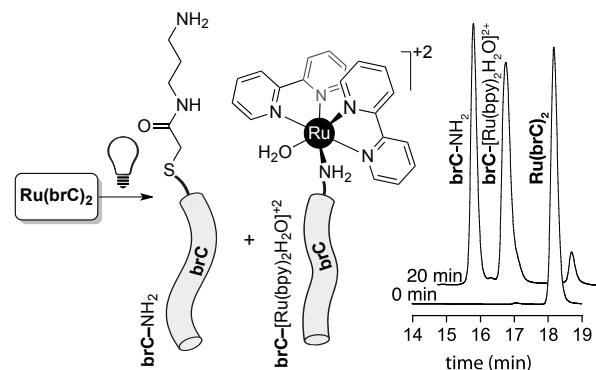


Fig. 2 Left: Photolysis of **Ru(brC)**₂ and observed products. Right: HPLC of the photolysis of **Ru(brC)**₂.

Importantly, the ruthenium dimer can also be efficiently photolyzed in the presence of the target DNA without causing damage of the DNA (see the ESI). Therefore EMSA analysis of the mixture of **Ru(brC)**₂ and the oligo **ATF/CREB** after 30 min of irradiation did not show retarded bands corresponding to the **Ru(brC)**₂/DNA complexes (Fig. 3, left), demonstrating the loss of DNA binding. These results were further supported by fluorescence anisotropy experiments that showed a progressive decrease in the fluorescence anisotropy of a TAMRA-labeled **ATF/CREB**

oligonucleotide in the presence of the **Ru(brC)₂** dimer upon continuous irradiation, so that after approximately 20 minutes the anisotropy value was in the range of that measured for the oligonucleotide alone (Fig. 3, right). Taken together, these results demonstrate the development of functional bZIP dimeric peptides whose DNA binding can be suppressed upon irradiation with visible light, and support the use of the bis-electrophilic reagent **2** as an effective visible-light photolabile dimerizer.

Since many biological interactions rely on the formation of heterodimeric assemblies, we were intrigued by the possibility of applying the same strategy for controlling the activity of functional heterodimers. In particular, we approached the synthesis of **Ru(brC)(ebpC)**, a metallopeptide construct featuring the basic regions of GCN4 and of the enhancer binding protein C/EBP.²⁶ The dimer was assembled in a two-step protocol involving an initial reaction between the dibromide **2** and a C-terminal Cys derivative of the C/EBP basic region (**ebpC**: *Aba*-NEYRVRRRERNNIAVRKSRD KAKQC, 1.3 equiv), followed by a second coupling with the previously described GCN4 basic region peptide (**brC**, 2.5 equiv).

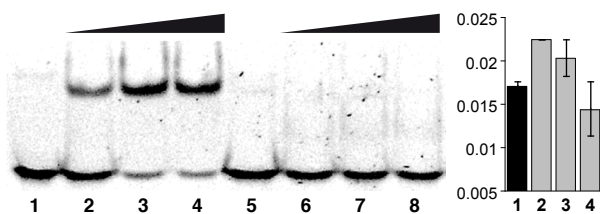


Fig. 3 Photolysis of **Ru(brC)₂**. Left: Photolysis of 8 μ M **Ru(brC)₂** in presence of the target **ATF/CREB** oligonucleotide (1 μ M) studied by EMSA (same conditions as described before). Lanes 1–8, 100 nM **ATF/CREB**; lanes 2–4: 200, 400, 600 nM **Ru(brC)₂** before irradiation; lanes 6–8: 400, 600, 800 nM **Ru(brC)₂** after irradiation (30 min). Right: Effect of the irradiation on the measured fluorescence anisotropy of a **Ru(brC)₂** and TAMRA-ATF/CREB mixture in 20 mM Tris-HCl buffer pH 7.5, 100 mM NaCl. bar 1: TAMRA-ATF/CREB; bar 2: 25 nM TAMRA-ATF/CREB and 175 nM **Ru(brC)₂**; bars 3 and 4: same as in 2, after 10 min and 20 min irradiation, respectively.

EMSA revealed that incubation of the dimer with an oligo containing the target composite sequence (**CE/CR**) generates slow-migrating bands consistent with the formation of the expected **Ru(brC)(ebpC)**/DNA complex. Moreover, as in the case of the homodimer, this complex can be effectively disassembled by irradiation with long-wavelength light (530–550 nm, 30 min) as shown by EMSA and circular dichroism (Fig. 4). Moreover, HPLC of the reaction mixture after photolysis of **Ru(brC)(ebpC)** in the absence of DNA revealed the formation of four products, two of which exhibited masses corresponding to the metal-free peptides, while the other two matched their ruthenium complexes, as expected for an asymmetric cleavage (see the ESI).

In conclusion, the biselectrophilic Ru(II) complex **2** is an excellent long-wavelength photosensitive linker for the homo- and heterodimerization of Cys-containing peptides. We have used this linker for the straightforward synthesis of high affinity DNA binding bZIP derivatives whose interaction with the DNA can be suppressed at will by irradiation with visible light. The strategy represents one of the first approaches to the conditional deactivation of DNA binders, and should be extendible to other systems that require a dimeric state for function.

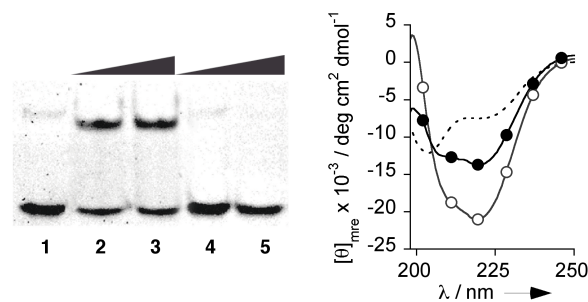


Fig. 4 Left: DNA binding of **Ru(brC)(ebpC)** studied by EMSA (same conditions as described before). Lanes 1–5: 100 nM **CE/CR** oligo; lanes 2–3: 400, 600 nM of **Ru(brC)(ebpC)**; lanes 4–5: 400, 600 nM after photolysis. **CE/CR** (binding site is in italics, only one strand shown): 5'-CGG *ATTGCG TCAT* TTTTTTTC-3'. Right: Circular dichroism of a 5 μ M solution of **Ru(brC)(ebpC)** in absence of DNA (dashed line); in the presence of 1 eq. of **CE/CR** before (\circ), and after photolysis (\bullet). The decrease in the negative ellipticity at 220 nm after photolysis supports the cleavage of the dimer and abolition of the DNA binding.

Acknowledgements

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Electronic Supplementary Information (ESI) available: Synthesis and characterization of **1**, **2**, peptide synthesis, in vitro uncaging and biochemical studies. See DOI: 10.1039/c000000x

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