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Deactivation of a dimeric DNA-binding peptide through a palladium-mediated self-immolative cleavage†

Jessica Rodríguez, [‡] Cibrán Pérez-González, [‡] Miguel Martínez-Calvo, [‡] Jesús Mosquera and José L. Mascareñas ^{‡*}

Herein, we describe an approach for the on-demand disassembly of dimeric peptides using a palladium-mediated cleavage of a designed self-immolative linker. The utility of the strategy is demonstrated for the case of dimeric basic regions of bZIP transcription factors. While the dimer binds designed DNA sequences with good affinities, the peptide–DNA complex can be readily dismantled by addition of palladium reagents that trigger the cleavage of the spacer, and the release of unfunctional monomeric peptides.

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In recent years, there has been a lot of progress in the development of uncaging reactions that release active drugs or biopolymers from inactive precursors.¹ The strategy lies on a temporary blocking of a key functional group of an active compound with a caging/protecting group, protection that cancels its biological function. The activity can be eventually recovered by removing the caging pendant, a process that is usually achieved upon application of a light pulse,² a chemical trigger³ or a metal-based reagent.⁴ This latter strategy has gained relevance in recent years, owing to its intrinsic catalytic potential and the variety of mechanisms offered by organometallic chemistry. Many of the biological applications of transition metal-mediated bioorthogonal uncaging reactions have relied on the use of allyl or propargyl derivatives as protecting groups, and ruthenium or palladium catalysts (Fig. 1a).⁵ In particular, palladium-mediated depropargylation reactions have been extensively used for “gain of function” processes based on the release of bioactive products containing amine or alcohol functionalities.⁶ Surprisingly, metal-promoted processes that lead to “loss of function” properties, which could find important applications in biosupramolecular chemistry, cell biology and/or biomedicine, are essentially unknown.⁷

Considering our previous work on the deactivation of dimeric DNA-binding peptides using light,⁸ we wondered whether it would be possible to cleave this type of dimers using

a metal-mediated protocol. This would be relevant not only in terms of developing a new methodology to regulate DNA binding processes, but also because of proposing a new type of

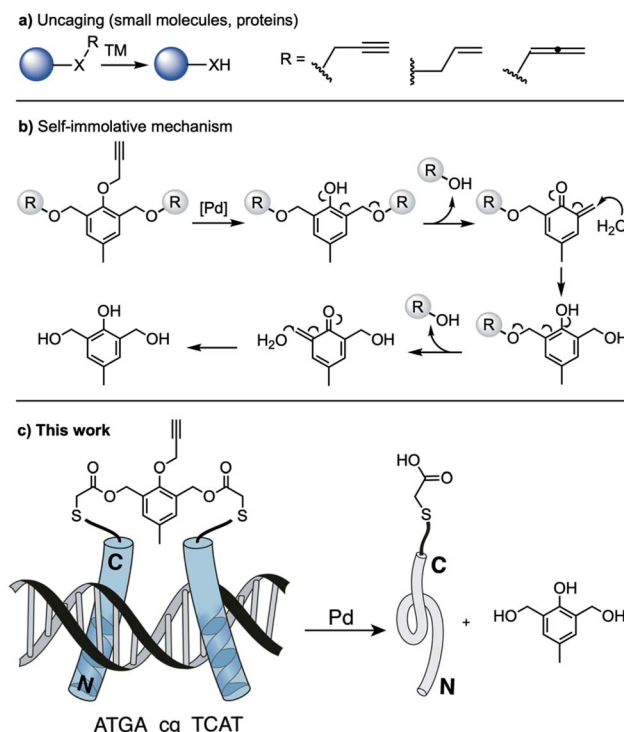


Fig. 1 (a) Representation of the metal-mediated uncaging strategies in chemical biology. (b) Mechanistic proposal for a palladium-mediated dedimerizing cleavage. (c) Illustration of the self-immolative GCN4 dimer, and palladium-mediated uncaging with suppression of the DNA interaction.

Centro Singular de Investigación en Química Biolóxica e Materiais Moleculares (CIQUS), Departamento de Química Orgánica, Universidade de Santiago de Compostela, Rúa Jenaro de la Fuente s/n, Santiago de Compostela, 15782, Spain. E-mail: joseluis.mascareñas@usc.es

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‡ These authors contributed equally.



uncaging approach based on the cleavage of a linker rather than on the release of a caging group. Although there are some precedents on the uncaging of temporary linked systems, they have been essentially centered on the dual release of two active molecules, rather than on the deactivation of biorelevant processes.⁹

Using as reference a report describing the viability of cleaving benzylic alcohols in *ortho* to aryloxy derivatives,¹⁰ we reasoned that introducing a propargylic protecting group in this type of phenols might provide for a Pd-mediated release of the pendants (Fig. 1b). Herein we demonstrate the viability of this approach, by describing the synthesis of DNA-binding bZIP-based peptides dimerized through a phenol-like linker that undergoes a self-immolative cleavage after treatment with a palladium reagent. This process results in the release of monomeric peptides and suppression of the DNA binding (Fig. 1c).

Basic leucine zippers (bZIP) are among the most important families of transcription factors (TFs). They are formed by an N-terminal basic region (br) that makes specific interactions with the major groove of the DNA, and a C-terminal leucine zipper domain that mediates dimerization.¹¹ Monomeric basic regions are not able to bind their consensus DNA sites with enough affinity, unless they are connected to other DNA binders,¹² or arranged into pre-folded constructs.¹³ Alternatively, the DNA binding can be rescued when the basic region peptides are engineered as dimeric systems using suitable synthetic connectors.¹⁴

To implement our on-off DNA binding system, we selected the GCN4 transcription factor, one of the best-characterized bZIP proteins, which specifically binds to the ATF/CREB (5'-ATGAcgTCAT-3') or AP1 (5'-ATGA(c/g)TCAT-3') consensus sequences.¹⁵ The DNA-binding peptide fragment (br) was chosen based on previous reports that determined the smallest fragment of the GCN4 basic region that preserves the DNA binding attributes of the full protein when engineered as a disulfide dimer.¹⁶

Considering the versatility and wide applicability of cysteine alkylations for the selective bioconjugation of peptides and proteins,¹⁷ we selected peptide fragments with a C-terminal cysteine unit. As aryl-containing linking moiety we synthesized product **2**, featuring thiol-reactive bromoacetyl units (Fig. 2a). The commercially available precursor 2,6-bis(hydroxymethyl)-*p*-cresol **1** was first protected as *bis*-TBS derivative. After reaction of the resulting phenol with propargyl bromide to afford **1b**, the TBS group was removed and the diol bis-acylated to give the desired dibromide (overall yield of the synthetic process: 22%).

The chemoselective coupling between this bis-electrophilic linker and the cysteine-containing peptide brC, was carried out in 100 mM aqueous phosphate buffer (pH 7.8) containing 25% of CH₃CN, using 2.1 equiv. of the peptide. HPLC-MS analysis of the reaction showed the complete disappearance of the dibromide after 1.5 h at rt, and the formation of a new major species identified as the expected dimeric peptide (Fig. S10†).

The DNA binding of the resulting dimeric construct Pr(brC)₂ was first analyzed by electrophoretic mobility shift assays

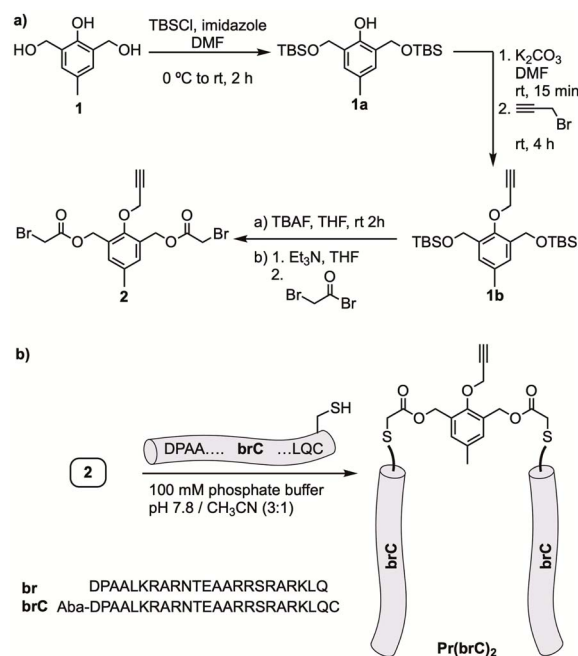


Fig. 2 Synthesis of (a) the bis-electrophilic linker **2** and (b) the GCN4 basic region dimer Pr(brC)₂ by bis-alkylation with the reactive linker **2**. Aba = acetamido benzoyl (included as a chromophore to quantify the peptide).

(EMSA) under non-denaturing conditions¹⁸ and using SYBR gold for DNA staining.¹⁹ Thus, when a double stranded oligonucleotide containing the target ATF/CREB site (ATF/CREB) was incubated with increasing concentrations of Pr(brC)₂ in Tris-HCl buffer at 20 °C, we observed the appearance of a new retarded band consistent with the formation of a specific DNA/Pr(brC)₂ complex (Fig. 3). The shift of this band is in line with that observed when the same oligonucleotide is incubated with the reference disulfide dimer (brGCC)₂ (Fig. 3, lane 6).¹⁴ A control EMSA experiment with an oligonucleotide containing a random sequence does not show retarded bands, thus confirming that Pr(brC)₂ is a highly selective DNA binder (Fig. S14†). Fluorescence anisotropy titrations using a FAM-

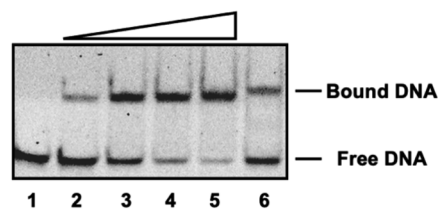


Fig. 3 DNA binding of Pr(brC)₂ studied by EMSA. Lanes 1–6: 50 nM target ATF/CREB; lanes 2–5: 250, 500, 750, 1000 nM Pr(brC)₂; lane 6: 400 nM (brGCC)₂. Peptides and dsDNA were mixed in 18 mM Tris-HCl pH 7.5, 90 mM KCl, 1.8 mM MgCl₂, 1.8 mM EDTA, 9% glycerol, 0.11 mg mL⁻¹ BSA, 2.25% NP-40 for 30 min at 20 °C, and loaded into gel. Gel was run on 10% nondenaturing polyacrylamide and 0.5× TBE buffer over 40 min, and stained with SyBrGold (5 µL in 50 mL of 1× TBE) for 10 min. ATF/CREB (binding site in italics, only one strand is shown): 5'-CGG ATGACGTCAT TTTTITTC-3'.

labeled ATF/CREB dsDNA confirmed the high affinity interaction of the peptide dimer **Pr(brC)₂** with the ds-oligonucleotide, with an apparent K_D of 321 nM at 20 °C (Fig. S16†).

We next studied the cleavage of **Pr(brC)₂** in the absence of DNA. As palladium reagents we selected commercially available complex $[\text{Pd}(\text{allyl})\text{Cl}]_2$ (**Pd1**) and pi-allyl derivative **Pd2**, both previously shown to mediate depropargylation reactions in water, and even in complex biological settings.^{6,20} To our delight, incubation of a solution of **Pr(brC)₂** (10 μM) in 20 mM Tris buffer (pH 7.5) with either **Pd1** or **Pd2** (100 μM) for 3 h at room temperature led to the disappearance of the starting dimer (peak at 18.7 min, see Fig. 4 and S13,† 81% conversion after 2 h).²¹ The chromatogram showed a new peak at 17.3 min which was identified as the monomeric fragment **3** by MS (Fig. S12†), which is consistent with the cleavage of the linker and the release of the peptide arms (**brC**) as terminal acids (Fig. 4).

Very importantly, the Pd-promoted process can be performed after formation of the peptide/DNA complex, leading to the expected disassembling. Therefore, when the target DNA (500 nM) was incubated in buffered solution (20 mM Tris buffer pH 7.5, 100 mM KCl) with **Pr(brC)₂** (5 μM), and the mixture was treated with either **Pd1** or **Pd2** (50 μM) for 3 h, we could clearly observe by EMSA the disappearance of most of the retarded band corresponding to the **Pr(brC)₂**-DNA complex (band b in Fig. 5, lanes 4–5 and Fig. S15†). A control experiment with the disulfide dimer (**brGCC**)₂ revealed that the DNA binding is retained after incubation with **Pd1** or **Pd2** (band a in Fig. 5, lanes 2–3 and Fig. S15†), which confirms that the cleavage/disassembly sequence only occurs with our responsive peptide dimer.

Moreover, in agreement with the EMSA, circular dichroism experiments revealed that addition of 1 equiv. of the dsDNA ATF/CREB to a 5 μM solution of **Pr(brC)₂** or (**brGCC**)₂ promotes a significant increase in the ellipticity of the negative bands at 208 and 222 nm, consistent with the expected folding of the basic regions into α -helices (Fig. 6).^{11,14} Treatment of the

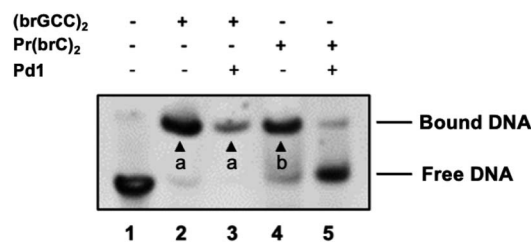


Fig. 5 Palladium-promoted cleavage of **Pr(brC)₂** studied by EMSA (same conditions as described before). Lanes 1–5: 50 nM target ATF/CREB; lanes 2–3: 500 nM (**brGCC**)₂; lanes 4–5: 500 nM **Pr(brC)₂**. Concentration of **Pd1** is, when present, 5 μM .

solution containing the **Pr(brC)₂**-dsDNA complex (5 μM) with **Pd1** or **Pd2** (50 μM) in 10 mM phosphate buffer (pH 7.5), for 3 h at rt, induced a significant decrease in ellipticity, as expected for single monomers.^{8a, 13c} In contrast, the same addition to the control mixture containing the (**brGCC**)₂ peptide did not promote any change. All these results are in total agreement with a Pd-promoted self-immolative cleavage of the propargylic phenol connector, and subsequent release of the two monomeric basic regions (**3**), which are not capable to efficiently bind to the ATF/CREB sequence.

In conclusion, we have designed and developed a new palladium-cleavable linker that can work as temporary dimerizing unit. The utility of the strategy was demonstrated for the construction of functional bZIP dimeric peptides which bind the consensus DNA sequences with high affinity. However, the DNA binding can be efficiently suppressed upon incubation with readily available palladium reagents under mild and physiological conditions. Together with its easy synthetic accessibility, these results support the use of the self-immolative linker **2** as an effective dimerizer that can be readily cleaved in aqueous buffers using transition metal reagents. The methodology, which is complementary to other uncaging strategies based on the use of light, should be

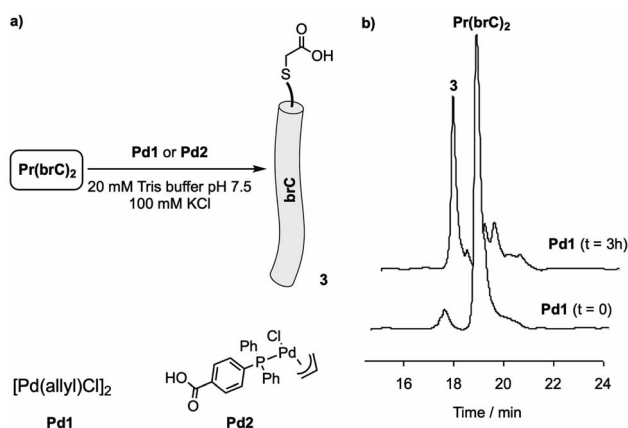


Fig. 4 (a) Representation of the Pd-triggered cleavage of the self-immolative peptide dimer **Pr(brC)₂**. (b) HPLC of the self-immolation of **Pr(brC)₂** promoted by the **Pd1** reagent. Gradient 10 to 50% B over 40 min.

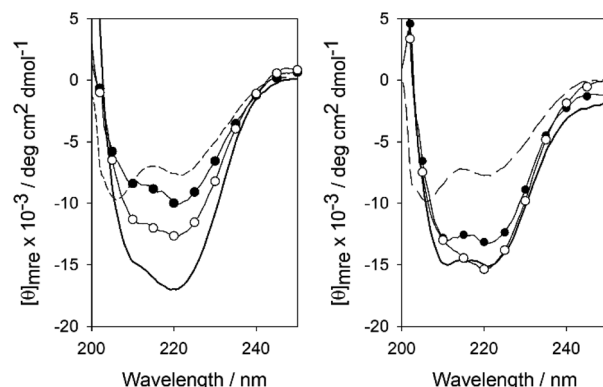


Fig. 6 Circular dichroism of a 5 μM solution of (left) **Pr(brC)₂** and (right) (**brGCC**)₂ in 10 mM phosphate buffer pH 7.5 100 mM of NaCl in the absence of ATF-CREB DNA (dashed line), and after subsequent addition of 1 eq. ATF/CREB DNA (solid line), followed by addition of 10 eq. of **Pd1** (○) or **Pd2** (●). The contributions of the DNA to the CD are subtracted for clarity. All experiments were carried out at 20 °C.



extendible to switch off other type of functions that depend on the formation of dimers, as well as for releasing active products from inactive bifunctional precursors. Furthermore, the known ability of the palladium catalyst to promote depropargylation reactions in living systems,^{6a} suggest that this type of switches can be translated to *in vivo* settings.

Conflicts of interest

There are no conflicts to declare.

Acknowledgements

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- 21 We tested different Pd/dimer molar ratios for the cleavage reaction, and concluded that a 10-fold molar excess of Pd complex over the dimer leads to the best performance (see Fig. S11†). 10 eq. of Pd is the amount commonly used in depropargylation reactions in water, see for example ref. 9b and 20.

