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RESEARCH ARTICLE

Intracellular reactions promoted by bis-histidine miniproteins stapled with Pd(II) complexes

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Dedication ((optional))

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Abstract: The generation of catalytically active metalloproteins inside living mammalian cells is a major research challenge at the interface between catalysis and cell biology. Herein we demonstrate that basic domains of bZIP transcription factors, mutated to include two histidine residues at i, i+4 positions, react with palladium (II) sources to generate catalytically active, stapled pallado-miniproteins. The resulting constrained peptides are efficiently internalized into living mammalian cells, where they can perform palladium-promoted depropargylation reactions, without cellular fixation. Control experiments confirm the requirement of the peptide scaffolding and the palladium staple for attaining the intracellular reactivity.

Introduction

Metalloproteins make up a substantial portion of the human proteome (between one-third and one-half) and play critical structural and catalytic role in living cells and whole organisms.[1] Catalytic metalloproteins, *i.e. metalloenzymes*, are involved in many fundamental metabolic reactions, including, among other, redox processes, isomerisations, hydrolysis or condensations.[2] Interestingly, despite the large number of alternatives offered by the periodic table, Nature has evolved to use just a few metals as cofactors to build the palette of metalloenzymes required to sustain life. Indeed, most metal-based proteins feature either alkali and alkaline earth elements, or first row transition metals like Fe, Cu, Mn, Co or Zn.[2,3]

The recognition that introducing other transition metals like Ni, Ru, Rh, Pd or Ir at designed sites of proteins might provide for non-natural transformations with enzymatic characteristics has led to very significant advances in the field of "artificial metalloenzymes".[4] Therefore, a number of transition metal protein hybrids capable of promoting otherwise difficult reactions have been recently reported.[5] Most successful results involve the use of prosthetic groups as metal coordinating units, albeit some examples in which the artificial metalloproteins result from direct metal coordination to amino acid side chains have also been described.[6]

Whereas these efforts are ascribed to the realm of synthetic chemistry, a challenging, remaining goal in the field consists of the introduction or generation of artificial metalloenzymes in living mammalian cells, which is the native environment of many natural enzymes.[7] Not surprisingly, progress in this matter has been extremely scarce, and essentially limited to one elegant report in the internalization of streptavidin-ruthenium constructs in HEK-293 cells.[8] This paucity contrasts with the increasing number of reports on the development of bioorthogonal and intracellular reactions promoted by discrete transition metal complexes, [9,10] or metal nanoparticles.[11]

Quite recently, we discovered that introducing two histidine residues in strategic i and i+4 positions of the basic region of a GCN4 bZIP transcription factor,[12] allows to trigger its DNA binding upon addition of $PdCl_2$(en) (en = ethylenediamine).[13] Interestingly, the palladium additive also sparks an efficient membrane translocation into mammalian cells. Specific control experiments confirmed that these properties result from stapling of the two histidines by the added metal (Figure 1).

This discovery raised intriguing questions related to the generality of the approach, the mechanism of the membrane crossing, and the stability of the peptide-palladium complex in the cell cytosol, among others. But more importantly, since the approach can also be viewed as a peptide-promoted internalization of a palladium complex, we wondered whether the resulting metallopeptide could promote designed reactions inside cells. This could represent a significant first step towards the challenging endeavour of developing intracellular, catalytic metalloproteins.

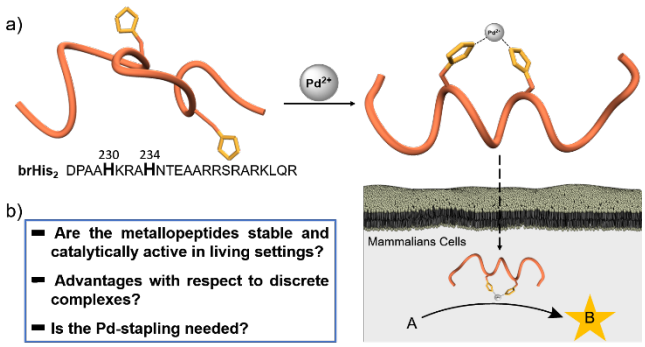

Figure 1. a) Representation of the bis-Histidine modified basic region protein, and conformational constrain resulting from the Pd(II) stapling. The clipped miniprotein is internalized into mammalian cells. b) Some questions approached in this work. *Note:* Amino acid numbering according to the natural bZIP protein.

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Herein we demonstrate the viability of the approach by describing the first metal-equipped, synthetic miniproteins made by natural amino acids that are capable of promoting abiotic reactions inside living mammalian cells. Our results indicate that the bis-histidine containing peptide and the palladium complex are both required for the cellular internalization and for the intracellular reactivity, which in this work is demonstrated for depropargylation reactions.

Results and Discussion

Before testing catalytic reactivities, we considered fundamental to obtain more information on the internalization requirements of the miniprotein. Our previous studies had demonstrated that mixing the peptide **brHis$_2$** with $PdCl_2$(en) produces relatively stable peptide palladium complexes (detected by ESI-MS), and that these metallopeptides go inside HeLa cells at concentrations as low as 5 μM.[13]

We have now found that keeping the six native arginines of the basic region fragment is key for the internalization, because replacing any of them by alanines leads to a significant decrease in the intracellular fluorescence of tetramethylrhodamine (TMR)-labelled derivatives (see the SI, Figure S32). This is consistent with previous studies with cell penetrating peptides, where arginines play a critical role to favour the cell uptake.[14] Not surprisingly, when the internalization experiments were carried out at 4ºC instead of at 37ºC, the intracellular fluorescence, as measured by flow cytometry, was considerably lower (Figure S35). This result suggests that the cell penetration is energy dependent, and therefore very likely occurring by endocytosis, with the resulting peptide accumulated in vesicular structures.

Importantly, the palladium complex used as stapling agent can feature other bidentate ligands than ethylenediamine (en), e.g. a bipyridine (bpy). We therefore envisioned the use of a bipyridine ligand equipped with a fluorophore other that TMR (already present in the peptide), to simultaneously track the localization of the peptide and the metal complex.

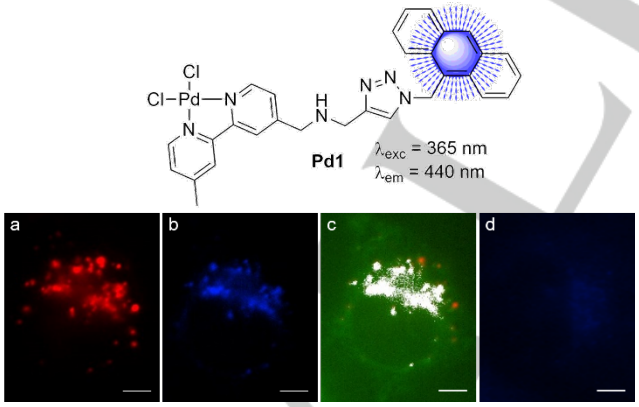

Figure 2. Upper: Structure of **Pd1.** Bottom: Fluorescence micrographies of HeLa cells. The palladopeptide complex was made by mixing TMR-**brHis$_2$** (10 μM) with **Pd1** (1:1 ratio) in water for 30 min. Cells were incubated with the mixture for 1 h at 37 ºC, washed twice with PBS, replenished with fresh DMEM, and observed under the microscope. a) Red emission channel corresponding to the TAMRA fluorophore; b) Blue emission channel corresponding to the anthracene; c) Colocalization image processed with Image J; d) Blue emission channel after incubation with **Pd1** alone. Incubations were made in Dulbecco´s modified Eagle medium (DMEM) completed with 5% of fetal bovine serum (FBS-DMEM). Red channel: λ_{exc} = 550 nm, λ_{em} = 590-650 nm. Blue channel: λ_{exc} = 385 nm, λ_{em} = 450-510 nm. Scale bar: 5 μm, the image shows a single cell.

Specifically, we synthesized the bipyridine-anthracene palladium derivative **Pd1** (Figure 2), and found that it also reacts efficiently with the bis-histidine peptide to give the expected complex, as corroborated by LC-MS (see Figure S21). HeLa cells were then incubated for 1 h with the complex resulting from mixing equivalent amounts of TMR-**brHis$_2$** and **Pd1** (30 min). After washing (PBS, 2X), cells were observed by microscopy at different times. Figures 2a and 2b show the intracellular emissions corresponding to both fluorophores after 1 h, while Figure 2c is a colocalization image. Observation after 4 h did not show perceptible changes in the colocalization of the fluorophores. A control experiment using only the metal complex (**Pd1**), at the same concentration, revealed a very poor internalization (Figure 2d), confirming the role of the peptide as palladium transporter.

With a method to internalize a relatively stable Pd(II) complex, we wondered whether this species could be engaged in catalytic reactions. As testing reaction, we explored the depropargylation of the probe HBTPQ (**1,** Figure 3a),[10i] which is not fluorescent but upon uncaging generates a product **2** that emits light at 635 nm when excited in the far-ultraviolet (far-UV) region (λ_{exc} = 330 nm). Importantly, previous work from our and other groups had shown that typical palladium sources like $PdCl(allyl)_2$ or $Pd(OAc)_2$ fail to promote this reaction inside HeLa cells,[10i,11d] likely because of a poor cell penetration of the palladium species, as well as a rapid deactivation in the presence of cellular components.

We first assessed the reactivities “in vitro”, using PBS as solvent. $Pd(OAc)_2$ and the palladium chloride sources used in the preparation of the hybrid bis-histidine metal complexes, $PdCl_2$(en) and $PdCl_2$(bpy), were able to promote the reaction (conditions: 200 μM solution of **1** and 10 mol % of the catalyst Figure 3b).

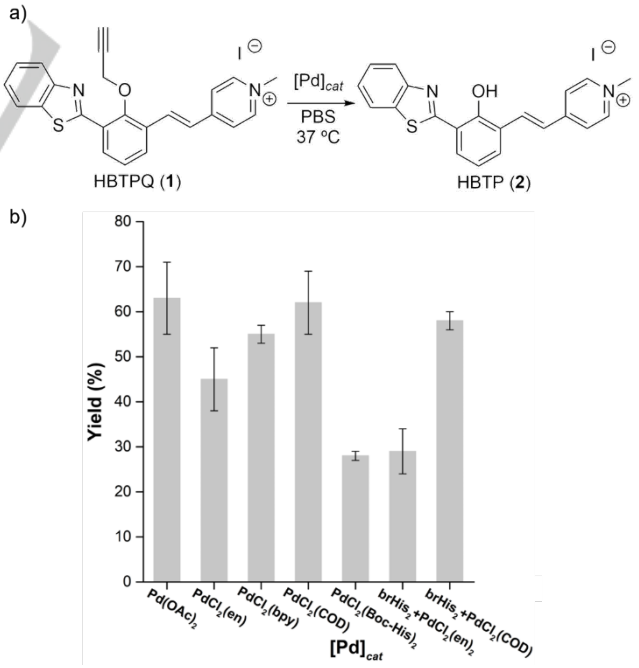

Figure 3. a) Uncaging of HBTPQ (**1**). b) Bar diagram representation of the yields obtained for each catalyst. Reaction conditions: HBPTQ (200 μM), Pd source (20 μM, 10 mol%) in 1 mL of PBS at 37 ºC, for 24 h. Yields were calculated by RP-HPLC-MS using internal standards (see the SI). Data are mean +/- SEM for experimental performed in triplicates and repeated in three independent times.

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Noticeably, the palladopeptide resulting from mixing stoichiometric amounts of peptide and $PdCl_2$(en) in water was much less effective (Figure 3b).[15] Despite this result could be discouraging, it is not so surprising, as in this complex the ethylendiamine ligand is not labile enough to allow an efficient coordination of the substrate. Indeed, a similar metallopeptide made from $PdCl_2$(COD) (COD = 1,8-cyclooctadiene),[17] gave the product in 65% yield after 24 h of reaction (Figure 3b). This result confirms the need of using Pd(II) reagents with suitable labile ligands to obtain good reactivities. As a control reagent we also explored the reactivity of a purposely made bis-histidine dichloride complex, **$PdCl_2$(Boc-His)$_2$** (see the Supporting Information) which gave the product albeit with lower yields (30%) than the metallopeptide, suggesting a positive effect of the peptide scaffold in the reactivity.

After these in vitro results, the stage was set for the intracellular studies. In consonance with the results observed with $PdCl_2$(en), the internalization of TMR-**brHis$_2$** can also be triggered by pre-treatment with the complex $PdCl_2$(COD) (Figure 4a) Interestingly, circular dichroism spectra of the peptide (TMR-**brHis$_2$**) after adding increasing amounts of the Pd(II) complex, at 4ºC, confirmed the propensity of the peptide to acquire an alpha-helical structure (Figure 4b).[18]

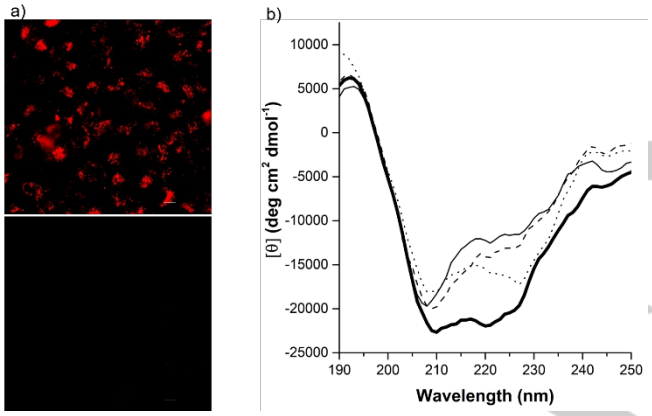

Figure 4. a) Fluorescence micrographies of HeLa cells (confocal). Upper: TMR-**brHis$_2$** (5 μM) was pre-mixed with $PdCl_2$(COD) (1:1 ratio) in water for 10 min and incubated with cells for 30 min at 37 ºC. Bottom: Incubation with TMR-**brHis$_2$** alone. The cells were washed with PBS before being observed under the microscope. All the incubations were made in FBS-DMEM. Red channel: λ_{exc} = 561 nm, λ_{em} = 620/60 nm. Scale bar: 10 μm. b) Circular Dichroism titration of a 5 μM solution of **brHis$_2$** (narrow solid line) and increasing concentrations of $PdCl_2$(COD): 1 equiv. (dashed line), 3 equiv. (dotted line) or 10 equivalents (thick solid line). The experiments were carried out at 4 °C, in 10 mM phosphate buffer pH 7.5 and 100 mM of NaCl. Mean residue molar ellipticity (MRE) was calculated considering a 24-mer.

The cellular reactions were carried out by incubation of HeLa cells with 50 μM of probe **1** for 30 min at 37 °C. The medium was removed, and the cells washed twice with FBS-DMEM to ensure removal of extracellular probe. The peptide-palladium reagents (50 μM) were then added in fresh media and, after 1 h and two washes with PBS, the cells were imaged by wide-field fluorescence microscopy. Gratifyingly, when cells were treated with the palladopeptide resulting from mixing TMR-**brHis$_2$** and $PdCl_2$(COD), we could observe a very intense intracellular fluorescence arising from the expected product **2** (Figure 5a). Not so surprisingly, with the metallopeptides made from $PdCl_2$(en) and $PdCl_2$(bpy), we didn't observe fluorescence, which must be a consequence of the lack of reactivity, because the palladopeptides are inside the cells, as can be deduced from the TMR fluorescence (Figures 5b-c, bottom row). Control experiments with a peptide lacking the TMR label gave similar reactivity results, confirming that the fluorophore label doesn't affect the process. As expected, using the peptide TMR-**brHis$_2$** alone, we didn't detect any fluorescence (see Figure S37).

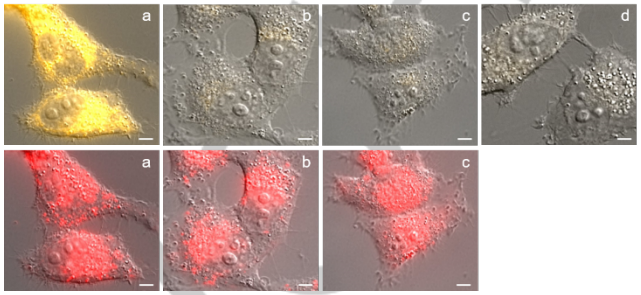

Figure 5. Intracellular depropargylation of **1** monitored by fluorescence microscopy in HeLa cells. Cells were treated with 50 μM of probe **1** for 30 min at 37 ºC, and washed twice to remove excess of the probe. Peptides (10 μM), or solutions of peptide-palladium complexes obtained by mixing equivalent amounts of the peptide and the palladium source for 10 min, were added in fresh media, and the cells were incubated for 1 h. a) Incubation with the palladopeptide obtained from $PdCl_2$(COD); b) Incubation with the palladopeptide obtained from $PdCl_2$(en); c) Incubation with the palladopeptide obtained from $PdCl_2$(bpy); (d) Incubation with $PdCl_2$(COD), in absence of peptide. The cells were washed with PBS before being observed under the microscope. All the incubations were made in FBS-DMEM. Bright fields images are superimposed to the red or orange emission channel (the orange colour is arbitrarily chosen to differentiate the emission with the red channel). Upper row: Orange channel: λ_{exc} = 385 nm, λ_{em} = 515-700 nm; Bottom row: Red channel: λ_{exc} = 550 nm, λ_{em} = 590-650 nm; Scale bar: 5 μm.

We observed a similar reactivity using lower amounts of the reactive metallopeptide (10 μM), which demonstrates the effectivity of the system (see Figure S38).

Given that the palladopeptide is preformed just before the cellular incubations, it could not be discarded that the intracellular reaction was promoted by discrete palladium entities derived from the palladium source. However, in the absence of the peptide, the complex $PdCl_2$(COD) is incapable of generating any intracellular fluorescence (Figure 5d and S38). Other palladium sources like $Pd(OAc)_2$ or $PdCl_2$(allyl) also failed to generate intracellular fluorescence under the above conditions (Figure S39).

ICP-MS analysis of cellular extracts revealed that the palladium content is substantially higher in cells treated with the preformed palladoprotein hybrid than with equivalent amounts of $PdCl_2$(COD) (Figure S42). Nonetheless, this doesn't account for the lack of reactivity observed with these palladium sources, which suggests that the stapled peptide structure also plays a key role to favour the intracellular reaction.

Additional insights were obtained using as reagents the peptides **brHis** (His 230 mutated to Leu), or **br** (native GCN4-basic region, without histidines), instead of **brHis$_2$**, which were not able to promote any intracellular reaction when premixed with $PdCl_2$(COD) (Figure S40). Using a mutated peptide that lack key arginines for cellular uptake (R249A, R245A), also led to similar fails. These results confirm the requirement of a bis-histidine

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stapling and an appropriate set of arginines for cellular uptake and intracellular reactivity.

Also importantly, the approach can be used in other human cells such as A549 and mammalian cells like Vero (see Figure S41), which supports the generality of the approach.

The chemistry can be extended to other challenging substrates, like the bis-propargyl derivative **3**, which upon deprotection produces a far-red emitting product. As in the case of the phenol derivative **1**, only the palladominiprotein was able to perform the reaction inside mammalian cells (Figure 6).

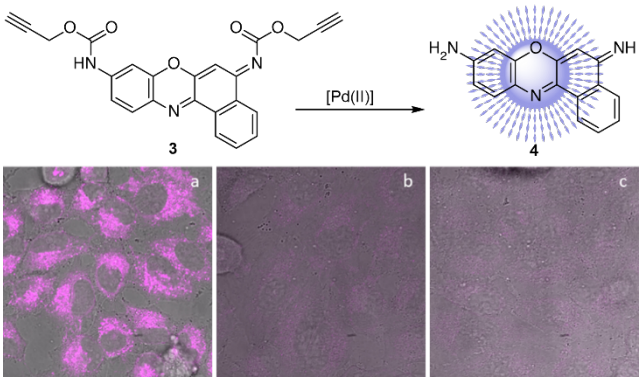

Figure 6. Fluorescence micrographies of HeLa cells (confocal). Standard conditions as in the previous cases, with 75 μM of probe **3** and 10 μM of the reagents. Bright field images are superimposed to far-red emission channel. a) Incubation with the metallopeptide made from **brHis$_2$** and $PdCl_2$(COD); b) Incubation with $PdCl_2$(COD) alone; c) Incubation with substrate **3** alone. λ_{exc} = 561 nm, λ_{em} = 620/60 nm. Scale bar: 10 μm.

It is important to note that cell viability tests (MTT in HeLa Cells, 24 h) demonstrated that the none of palladopeptide hybrids or palladium complexes were toxic below 50 μM (more than 90% viability after 24 h, Figure S43).

Altogether, the above results confirm that engineering a bis-histidine handle in a basic miniprotein can provide for cellular internalization and cytosolic palladium catalysis owing to its ability to form palladium stapled derivatives. An ensuing question is whether the palladium stapling strategy is also effective for other, shorter peptides.[18] Considering the well-known integrin targeting motif RGD (Arg-Gly-Asp), we wondered whether it could be also converted into a reactive metallopeptide by adding two histidines at the edges (Figure 7, panel 1). A TMR-labelled derivative revealed that while the peptide HRGDH presents a relatively poor cellular uptake in HeLa cells, the internalization is significantly improved when pre-treated with $PdCl_2$(COD), albeit the uptake is not as efficient as with the above basic miniprotein (Figures S33 and S34).

Not surprisingly, the peptide-palladium complex is also capable of performing the intracellular depropargylation of substrate **1** (Figure 7, panel 2b), however the reactivity was lower compared with the pallado-miniprotein (Figure 7, panel 3). A similar peptide with only one histidine (HRGDA) presents some capacity to internalize (the same with or without adding $PdCl_2$(COD), see Figure S34), but failed to act as a catalyst (Figure 7, panel 2c). Interestingly, the non-cyclic bis-histidine palladium complex **$PdCl_2$(Boc-His)$_2$** also failed to generate the intracellular product (Figure 7, panel 2d).

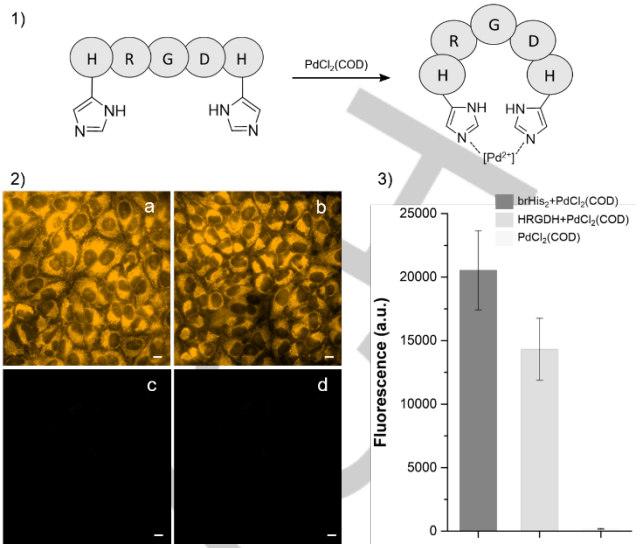

Figure 7. 1) Bis-Histidine RGD derivative and stapling produced by treatment with the palladium complex. 2) Intracellular depropargylation using the peptide TMR-**HRGDH** or control derivatives. HeLa cells were treated with 50 μM of probe **1** for 30 min at 37 ºC and washed twice. Peptides (10 μM) or solutions of peptide-palladium complexes obtained by mixing equivalent amounts for 10 min, were added in fresh media, and the cells were incubated for 1 h. a) Pallado-miniprotein resulting from $PdCl_2$(COD) and **brHis$_2$**; b) Mixture of TMR-**HRGDH** and $PdCl_2$(COD); c) Mixture of TMR-**HRGDA** and $PdCl_2$(COD); d) **$PdCl_2$(Boc-His)$_2$**. The cells were washed twice with PBS before being observed under the microscope. All incubations were in FBS-DMEM. λ_{exc} = 385 nm, λ_{em} = 515-700 nm. Scale bar: 10 μm. 3) CTFC measurements after the intracellular reaction. Data are mean +/- SEM for experimental performed in triplicates and repeated in three independent times.

Finally, given that the cellular uptake of the palladium chelated complex of HRGDH in cells might be partially associated to the presence of integrin receptors, we anticipated that its cell penetration would depend on the type of cells and the presence of these receptors. Indeed, we were glad to observe that while in A549 or HeLa cells there is intracellular catalysis, in the MCF-7 breast cancer cell line, which expresses integrin at low levels, we barely detect any fluorescence. This preliminary result prompts well for the development of target selective intracellular metal catalysis (Figure 8).

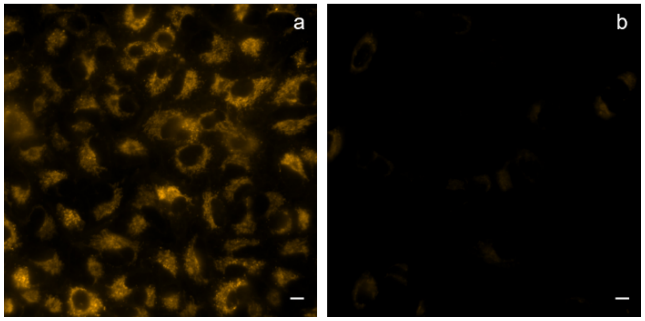

Figure 8. Fluorescence micrographies of A549 (a) and MCF-7 cells (b), using standard incubation conditions with the peptide-palladium complex resulting from TMR-**HRDGH** and $PdCl_2$(COD) (1:1 ratio). λ_{exc} = 385 nm, λ_{em} = 515-700 nm. Scale bar: 10 μm.

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Conclusion

In summary, arginine-rich miniproteins made of natural amino acids, and equipped with i, i+4 bis-histidine residues, can efficiently react with different Pd(II) salts to form stapled derivatives that show enhanced cell internalization properties. More importantly, the pallado-miniproteins obtained when using $PdCl_2$(COD) as palladium source, work as effective metalloreactors to promote depropargylation reactions inside living mammalian cells; transformations that cannot be performed using just the palladium sources. The effectivity of approach is likely associated to a synergistic beneficial effect of the conformational constrain introduced by the metal bridge, and a protective role of the peptide scaffolding, which avoids a rapid deactivation of the metal.

Our results represent a first step towards the development of a "bottom-up" strategy for the generation of artificial catalytic metalloproteins capable of working in the native living environment of enzymes. Additionally, the well-known transformative potential of palladium catalysis prompts well for further applications of the strategy in other type of reactions.

Acknowledgements

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Keywords: Metalloproteins • Intracellular catalysis • Palladium promoted uncaging • Stapling • bZIP proteins

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[16] The peptide TMR-**brHis$_2$** features an aminohexanoic unit between the TMR and the N-terminal amino acid of the sequence (Asp, see the Supporting Information).

[17] Not surprising, while in the MS of the palladapeptide resulting from mixing **brhis$_2$** and $PdCl_2$(en) the ethylenediamine ligand is conserved, when using $PdCl_2$(COD) the MS fits with a palladapeptide without ligands (see the SI for more details).

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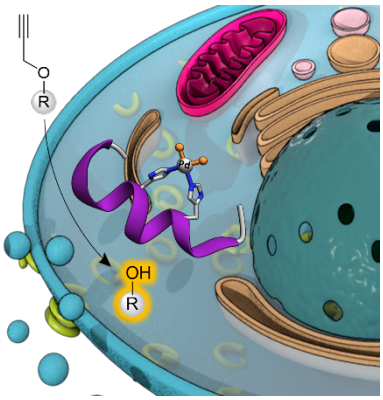

Stapling bis-histidine equipped basic miniproteins with Pd(II) produces cell-permeable derivatives that enable depropargylation reactions in the interior of living mammalian cells. Both the metal bridge and the peptide scaffolding are essential for the internalization, and for observing the intracellular transformations.

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