Controlled In-Cell Generation of Active Palladium(0) Species for Bioorthogonal Decaging

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Abstract: Owing to their bioorthogonality, transition metals have become very popular in the development of biocompatible bond-cleavage reactions. However, many approaches require design and synthesis of complex ligands or formulation of nanoparticles which often perform poorly in living cells. This work reports on a method for the generation of an active palladium species that triggers bond-cleaving reactions inside living cells. We utilized the water-soluble Na₂PdCl₄ as a simple source of Pd⁰ which can be intracellularly reduced by sodium ascorbate to the active Pd⁰ species. Once generated, Pd⁰ triggers the cleavage of allyl ether and carbamate caging groups leading to the release of biologically active molecules. These findings do not only expand the toolbox of available bioorthogonal dissociative reactions but also provide an additional strategy for controlling the reactivity of Pd species involved in Pd-mediated bioorthogonal reactions.

Most of the bioorthogonal applications are focused on ligation reactions [1], however, bond-cleavage reactions [2] have recently emerged as useful bioorthogonal chemical transformations in research areas such as targeted payload delivery, [3] activation of prodrugs [4] and studies on the gain-of-function of proteins. [5] Bond-cleavage reactions are often initiated by the addition of an exogenous small molecule [6] or a transition metal complex [7] as triggering agents. Some of the advantages of transition-metal-mediated processes are their catalytic performance and almost guaranteed layer of bioorthogonality. Among all transition metals which have been employed in bioorthogonal bond-cleavage reactions, palladium stands out as the most thoroughly studied metal trigger. Strategies based on biocompatible Pd-mediated reactions have been utilized for on-demand cleavage of functional groups such as allyl carbamates, [8] propargyl carbamates, [9] propargyl ethers, [10] propargyl amines, [11] allenyl ethers [12] and thioethers [13]. Whereas extracellular approaches have relied mostly on Pd-resins which could be implanted on the sites where the desired reaction is expected to take place, [14] intracellular Pd-mediated cleavage reactions have been more challenging to achieve. This is mainly associated with poor cellular uptake, solubility or instability of many Pd catalytic systems under biological conditions and their interference with major cellular functions. [15] Nevertheless, commercially available Pd catalysts Pd(dba)₃ and [Pd(allyl)Cl]₂ [16] (Figure 1a) or Pd complexes bearing purposely designed phosphine ligands [17] (Figure 1b) have been successfully used to promote bond-cleavage reactions inside living cells. To further expand on this, we envisioned a strategy which would overcome the limitations of available methods. Herein, we demonstrate that the biological activity of small molecules containing allyl ether and carbamate masking groups can be efficiently...
restored using catalytically active Pd\(^0\) species formed by reduction of a water soluble Pd\(^{II}\) salt with sodium ascorbate in living cells. (Figure 1c) This simple, yet effective strategy allows to control the oxidation state of the Pd catalyst and the corresponding Pd-mediated bioorthogonal cleavage reaction in a spatiotemporal manner.

The initial interest in the development of a new allyl-Pd bioorthogonal bond-cleavage pair was based on our previous work on the tetrazine-mediated decaging of vinyl ethers.\(^{[15]}\) While the release of alcohols from vinyl ether containing molecules proceeded under biocompatible conditions,\(^{[15]}\) the kinetics of this decaging reaction are much slower than those obtained from strained alkenes.\(^{[4a]}\) We anticipated that some drawbacks of the vinyl ether moiety could be resolved using a different masking group. Allyl ether was previously reported as a Ru-\(^{[16]}\) or Pt-labile\(^{[17]}\) caging group in living cells, however, decaging of allyl ethers mediated by Pd under biocompatible conditions has not been reported yet.

In order to find optimal reaction conditions, various types of Pd catalysts were screened for the deallylation of a model 2-(allyloxy)naphthalene 2 under aqueous conditions (Figure 2a and Table S1). Among these, Na_2PdCl_4 in combination with a TPPTS ligand and morpholine provided the best result (Figure 2a, Entry 4 and Table S1). Other sources of Pd in the absence of ligands and presence of external nucleophiles (see Table S1 for all tested conditions) only gave low yields of the isolated alcohol 1. Since reduction of Pd\(^{II}\) to Pd\(^0\) is facilitated in the presence of phosphine...
ligands,[7] these observations are in accordance with previously published works,[18] where the combination of a Pd⁰ catalyst and an external nucleophile was necessary to ensure a successful decaging reaction.[19] On the other hand, a simple Pd⁰ ↔ Pd II reduction of Na₂PdCl₄ mediated by sodium ascorbate[20] (Figure 2a, Entry 9 and Table S1) did not prove to be efficient in this type of bond-cleavage reaction. This is probably due to the observed formation and aggregation of catalytically inactive Pd black[21] under the reaction conditions and in the absence of a stabilizer.[22] The optimized decaging conditions were then used for the deallylation of various small molecules containing allyl ethers 2, 4, 6, 8, 10 and even an allyl ester 12 (Figure 2b).

Next, we studied this Pd-triggered bond-cleavage reaction by fluorescence recovery experiments. Formation of the allyl ether bond effectively quenched the fluorescence of umbelliferone 7 which allowed monitoring of the cleavage reaction in response to a Pd trigger (Figure 2c,d). We observed that the presence of morpholine did not significantly affect the Pd⁰-mediated decaging of allyl ether 8 when the decaging reaction was performed in PBS buffer pH 7.4 at 37 °C (Supporting Information, Figure S1). This suggests that under tested conditions, no external nucleophile is needed and perhaps water or TPPTS can act as a nucleophile in the release of the desired alcohol 7 from the Pd⁰-allyl complex.[18a] In fact, this could be beneficial in more complex biological settings, e.g., in cell culture media which will contain various nucleophiles that could promote this type of cleavage reactions. Although the Pd⁰/TPPTS complex performed well in these decaging experiments, it was previously shown that such complex could not cross cell membranes and thus was not available for intracellular reactions.[15a, 23] Therefore, we sought for simpler or ligandless sources of Pd⁰ species which would facilitate its cellular entry.

Earlier works showed that active Pd⁰ catalyst can be obtained by Pd⁰ ↔ Pd II reduction by sodium ascorbate[15a, 9, 25] in form of nanoparticles with different sizes, shapes and catalytic activities.[20] In contrast to the catalyst optimization (Figure 2a and Table S1), Pd⁰ generated by reduction of Na₂PdCl₄ Pd II salt with sodium ascorbate promoted the cleavage of allyl ether 8 in PBS buffer at 37 °C almost as efficiently as the Pd⁰/TPPTS complex with about 60 % fluorescence recovery observed (Figures 2c and S2). The non-quantitative yield of the recovered fluorescent signal was associated with partial fluorescence quenching of the released fluorophore, probably caused by microprecipitated metallic Pd (SI, Figure S6). In the absence of sodium ascorbate, decaging of 8 was not observed, suggesting that reduced Pd⁰ is the active species in this cleavage reaction. Poisoning experiments with Hg, CS₂ and EDTA further support our hypothesis that the uncaging is indeed mediated by Pd⁰, and kinetics experiments suggest the reaction has an heterogeneous (nanoparticles) contribution (see Supporting Information, Figures S16–18). Additionally, reduction of Pd⁰ to Pd II does not occur in the presence of other bioadditives, assuming that the reducing capability is exclusive to sodium ascorbate also in biological media (Table S3). However, when carried out in cell culture medium, it was only the Pd⁰/TPPTS complex that efficiently promoted the decaging of 8 and higher amount of the catalyst was required to obtain acceptable levels of decaging (Figures 2d and S3). The reason for this may be the deactivation of the formed Pd⁰ catalyst by the additional compounds present in the cell culture medium.

Having established the Pd⁰-mediated decaging for allyl ethers, we next attempted to extend this methodology to the cleavage of allyl carbamates. In PBS buffer at 37 °C, about 40 % increase in the fluorescence signal was observed upon the treatment of the allyl carbamate caged 7-amino-4-methylcoumarin 14 with either the Pd⁰/TPPTS complex or the combination of Na₂PdCl₄ and sodium ascorbate (Figures 2e and S4). Similarly to the experiments with allyl ether 8, no uncaging reaction was observed in the presence of Pd II and again, only the Pd⁰/TPPTS complex was effective in the cleavage of allyl carbamate bond in cell culture medium (Figures 2f and S5). Fluorescence recovery experiments have demonstrated that both Pd⁰/TPPTS complex and Pd⁰ species generated by sodium ascorbate-mediated Pd⁰ ↔ Pd II reduction can be used for the cleavage of allyl ether and carbamate bonds under biocompatible conditions. Notably, these experiments also confirmed the stability of both caging groups in cell culture medium ruling out spontaneous deallylation reactions.

Our final goal was to apply the developed strategy for Pd-mediated bond-cleavage to the release of biologically active small molecules in living cells (Figure 3). Duocarmycin 15[25] and doxorubicin 17[26] were chosen as examples of cytotoxic drugs containing functional groups to be uncaged by our method. We showed that the masking of the aromatic hydroxyl of 15 and amino group of 17 leads to about 19- and 72-fold (respectively) decrease in the cytotoxic activity in SKBR3 breast cancer cells (Supporting Information, Figure S9). These results correspond to previous reports[18b, 9, 15] and provide therapeutic windows suitable for use in the prodrug activation therapy concept. Using LC-MS analysis, it was confirmed that both prodrugs 16 and 18 can be efficiently decaged via bond-cleavage reactions mediated by Pd⁰ catalysts under biocompatible conditions at 37 °C in PBS buffer (Supporting Information, Figures S7, S8).

We then proceeded with experiments with prodrug activation in living cells. The inspiration for generating active Pd⁰ species inside of living cells was found in works which have reported production of Pd nanoparticles in bacteria.[27] We hypothesized that a similar approach employing sodium ascorbate-promoted reduction of Na₂PdCl₄ could be used to generate active Pd⁰ species in living cells. Various metabolites and biomolecules present in cells could potentially act as capping agents or stabilizers providing catalytically active Pd⁰ species. Concentrations of Pd catalysts and sodium ascorbate used in these experiments were optimized based on performed cytotoxicity assays (Supporting Information, Figure S10). Incubation with prodrug 16 or 18 and Na₂PdCl₄ did not significantly decrease the viability of SKBR3 breast cancer cells (Figures 3a, b). However, preincubation with prodrug and Pd⁰, followed by incubation with sodium ascorbate recovered the cell killing activity for both prodrugs (Figures 3a, b). As anticipated,
cytotoxic activity of the uncaged drugs as well as the unwanted toxic effects of the bond-cleavage reaction triggers increased in a dose dependent manner. Such significant cell-killing effect was not observed using Pd\textsubscript{0}/TPPTS complex which confirms the poor cellular uptake of the bulky and charged Pd\textsubscript{0}/TPPTS complex (Figures 3c, d and S19). Nevertheless, when the prodrug is administered together with Pd\textsubscript{0}/TPPTS, bond-cleavage reaction takes place extracellularly and the liberated drug can then enter cells where it exerts its cytotoxic activity (Supporting Information, Figure S11). Noteworthy, in control experiments, simultaneous addition of the prodrug, Na\textsubscript{2}PdCl\textsubscript{4} and sodium ascorbate did not exhibit any significant levels of cell cytotoxicity (Supporting Information, Figure S13) indicating that the active Pd\textsuperscript{0} species required for the release of cytotoxic drugs is effectively generated in the intracellular environment. These observations, together with the evidence that sodium ascorbate promotes the reduction of Pd\textsuperscript{II} to the active Pd\textsuperscript{0},

![Figure 3](image-url)

Figure 3. Pd-mediated decaging experiments in SKBR3 cells. Decaging of prodrugs 16 (a) and 18 (b) by in-cell generated Pd\textsuperscript{0} species compared to their decaging by Pd\textsuperscript{0}/TPPTS complex (c) and (d), respectively. Error bars represent SEM. ns (P > 0.05), *** (P ≤ 0.001). Decaging of caged fluorophores 21 (e) and 23 (f) followed by fluorescence microscopy. Scale bars represent 100 μm. For full experimental details see Supporting Information.
suggest that spatiotemporal control can be achieved using this Pd-mediated bioorthogonal bond-cleavage strategy.

We also investigated another prodrug of doxorubicin, a propargyl carbamate 19, and observed that the Pd-mediated decaging of 19 is less efficient under these conditions (Supporting Information, Figure S12). This suggests that the depropargylation reaction requires different Pd species. Finally, fluorescence microscopy imaging experiments were performed to provide more evidence in the favor of the presented method for in cell generation of the active Pd species (Figures 3e, f). Caging with either allyl ether or carbamate group resulted in fluorescence quenching of resorufin allyl ether 21 and bis-N,N'-allyloxyacarbonyl-Rhodamine 110 23. As expected, no fluorescent signal was recovered in the presence of Na2PdCl4, however, upon addition of sodium ascorbate “turn-on” of the fluorescent signal was detected inside of SKBR3 cells in both cases (Figures 3e, f). Furthermore, experiments to support the mechanism for extracellular decaging employing the Pd/TPPTS complex were performed and fluorescence of both fluorophores was recovered in this instance as well (Supporting Information, Figures S14, 15). ICP-MS analysis of the cellular extracts revealed the intracellular amount of Pd (ca. 230 ppm) after incubation of Na2PdCl4 following several washing steps and lytic treatment (Figure S19). Altogether, our findings demonstrate that it is the Pd species that are responsible for the restoration of the biological activity of caged allyl ethers and car bamates.

In summary, presented results show that intracellular PdII—Pd0 reduction provides spatiotemporal control over Pd0-catalyzed bioorthogonal chemical reactions. Our simple yet elegant method utilizes the commercially available and water soluble Na2PdCl4 which is reduced to the active Pd0 species by the action of sodium ascorbate inside of living cells. We show that the active catalyst effectively triggers the cleavage of allyl ether and carbamate caging groups, leading to the release of parent small molecules such as fluorophores or cytotoxic drugs. The present study is a proof-of-concept that opens up several possibilities to perform not only bond-cleavage but also other transition metal-assisted bioorthogonal reactions. It is yet not clear how different cell lines, cell growth medium composition and additives influence the production and reactivity of the formed Pd0 species. Further investigations will determine what type of catalytically active Pd0 species is generated inside of living cells and new applications of this strategy towards other types of biocompatible reactions are currently underway in our laboratory.

Acknowledgements

This project has received funding from the European Research Council (ERC) under the European Union’s Horizon 2020 research and innovation programme (Grant agreement No. 676832), the European Commission (Marie-Sklodowska-Curie Actions IEF to E.J.M., Grant agreement No. 701473), FCT Portugal (Stimulus CEECIND/00453/2018 to G.J.L.B) and CAPES PrInt Call—Program for Institutional Internationalization (88887.310560/2018-00). The authors would also like to thank Dr. Vikki Cantrill for her help with the editing of this manuscript and Claudia Flandoli (draw.science) for editing the figures.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: Bioorthogonal · Bond-cleavage · Cancer · Decaging · Palladium

A simple, cell-permeable source of Pd\(^{II}\) is intracellularly reduced to the catalytically active Pd\(^{0}\) through the addition of sodium ascorbate. Controlled in-cell generation of Pd\(^{0}\) enables Pd-mediated bioorthogonal intracellular activation of caged cytotoxic drugs and fluorophores in cancer cells.