Organometallic palladium reagents for cysteine bioconjugation

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Reactions based on transition metals have found wide use in organic synthesis, in particular for the functionalization of small molecules1–3. However, there are very few reports of using transition-metal-based reactions to modify complex biomolecules4–6, which is due to the need for stringent reaction conditions (for example, aqueous media, low temperature and mild pH) and the existence of multiple reactive functional groups found in biomolecules. Here we report that palladium(II) complexes can be used for efficient and highly selective cysteine conjugation (bioconjugation) reactions that are rapid and robust under a range of biocompatible reaction conditions. The straightforward synthesis of the palladium reagents from diverse and easily accessible aryl halide and trifluoromethanesulfonate precursors makes the method highly practical, providing access to a large structural space for protein modification. The resulting aryl bioconjugates are stable towards acids, bases, oxidants and external thiol nucleophiles. The broad utility of the bioconjugation platform was further corroborated by the synthesis of new classes of stapled peptides and antibody–drug conjugates. These palladium complexes show potential as benchtop reagents for diverse bioconjugation applications.

Post-translational modifications greatly expand the function of proteins7. Chemists aim to mimic the success of such natural transformations through the development of chemo- and regioselective reactions of proteins. The diversity of potentially reactive functional groups present in biomolecules (for example, amides, acids, alcohols, amines) combined with the requirement for fast kinetics and mild reaction conditions (for example, aqueous solvent, pH 6–8, temperature T < 37 °C) make challenging the development of new techniques to functionalize proteins. Nevertheless, methods have emerged for bioconjugation with natural and unnatural amino acids in protein molecules8,9. Cysteine is a key residue for the chemical modification of proteins owing to the unique reactivity of the thiol functional group and the low abundance of cysteine residues in naturally occurring proteins8,9. Michael addition to maleimides and S$_2$2 reaction with alkyl halides are commonly used for cysteine modification. The resulting conjugates tend to decompose in the presence of external bases or thiol nucleophiles10, which prompts the recent development of advanced cysteine bioconjugations for the improved stability of conjugates (see ref. 11, and references therein).

The ability to achieve high levels of chemo- and regioselectivity through the judicious choice of metal and ligand design suggest that metal-mediated processes could be very attractive for the development of new bioconjugations. Existing metal-based transformations often rely on the use of functional handles12 or unnatural amino acids, such as 4-iodophenylalanine and aldehyde- or alkyne-containing amino acids13–15, and require high concentrations (mM) of derivatizing agents, which can cause off-target reactivity or purification problems. We considered that palladium complexes resulting from the oxidative addition of aryl halides or trifluoromethanesulfonates14 could be used for the transfer of aryl groups to cysteine residues in proteins (Fig. 1a). (For existing transition-metal-catalysed C–S bond-forming reactions, see ref. 15.) The efficiency and selectivity of the proposed reaction with the highly active palladium species may be hampered by the presence of a variety of functional groups within complex biomolecules. Furthermore, the presence of free thiols has been previously shown to inhibit palladium-catalysed cross-coupling reactions on peptides16, while Pd(II)-complexes have also been shown to exhibit protease-like behaviour with certain peptides17. However, we envisioned that the careful choice of ligand would provide stable, yet highly reactive reagents for the desired transformations (Fig. 1b), while the interaction between the soft nucleophile cysteine thiol and the aryl palladium(II) species would guide its selectivity.

We began our study with a palladium–tolyl complex (1A–OTf; Fig. 1c) using 2-dicyclohexylphosphino-2,6-dipropargylphenyl (RuPhos) as the ligand and trifluoromethanesulfonate as the counterion. A model peptide (P1; Fig. 1c) was used for the optimization of the reaction conditions and for the exploration of the substrate scope. Full conversion of the starting peptide to the corresponding aryl product was observed in less than 5 min at low micromolar concentrations of reagents (Fig. 1c). Further, the reaction was selective for cysteine. No reaction was observed using a control peptide with cysteine mutated to serine (Supplementary Information), in contrast to the palladium-mediated protein allylation, which is selective for tyrosine (O-allylation) over lysine and cysteine (N- and S-allylation). (The new aryl–palladium reagents are less electrophilic than the allylpalladium species used in ref. 18, which tunes their selectivity towards cysteine residues while making them completely unreactive towards alcohol-based species like tyrosine.) These results highlight the importance of choosing the right ligand, which facilitates C–S reductive elimination and, together with the overall electrophilicity of the palladium centre, tunes the selectivity of the transformation.

Most cysteine conjugation reactions operate at nearly neutral to slightly basic pH. Further evaluation of the reaction conditions using palladium reagents revealed quantitative conversion of the starting peptide to the corresponding S-aryl cysteine conjugate within a broad pH range (5.5–8.5) using common organic co-solvents (5% of N,N-dimethylformamide (DMF), dimethylsulfoxide (DMSO), acetonitrile (CH$_3$CN)) in various buffers (Supplementary Information, Supplementary Table 1). Remarkably, even in 0.1% trifluoroacetic acid (TFA) solution (pH 2.0) the reaction yielded 59% of the S-arylated product after 7 h. The process was also compatible with the protein disulfide reducing agent tris(2-carboxyethyl)phosphine (TCEP) that has been shown to hamper bioconjugations by reacting with maleimide and α-haloacyl groups19.

The palladium-mediated conjugation is fast, and complete product formation occurs within 15 s at 4 °C. The reaction rate was estimated by competition experiments against the commonly used N-methyl maleimide cysteine ligation. (The reported20 kinetics for maleimide conjugation are in the range $10^3$–$10^5$ M$^{-1}$ s$^{-1}$ at pH 7.5.) At pH 7.5
the rate of the palladium-mediated reaction was comparable to that of the maleimide ligation, where 70% of the products resulted from the reaction with palladium–tolyl complex (1A–OTf). Notably, the palladium-mediated conjugation outperformed the maleimide ligation at pH 5.5 where only the arylated product was formed.

The optimized conditions (0.1 M Tris buffer, 5% CH$_3$CN, pH 7.5, room temperature) were used for further evaluation of the substrate scope. Palladium complexes containing chloride, bromide and iodide counterions were all found to produce the desired product (Fig. 2, 1A–Cl, 1A–Br and 1A–I). This method can be used to functionalize unprotected peptides with a variety of important groups, including fluorescent tags (1C, 1D), affinity labels (1E), bioconjugation handles (aldehyde 1F, ketone 1G and alkyne 1H) and photochemical crosslinkers (1I), as well as complex drug molecules (1J). Vinyl palladium complexes were also shown to be competent in this transformation (Supplementary Information). Importantly, the palladium(ii) complexes are stable under ambient conditions, and can be stored in closed vials under air at 4 °C for over four months. Long-term stability of 1A–I, 1A–Br, 1A–Cl and 1A–OTf was evaluated; only the complex bearing the trifluoromethanesulfonate counterion (1A–OTf) showed some degradation (≤15%) after 20 weeks (Supplementary Information). Nevertheless, the ‘aged’ reagent still exhibited reactivity comparable to the freshly made complex.

The stability of our arylated peptides was compared to that of conjugates formed from reactions with reagents including N-ethyl maleimide, 2-bromoacetamide and benzyl bromide. The S-arylated peptide was shown to be stable towards acids, bases and external thiol nucleophiles (Supplementary Information). In contrast, the corresponding acetamide derivative was unstable under acidic and basic conditions, and the maleimide conjugate decomposed in the presence of base and...
exogenous thiol. Finally, comparable stability of both aryl and benzyl conjugates to treatment with the periodic acid oxidant at 37°C was observed. However, additional tuning of the electronic properties of the aromatic ring of the arylated peptide could be achieved by installing an electron-withdrawing cyano group in the \textit{para} position. This modification significantly decreased the amount of oxidation, producing the most stable peptides across all the evaluated conjugates.

Notably, installing the \textit{para} cyano group in the benzyl conjugates had no effect on oxidation (Supplementary Information).

We further explored this reaction with proteins. Three antibody mimetic proteins (P4–P6; Fig. 3) were expressed that contained a cysteine at structurally distinct positions including the amino-terminus, the carboxy-terminus and a loop. The same proteins without cysteines were used as controls to confirm the selectivity of the reaction.

\[ \text{Protein} + \text{Pd complex} \rightarrow \text{Arylated Protein} \]

Figure 2 | The substrate scope of cysteine arylation using organometallic palladium reagents. Top, the reaction studied. Bottom, a selection of palladium reagents was used to test the effect of the leaving group (X) on the reactivity and explore the substrate scope with regard to biologically relevant groups (fluorescent tags, bioconjugation handles, affinity tag and a drug molecule). Full conversion of starting peptide P1 into the corresponding arylated products was observed in all the cases shown, as confirmed by LC-MS. For exact reaction procedures and conditions, see Supplementary Information.

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Figure 3 | Protein modification using the developed palladium reagents. Cysteine residues at the N terminus (P4; a), a loop (P5; b) and the C terminus (P6; c) of proteins are quantitatively modified with coumarin after the reaction with palladium complex 1D (top). Deconvoluted mass spectra of the full protein peaks are shown for the starting proteins (P4–P6) and reactions with coumarin-palladium complexes after 30 min (bottom). Three-dimensional structures of proteins P4–P6 and the arylated products (P4-D, P5-D and P6-D) are presented next to the corresponding mass spectra.
The developed protocol was used to arylate an engineered cysteine residue in the C-terminal region of diphtheria toxin A-chain (DTA) fused to the lethal factor N-terminal domain (LFN-DTA-Cys, see Supplementary Information)\(^1\). The modified LFN-DTA-Cys variant was readily separated from the remaining palladium species, ligands and other small molecules using commercially available size-exclusion chromatography columns (91% of palladium was removed, as determined by inductively coupled plasma mass spectrometry (ICP-MS) analysis of the purified protein sample, see Supplementary Information). The modified and purified LFN-DTA-Cys variant displayed similar activity (half-maximal effective concentration \(EC_{50} = 0.40 \pm 0.09 \text{nM}\)) in a cell-based protein synthesis inhibition assay compared to the control (a serine mutant LFN-DTA-Ser showed \(EC_{50} = 0.25 \pm 0.05 \text{nM}\) (Supplementary Information)).

Stapled peptides have shown significant promise as next-generation therapeutics\(^2,3\). However, there are limited methods for the synthesis of these bioconjugates with structurally diverse linkers\(^4\), which hinders the systematic investigation of the effect of the linker on the properties of the stapled peptides\(^5\). We envisioned that palladium reagents containing two electrophilic metal centres could be efficiently used to cross-link two cysteine residues on a peptide chain, thereby providing access to stapled peptides with various aryl linkers (Fig. 4a). Indeed, running the reaction at 10 \(\mu\)M concentration of peptide in a 1:1 (v/v) acetonitrile/water solvent mixture at pH 7.5 using a twofold excess of the bis-palladium complex 2A resulted in quantitative formation of the target stapled peptide within 10 min (Fig. 3a and Supplementary Information). Considering the availability of commercially or otherwise easily accessible diarylhalide reagents, this approach provides facile access to a diverse aryl-linker space for stapled peptides\(^6\).

Antibody–drug conjugates (ADCs) are a promising class of biotherapeutics, which combine the potency of cytotoxic drugs with the target specificity of monoclonal antibodies\(^7\). We aimed to attach drug molecules directly to cysteine residues in antibodies through the developed palladium conjugation chemistry. The drug payload vandetanib was used to form palladium complex 1J (Fig. 2) by making use of the aryl bromide present in its structure. Treating partially reduced trastuzumab antibody\(^8\) with 1J readily produced ADCs with a 4.4 drug to antibody ratio (DAR; Fig. 4b). The purified arylated ADCs (94% of palladium was removed, as determined by ICP-MS analysis of the purified ADCs, see Supplementary Information) retained binding affinity (dissociation constant \(K_d = 0.3 \pm 0.2 \text{nM}\) to recombinant HER2 compared to the unmodified trastuzumab antibody (Supplementary Information). While traditional ADCs use various linkers to attach drug molecules to antibodies, our method significantly expands the structural space of ADCs by providing the capability to directly attach drug molecules containing native or pre-installed aryl halide or phenol functional groups. The therapeutic potential of this class of 'linker-free' ADCs will be investigated in the future.

We note that the ease of preparation, storage and use of the present palladium reagents make them particularly attractive for routine application in chemistry, biology, medicine and materials science. Further evolution of the metals and ligands employed will probably provide an extended set of organometallic bioconjugation reagents with altered selectivity and efficiency, allowing for functionalization of other amino acid residues.
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Supplementary Information is available in the online version of the paper.

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