Organometallic Oxidative Addition Complexes for *S*-Arylation of Free Cysteines

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Abstract

Development of bioconjugation strategies to efficiently modify biomolecules is of key importance for fundamental and translational scientific studies. Cysteine *S*-arylation is an approach which is becoming more popular due to generally rapid kinetics and high chemoselectivity, as well as the strong covalently bonded *S*-aryl linkage created in these processes. Organometallic approaches to cysteine *S*-arylation have been explored that feature many advantages compared to their more traditional organic counterparts. In this Viewpoint, progress in the use of Au(III) and Pd(II) oxidative addition (OA) complexes for stoichiometric cysteine *S*-arylation is presented and discussed. A focus is placed on understanding the rapid kinetics of these reactions under mild conditions, as well as the ability to generate biomolecular heterostructures. Potential avenues for further exploration are addressed and usefulness of these methods to the practitioner are emphasized in the discussion.

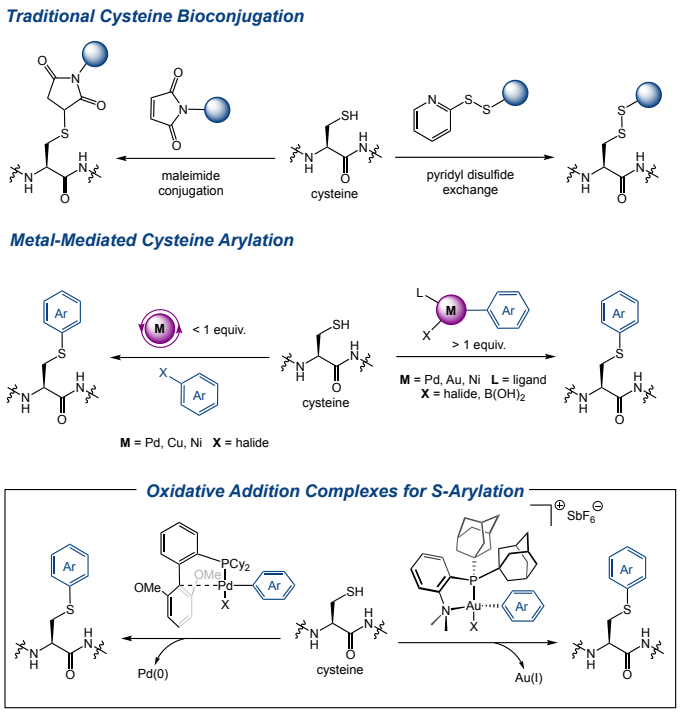
Introduction

Installation of synthetic post-translational modifications on proteins and peptides provides tremendous value to many fields that utilize these biomolecules. These modifications are typically made to comprehensively study or modulate the activity and other physical, material, and pharmacokinetic properties of these biomolecules, often for potential therapeutic purposes. Researchers at the interface of chemistry and biology, specifically those working in the field of bioconjugation, have developed a plethora of strategies to do so, each with their own unique benefits and challenges.[1]

Generally, bioconjugation reactions rely on modification of biological nucleophiles, particularly nucleophilic amino acid residues such as lysine, cysteine, serine, and histidine, as well as the *N*-terminus of proteins and peptides.[2,3] While these perform well as reactive handles, there are many different nucleophilic species usually present in a biomolecule; therefore, selectivity for the functional group of interest is an important consideration when developing a bioconjugation technique. Cysteine (Cys) is one of the least abundant amino acid residues, exhibits a relatively low pK_a (~8.3), and is highly nucleophilic, making it an effective target for selective bioconjugation.[4,5] The most commonly utilized bioconjugation strategies with cysteine include disulfide exchange and alkylation with iodoacetamide or maleimide Michael addition (**Scheme 1**, top). While these strategies have proven useful, the bonds formed in conjugations utilizing maleimide and disulfide chemistries tend to be labile *in vivo* and to acidic or reducing conditions. While reversible conjugation strategies can be beneficial when there is a desire to release the payload of interest, in many cases, early or non-specific release can cause major issues with biological delivery and circulation time, particularly in the context of long acting therapeutics which utilize toxic payloads.[6,7] When a stronger, non-labile covalent bond is preferred, an iodoacetamide reaction or arylation approach can be utilized. Over the past decade, many examples of the latter, Cys *S*-arylation have been developed, resulting in a stable S-C(sp)^2 bond between the biomolecule and the synthetic molecule of interest. This arylation approach has been increasingly growing including advances in organic S_NAr chemistry, and more recently organometallic bioconjugation strategies.[8]

Organometallic Bioconjugation

Organometallic bioconjugation describes methodologies which employ transition metal complexes for modifying biomolecules, and includes both catalytic and stoichiometric examples. Transition metal catalysts and reagents can be highly reactive, leading to the notion that they are difficult to handle and not appropriate for use in the aqueous conditions essential for biomolecules. However, with careful ligand design and metal selection, many bench stable and easy to handle transition metal catalysts and stoichiometric reagents have been developed for bioconjugation and are now available for use to a wide range of practitioners.[9,10]



Scheme 1: Common examples of traditional cysteine bioconjugation, metal-mediated cysteine arylation, and representative examples of the first OA complexes for cysteine arylation.

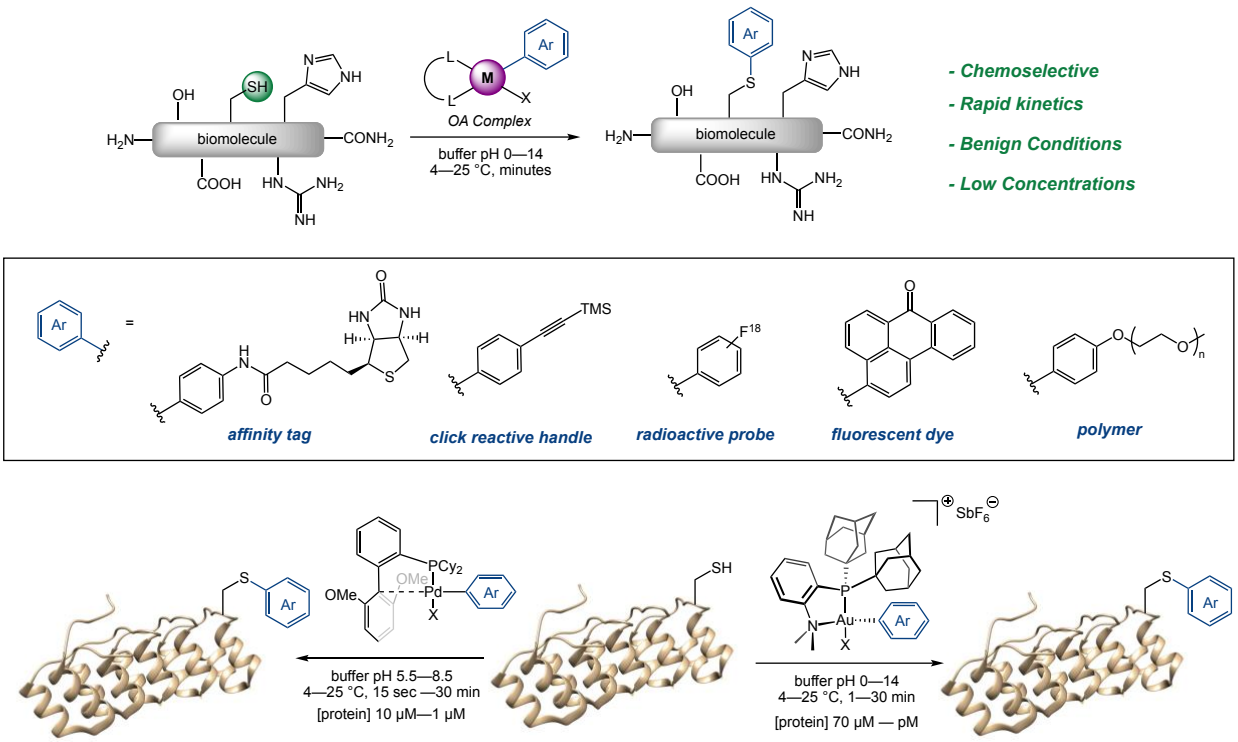
Catalytic transition metal bioconjugation strategies take inspiration from a large body of widely developed and highly valuable small molecule transition metal catalysis literature.[11] Stoichiometric examples have also been explored where one or more equivalents of transition metal complex are needed to complete the reaction (**Scheme 1**, middle). These reactions generally occur rapidly and selectively under more benign conditions in comparison to catalytic examples. Within these stoichiometric examples, two main classes of reactions have been explored: *in situ* metal-mediated conjugation and use of preformed oxidative addition (OA) complexes. While many catalytic and *in situ* stoichiometric transition metal-mediated bioconjugation strategies have been developed, the remainder of this Viewpoint will discuss the development of these OA complexes for *S*-arylation of cysteine residues and their unique qualities for rapid, efficient, and tolerant bioconjugation. The authors direct the reader to representative literature and several comprehensive reviews on other organometallic bioconjugation strategies for more information on those systems.[9,10,12–18]

Oxidative Addition Complexes

Organometallic OA complexes are reagents wherein a transition-metal-ligand complex has already undergone oxidative addition with an aryl-halide or aryl-triflate coupling partner.[19,20] These reagents can be isolated, purified, and stored for later use for modification of biomolecules. The oxidative addition step in a traditional catalytic cycle is generally considered the relatively slow, turnover-limiting step, often necessitating heat and high concentrations to achieve complete conversion. While these harsh reaction conditions are not generally amenable to delicate biomolecules, the preformation and isolation of OA complexes allows one to separate the use of more harsh reaction conditions from the bioconjugation step. Then, the OA complexes can be introduced to the biomolecule under reaction conditions amenable for their stability, and the bioconjugation process proceeds rapidly through the remaining elementary steps in the reaction. While turnover of the transition metal is not achieved in this system, the starting metal species is a byproduct in the reaction, and in some cases, this complex can be reisolated to undergo further oxidative addition reactions to enable its use in a subsequent bioconjugation reaction.[21] This strategy is highly modular, as a library of OA complexes can be synthesized and stored for subsequent bioconjugation to a variety of biomolecules containing cysteine.

In 2015, a collaboration between the Buchwald and Pentelute groups resulted in the first example of specific cysteine bioconjugation using a Pd(II) OA complex following the logic described above (**Scheme 1**, bottom left).[22] Utilizing the RuPhos ligand, the authors synthesized Pd(II)-aryl reagents by reacting Pd(0) precursor with aryl halides and aryl triflates in a nitrogen-filled glovebox for 16 hours. The resultant OA reagents were isolable and stable to open air, and performed rapid conjugation to cysteine in peptides and proteins, while successfully avoiding side reactivity with other nucleophilic residues. The authors observed that careful choice of ligand not only allowed for the observed high levels of reactivity and selectivity, but also enabled these reagents to be isolable and bench stable. Reaction times ranged from seconds to 30 minutes for these *S*-arylations, which coupled small molecules to various peptide and protein substrates with quantitative levels of conversion.

In 2018, the Spokoyny and Maynard groups developed Au(III) OA complexes[22] for bioconjugation to cysteine (**Scheme 1**, bottom right).[23] The inherent thiophilicity of Au(III), as well as the generally facile reduction from Au(III) to Au(I), lead to the hypothesis that these Au(III) OA complexes may perform well within the complex biological context with low propensity for background reactivity. These OA complexes utilized the hemilabile P,N DalPhos ligand scaffold and underwent oxidative addition in minutes open to air without the need for explicit exclusion of moisture. These bench stable Au(III) reagents were also able to achieve rapid and selective cysteine bioconjugation, thereby expanding the toolbox of these OA complexes in a complementary approach. Both the Au(III) and Pd(II) OA complexes create *S*-aryl bonds. The S-aryl bond has been shown to be stable at pH 0-14 and in highly reducing conditions (5% v/v% BME at 90 °C), a potential advantage of the chemistry for therapeutics. Therefore, the S-aryl bond has proven more stable in the test tube; however, the irreversibility of these bonds *in vivo* has not yet been proven.



Scheme 2: Top: General representation of the chemo-compatibility of bioconjugation process and selectivity for thiols. Middle: A wide range of OA complexes featuring biologically relevant substrates has been shown with a few selected examples shown here which can be transferred by either Au(III) or Pd(II) chemistry. Bottom: Highlighted examples of the benign reaction conditions employed in both Pd(II) and Au(III) bioconjugation to proteins (model protein DARPin shown as a representative example).

Chemoselectivity

In the context of many biological functional groups, selectivity for the conjugation handle of choice is of great importance, particularly for predictable and consistent conjugation to molecules with therapeutic relevance. When these OA complexes were introduced to biomolecules containing a range of reactive amino acids (Lys, Tyr, Ser, etc.), excellent selectivity for thiols was observed (**Scheme 2**, top). Additionally, both Pd(II) and Au(III) OA complexes tolerate phosphine reducing agents, such as TCEP, which are routinely included in the reaction mixture in order to reduce potential inter-protein disulfides. In the context of the Au(III) OA complexes, up to five equivalents of TCEP is tolerated before ligand exchange is observed, ablating the complex's ability to perform bioconjugation. An additional source of chemo-compatibility is observed in the context of the histidine tags commonly used to purify recombinant proteins. It was found that bioconjugation using these Au(III) OA complexes is tolerant to the presence of a His_6 tag on recombinant DARPin, despite the coordinating ability of histidine as a metal binding ligand. [24]

The oxidative addition step is also highly compatible with a wide range of functionalities including carboxylic acids, alcohols, amines, heterocycles, sulfones, sulfonyl fluorides, NHS esters and extraneous halides, including ^{18}F radiolabeled substrates.[22,23,25] Many molecules used in studies of bioconjugates possess a physical property, chemical reactivity or biologically activity that is critical to retain throughout the bioconjugation process and is necessary for further studies of the desired conjugate. Thus, this feature is a significant advantage of the approach. Examples of Pd(II) and Au(III) OA complexes include fluorescent dyes, affinity tags, drugs, and polymers (**Scheme 2**, middle).

Benign Reaction Conditions

Another important factor is the general instability of biomolecules in comparison to their small molecule and organic counterparts. Many biomolecules are only stable in buffered aqueous solutions at or below room temperature and deviation from these benign conditions results in protein degradation and aggregation. The rapid kinetics of these *S*-arylation processes allows for high levels of conversion quickly, in dilute aqueous solutions, at or below room temperature.[22–24,26] These exceptionally mild reaction conditions allow for rapid and efficient bioconjugation to substrates which may otherwise be unstable to harsher reaction conditions. Additionally, these reactions are well suited for biomolecules which are only stable or soluble at non-physiological pH, as these reactions also perform well in reaction conditions ranging from pH 0-14 (**Scheme 2**, bottom) as well as high ionic buffer strength. The limited aqueous solubility of some of these OA complexes often necessitates the addition of <10% polar organic solvent.

However, further ligand selection allowed for the synthesis of Pd(II) OA complexes with sufficient reactivity and improved water solubility through sulfonation of the aryl ring on the ligand (**Scheme 3**, a-c).[27] Furthermore, depending on the OA substrate, the resulting complex can be soluble in water.[23,24]

Moreover, the complex secondary structure of many biomolecules, particularly proteins, creates a challenging steric environment around the targeted nucleophilic residue, necessitating a reaction with rapid kinetics to overcome the decreased reaction rate. Many bioconjugation methods attempt to circumvent this challenge by using large excess of coupling reagent to ensure high level of conversion, and while this strategy does tend to increase conversion, it can be difficult to purify the excess reagent away from the conjugate. Alternatively, optimization of these Pd(II) and Au(III) OA complexes resulted in standard conditions of near equimolar amounts of the reagent while still achieving complete reactions within minutes at low µM concentrations and low temperatures.[22–24,26]

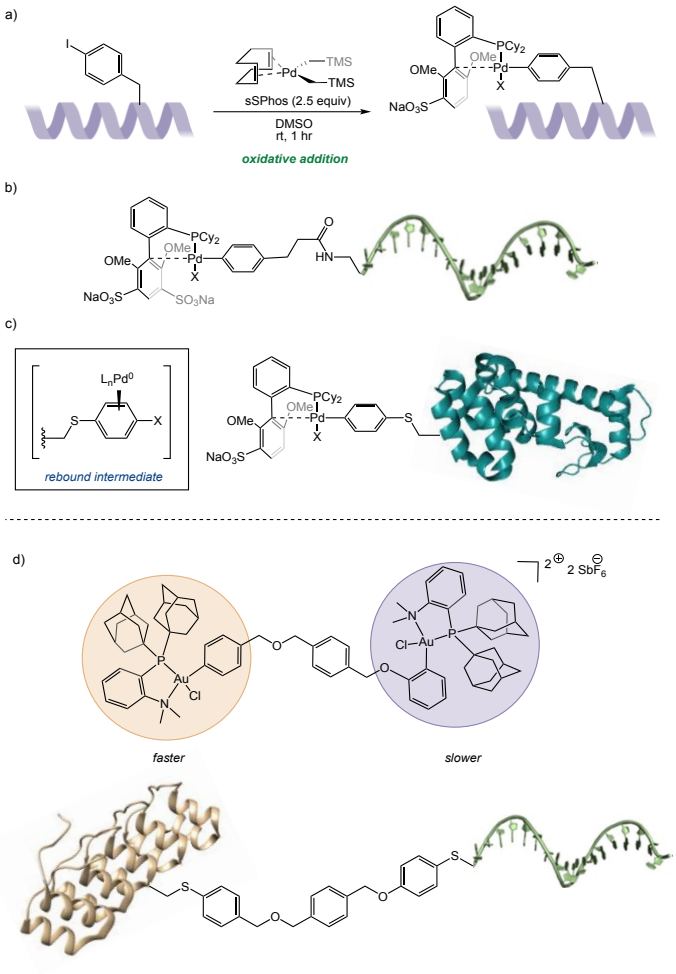
Challenging the Kinetic Limits

In order to further explore the rapid kinetics of the Au(III) OA complexes, the Spokoyny group challenged the system by increasing the steric crowding of the complex. An atomically precise boron cluster with Au(III) OA complexes extending from all 12 vertices was synthesized. This OA complex successfully achieved 12-fold *S*-arylation with glutathione, creating a densely functionalized nanocluster with high precision.[28] The Spokoyny and Maynard groups then increased the size of the OA complex further by preparing polymers. Conjugation of polymers to the surface of biomolecules has therapeutic relevance, and synthesis of protein-polymer conjugates can be challenging due to the increased steric bulk associated with both coupling partners. It was demonstrated that conjugation of polymers to the surface of proteins at 7 µM and 4 °C with only 1.3 equivalents of Au(III) mPEG reagent occurred with > 90% conversion in only one minute.[24]

Further exploration of ligand design in this Au(III) system resulted in the ability to tune the kinetics of the bioconjugation reaction. In this work, it was found that modifying the Me-DalPhos ligand to contain dicyclohexylphosphine instead of diadamantylphosphine allowed for a nearly 10-fold increase in the second order rate constant of the bioconjugation. This ultra-fast conjugation was employed to conjugate a fluorescent small molecule to a protein at high pM concentrations, representing an extreme example of the power of these reagents to modify proteins rapidly and under mild conditions.[26]

Construction of Biomolecular Heterostructures

The ability to dramatically increase the rates of these reactions simply by modulating the ligand environment also allows for one to slow down these reactions in a controlled fashion in order to tune selectivity and without compromising the useful kinetic range of the overall system. For example, modulation of the local steric environment around the Au(III) center resulted in selective bioconjugation with a *para*-substituted OA complex in the presence of an otherwise identical *ortho*-substituted OA complex. To this end, bifunctional Au(III) OA complexes have been developed to generate biomolecular heterostructures of various identities utilizing a linker containing the two, orthogonal Au(III) centers for selective and modular bioconjugation (**Scheme 3**, d).[26] Examples of these structures include but are not limited to peptide-



Scheme 3: a) Pd(II)-peptide OA complexes synthesized *via* oxidative addition with 4-iodophenylalanine amino acids.[30] b) Oligonucleotide OA complexes synthesized *via* orthogonal NHS ester coupling with aqueous complex.[31] c) Pd(II)-protein OA complexes synthesized *via* a Pd(II) rebound process *in situ*.[29] OA complexes a-c can be subsequently reacted with thiol-containing biomolecules to form heterodimers (PDB ID: 2HUK). d) Example of a Au(III) bifunctional OA complex for the highly modular construction of biomolecule heterodimers. The Au(III) center highlighted in orange is more sterically available, causing it to react selectively with the first equivalent of thiol-containing biomolecule, then the Au(III) center in purple reacts with a second equivalent of thiol added to solution in this one-pot system.

oligonucleotide, peptide-peptide, protein-polymer and protein-oligonucleotide biomolecular heterostructures.

Pd(II) OA complexes have also been developed for the construction of biomolecular heterostructures utilizing a different approach. Pd(II) protein, peptide, and oligonucleotide OA complexes have been synthesized for subsequent reaction with a library of thiol-containing biomolecules for heterodimerization (**Scheme 3**, a-c).[29–31] In some examples, the OA complexes are employed as rebound reagents wherein the reagent underwent a single bioconjugation to Cys, followed by subsequent reinsertion into the same aryl ring at a different site, which was then poised to react with a second thiol-containing molecule.[15,29,32] The speed and selectivity of the reinsertion of these Pd(II) complexes into the aryl ring at close proximity despite a surrounding sea of nucleophiles is particularly impressive, and the substrates which are able to react in this way open a new avenue for heterostructure synthesis.

Outlook

The rapid and selective nature of the developed OA reagents based on organometallic palladium and gold chemistry make these systems an ideal emerging choice for the construction of complex biomolecule conjugates for various materials and therapeutic applications. The speed of this chemistry has been utilized for the synthesis of ^{18}F labeled bioconjugates, however, there remains room for further exploration into the conjugation of other functional molecules with short lifetimes. The synthesis of even larger bioconjugate structures is also a potential future direction for exploration with these OA complexes, for example protein dimers or cages. The construction of branched or crosslinked polymer conjugates or even biomolecule-polymer frameworks may be accessible utilizing this method, especially where other conjugation methods fail to achieve high levels of bioconjugation in such sterically demanding environments. In order to test the limits of these bioconjugation reactions further, expansion of ligands and utilization of other metals for OA complexes may result in additional tools, particularly with the aid of computational understanding of the mechanism. Notably, OA complexes can be potentially accessed not only through a direct oxidative addition process of an aryl-based electrophile but also indirectly *via* other routes.[33–35]

One of the most interesting areas for future development rests with the ability to tune these reagents further to perform site-selective chemistry on Cys residues in the presence of other Cys within the same biomolecule. This can be accomplished by engineering more sophisticated ligands capable of selectively recognizing amino acid sequences through a combination of electrostatic and non-covalent interactions. This should in principle lead to the successful abiotic chemistry conceptually reminiscent of the enzymatic machinery capable of performing residue specific post-translational modification. Importantly, the high levels of stability of these systems may also allow for the general adoption of OA complexes by individuals with a range of expertise and interests, fueling advances and discoveries in fields far beyond organometallic chemistry. The remaining metal and ligand by-products do need to be removed from these reactions, but this is easily accomplished utilizing standard techniques such as HPLC or FPLC. Furthermore, the resulting conjugate is linked through an aryl carbon-sulfur bond, which could be more stable for conjugates with long *in vivo* half-lives such as antibody drug conjugates. Collectively these features make OA complexes attractive to the biomedical community. Therefore, there is a lot of potential future work with OA complexes for thiol conjugation to prepare simple protein-polymer conjugates to complex biomolecule architectures.

Acknowledgements

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