Invited paper

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Organometallic chemical biology: an organometallic approach to bioconjugation

https://doi.org/10.1515/pac-2017-0207

Abstract: This review summarizes the history and recent developments of the field of organometallic chemical biology with a particular emphasis on the development of novel bioconjugation approaches. Over the years, numerous transformations have emerged for biomolecule modification with the use of organometallic reagents; these include [3+2] cycloadditions, C–C, C–S, C–N, and C–O bond forming processes, as well as metal-mediated deprotection ("decaging") reactions. These conceptually new additions to the chemical biology toolkit highlight the potential of organometallic chemistry to make a significant impact in the field of chemical biology by providing further opportunities for the development of chemoselective, site-specific and spatially resolved methods for biomolecule structure and function manipulation. Examples of these transformations, as well as existing challenges and future prospects of this rapidly developing field are highlighted in this review.

Keywords: bioconjugation; biotherapeutics; chemical biology; chemical decaging; click chemistry; cross-coupling reactions; IUPAC-SOLVAY International Award for Young Chemists; organometallic chemistry.

Introduction

The fast pace and interdisciplinary nature of current research, challenges the broad, all-encompassing definitions of scientific disciplines, and calls for redefining specific research areas in order to highlight the essential details relevant to each particular subdiscipline. To this end, we have witnessed the recent emergence of chemical biology as a separate discipline [1, 2], which has long been part of the more general field of biochemistry. This appreciation of chemical biology can be gleaned from the emergence of specific chemical biology journals (e.g. *Nature Chemical Biology*, *ACS Chemical Biology*), conferences dedicated to the research in the field, and even university departments.

Another remarkable example is the field of bioinorganic chemistry [3, 4], which over the past 50 years has grown to encompass studies on the role of metals in biology (as metabolic cofactors and signaling mediators) [5, 6], medicine [7, 8], and toxicology [9], as well as metalloprotein engineering [10–14]. Each of these expanding areas involves multiple academic groups, deserves a review every year, and can be considered as a well-established subdiscipline of their own.

Similar to metal coordination complexes, organometallic compounds have found diverse applications in the field of biology [15], leading to the evolution of new subdisciplines, including "medicinal organometallic chemistry" [16] and "bioorganometallic chemistry" [17–19]. This review will focus on a relatively new subfield at the interface of organometallic chemistry and chemical biology, which has witnessed a rapid growth over the past decade – organometallic chemical biology. The wide ranging reactivities presented by organometallic compounds through the judicious choice of metal/ligand combinations have ignited interest in expanding

Article note: A collection of peer-reviewed articles by the winners of the 2016 IUPAC-SOLVAY International Award for Young Chemists.

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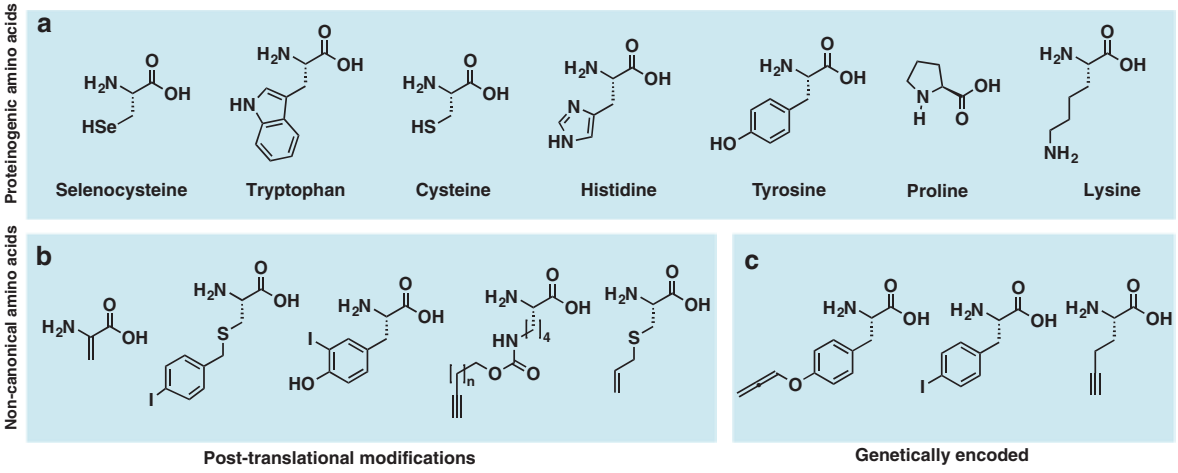

Fig. 1: A selection of proteinogenic (a) and non-canonical (b, c) amino acids used in organometallic chemical biology.

the scope of biological problems that can be addressed with organometallic chemical tools. As a result, there has been a steady presence of these transformations in recent reviews covering developments in the field of bioconjugation [20–23]. A representative selection of amino acids (proteinogenic and non-canonical), which have been used in organometallic chemical biology, is highlighted in Fig. 1.

Organometallic chemical biology: early days

One of the earliest examples of the use of organometallic compounds in the context of biomolecule functionalization can be traced back to Perutz and Kendrew (1950s), who used organomercury complexes to facilitate X-ray structure determination of globular proteins – myoglobin and hemoglobin [24–26]. Attachment of heavy mercury atoms to cysteine residues allowed the authors to apply the method of isomorphous replacement to protein substrates, which resolved the phase problem in protein X-ray structure determination, ultimately leading to Perutz and Kendrew receiving the 1962 Nobel Prize in chemistry.

Another early and often overlooked example of covalent labeling of biomolecules using organometallic complexes goes back to the early 1990s, when two groups independently published their work on the labeling of nucleophilic amino acid residues in peptides [27] and proteins [28] with in situ formed (dienyl)iron tricarbonyl cations (Fig. 2). Iron tricarbonyl remains bound to the final bioconjugate, which allowed both groups to study this transformation and its selectivity by IR spectroscopy combined with ^{1}H and ^{13}C NMR. The reactions were shown to be relatively promiscuous with respect to peptide and amino acid labeling (cysteine [27], histidine [27], amine-containing amino acid [28], and even glutamic acid [27] labeling was observed), however it was possible to achieve higher levels of selectivity using this transformation for protein labeling (e.g. lysozyme [27] and α-chymotrypsin [28]).

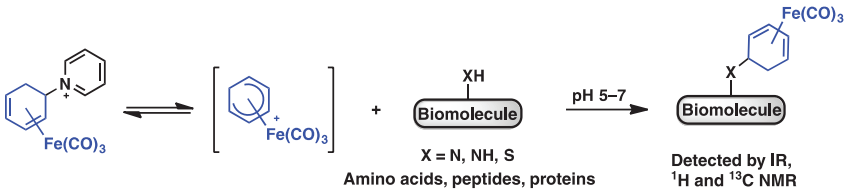

Fig. 2: One of the initial reports using organometallic compounds for covalent labeling of proteins [27, 28].

The developments in the field of organometallic chemical biology covered in this review can be divided into three categories:

1. **Organometallic chemistry for bioconjugation reactions, with the aim of building chemical and biological complexity.** The applications of this approach include synthesis of antibody-drug conjugates, stapled peptides and other functionalized peptide therapeutics. The tremendous success of the Cu-catalyzed [3 + 2] azide alkyne cycloaddition reaction has inspired further research in organometallic chemical biology, including the introduction of olefin metathesis, C–H arylation, C–C, C–S, and C–N cross-coupling reactions into the chemical biologist's toolbox. The challenges associated with these approaches, including the development of site-selective methods, expansion of the repertoire of the reactions and substrate complexity, transition metal removal, and the requirement for mild reaction conditions will be discussed.
2. **Reactions in living cells catalyzed by organometallic complexes.** These studies have allowed scientists to manipulate biochemical processes by generating active species (proteins or drugs) from the corresponding inactive pro-forms within intact cells. Reactions in cellular systems impose a set of new challenges for their development and application. Research in this area has been aimed at the design and synthesis of more efficient reaction catalysts, development of site and subcellular compartment specific transformations, as well as stimuli-responsive chemistries. To this end, the use of palladium and ruthenium catalysts for protein and pro-drug "decaging" has found the most success (decaging: a term, introduced to describe protecting group removal from pro-drugs and artificial pro-proteins).
3. **Organometallic complexes and transformations for small molecule detection in chemical biology.** This relatively new area of organometallic chemical biology is aimed at the development of new organometallic tools for small molecule detection in cells and elucidation of their role in diverse cellular processes, including cell signaling. To this end, reaction-based organometallic fluorescent probes have been used for imaging of carbon monoxide in living cells. However, many other applications can be anticipated. Enhancement of reaction kinetics, expansion of substrate scope, stringent control of potential side reactions and metal complex toxicity, as well as design of reversible probes will ensure successful future developments of this area.

Each of these categories will be mentioned throughout the review when specific types of bioconjugation reactions are discussed. It is important to note, that while the distinction between organometallic complexes and coordination compounds is not always clear cut, in order to comply with the strict definition of organometallic chemistry (where an organometallic compound is defined as a chemical species with at least one bond between a metal and a carbon atom in an organic ligand), reactions catalyzed by coordination complexes of metals with organic ligands (e.g. metal-catalyzed hydrolysis of peptide bonds [29, 30] and single-electron transfer reactions [31, 32]) remain outside of the scope of this review.

Transition metal-catalyzed bioconjugation reactions

Transition metal catalysis has been instrumental in revolutionizing existing approaches toward small molecule synthesis for both industry and academia. The past 30 years of intensive research in this area have produced a number of robust, cost-efficient processes that are effective across a broad range of substrates; this includes widely used palladium-catalyzed cross-coupling reactions, the development of which led to the 2010 Nobel Prize in Chemistry awarded to Richard F. Heck, Akira Suzuki and Ei-ichi Negishi. Perhaps a true testament to the extent of progress in this area over the years is the increasing use of organometallic transformations for chemical biology applications [33–39]. The highly complex structure of biomolecules and their environment imposes strict requirements on the transformations and catalysts for bioconjugations. Catalysts must possess high activity towards the desired reaction partner (e.g. a specific amino acid residue) under biologically relevant conditions (aqueous solvents, pH 7.0–7.5), low substrate concentrations, and in the pres-

ence of numerous other potentially reactive residues. At the same time, catalysts should also be water-soluble and stable under the reaction conditions. Furthermore, the application of bioconjugation reactions to live cells imposes additional requirements on catalyst stability (e.g. high millimolar concentrations of thiols or variable pH in different subcellular compartments), as well as maintaining cellular homeostasis in the presence of reagents and catalysts.

Copper-catalyzed azide-alkyne [3 + 2] cycloaddition

Arguably the most widely used organometallic transformation in the field of chemical biology is the regioselective copper(I)-catalyzed Huisgen [3 + 2] cycloaddition of azides and alkynes. The reaction was first reported independently by the groups of Rostovtsev et al. [40] and Tornøe et al. [41], where the latter showed that the chemistry could be used on protected peptide substrates as part of solid phase peptide synthesis (SPPS). The high stability of both reaction partners, mild reaction conditions, compatibility with water, and the lack of byproducts formed make it an ideal transformation for chemical biology applications. In their pioneering work, Finn and co-workers realized the great potential of this transformation for bioconjugation of complex biomolecules, and were able to develop a set of conditions for bioconjugation of the cowpea mosaic virus pre-functionalized chemically with an azide or alkyne functional groups [42]. Shortly after, Speers and Cravatt [43] showcased the use of this approach in a more complex biological setting in cell lysates for activity-based protein profiling applications [43]. Since then, "click" chemistry has found numerous applications in chemical biology [44–46], materials science [47, 48], medicinal chemistry, and organic synthesis [49–51]. Some of the most important applications of copper-catalyzed "click" chemistry in chemical biology include activity-based protein profiling [43], DNA and protein labeling with fluorescent probes, and cross-linking of neighboring peptide side-chains (also known as peptide stapling) [52]. This topic has been extensively covered in a number of reviews [53–55] and will therefore only be briefly discussed here.

Continuous optimization of the conditions for the bioorthogonal copper(I)-catalyzed "click" [56] reaction has resulted in a number of guidelines with respect to the concentrations and stoichiometries of the reagents, preferred reducing agents, and ligands facilitating this transformation [57–60]. Typically, reactions employ sodium ascorbate or TCEP (tris(2-carboxyethyl)phosphine) as the reducing agent, and require an excess of ligand for stabilizing the Cu(I) species under aerobic aqueous conditions. Although phenanthroline derivatives

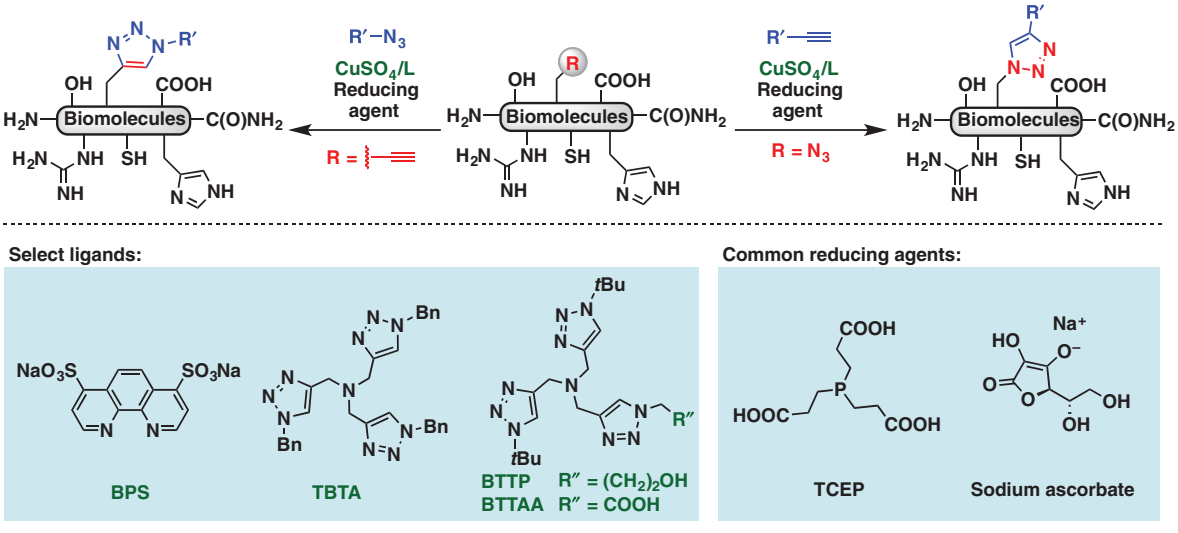

Fig. 3: Cu(I)-catalyzed [3 + 2] azide-alkyne cycloaddition reactions and the common ligands used to facilitate this transformations in vitro and in live cells. TCEP, Tris(2-carboxyethyl)phosphine; BPS, bathophenanthroline disulphonate disodium salt; TBTA, tris-((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine; BTTP, 3-[4-({bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]propanol; BTTAA, 2-[4-{(bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl]-acetic acid.

were among the first ligands to be identified to efficiently accelerate copper(I)-catalyzed "click" chemistry [61], TBTA (tris-((1-benzyl-1H-1,2,3-triazol-4-yl)methyl)amine) [62] remains one of the most widely used supporting ligands for this class of transformation, even though it has poor water solubility and the common requirement of *tert*-butanol as an additive or a co-solvent. A number of other ligands based on the same polytriazole core have been identified, including BTTP (3-[4-({bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino}methyl)-1H-1,2,3-triazol-1-yl]propanol) and BTTAA (2-[4-{(bis[(1-*tert*-butyl-1H-1,2,3-triazol-4-yl)methyl]amino)methyl}-1H-1,2,3-triazol-1-yl]-acetic acid), which are more water-soluble and have been developed specifically for labeling applications in intact cells [63] (Fig. 3). These ligands have been shown to alleviate the longstanding problem of Cu(I)-catalyzed "click" reaction cytotoxicity in cells [64, 65], typically associated with the formation of reactive oxygen species due to the presence of Cu(I) reducing agent and atmospheric oxygen [66, 67]. Another approach to overcoming issues with copper(I) cytotoxicity was developed by Ting and coworkers and involved the use of copper-chelating self-ligating azides to accelerate the reaction [68]. A summary of the common reaction conditions and catalyst systems, which have evolved over the years, is shown in Fig. 3.

Organometallic approaches to biomolecule alkylation

Alkylation of proteinogenic amino acids

Acylation and alkylation strategies for biomolecule functionalization are extremely widespread in the field of chemical biology, however most of these transformations are targeting a relatively small selection of amino acids (e.g. lysine and cysteine), and sometimes suffer from low stability of the resulting conjugates and/or non-selective reactivity outside of the relatively narrow working pH range. In particular, the use of maleimide reactive "warheads" is a very common way to append a variety of synthetic cargos to cysteines, albeit the well-known stability issues associated with the reversibility of the Michael addition to maleimides. The use of transition metal-based alkylation approaches toward protein functionalization offers an opportunity for diversification of the selection of available chemoselective alkylation protocols to other less nucleophilic, and therefore less reactive, residues. For example, Antos et al. [69, 70] were able to develop conditions for tryptophan alkylation in the presence of tyrosine, serine, lysine and histidine residues (however, in the absence of cysteine) using in situ generated rhodium(II)-carbenoids (Fig. 4-1a). Tryptophan is one of the most rare amino acids encountered in proteins in nature and is therefore very attractive for the development of selective functionalization techniques; however, it has been a relatively elusive target in the field of chemoselective protein modification. The selectivity of the rhodium-catalyzed process was hypothesized to arise from the competitive decomposition of the carbenoid species in water coupled with accelerated reactivity of tryptophan due to its hydrophobic interactions with the alkylating reagent. Unfortunately, the reactions with proteins studied in both papers [69, 70] (myoglobin, subtilisin Carlsberg, and lysozyme) required protein denaturation for the desired tryptophan residues to become accessible for modification, potentially due to the overall steric constraints of the rhodium-carbenoid chemistry [71]. While this observation narrows the potential substrate scope of the currently applied catalyst systems, it also provides an opportunity to achieve highly selective functionalization of surface-exposed tryptophan residues. Ball and coworkers used a proximity-driven molecular recognition approach with peptide-based ligands as the recognition elements to accelerate the rhodium carbenoid chemistry at physiological pH for peptide [72] and protein alkylation [73], while expanding the substrate scope to aromatic amino acids [74], glutamine, asparagine, arginine and glutamic acid [75].

Rhodium(II)-catalyzed alkylation of tryptophan residues introduced by Francis et al. and its more recent variations have highlighted the opportunities for organometallic chemistry presented by existing challenges in chemical biology. While there are still prospects for further optimization of the catalysts, substrates, and reaction conditions, the addition of this rhodium-mediated process to the toolbox of tryptophan bioconjugation methods is of great value and potential. It is, however, important to note that Kundu and Ball [76] have subsequently shown that the same reaction conditions in the presence of cysteine residues lead to S-H insertion, which was also selective for surface-exposed residues due to steric factors (Fig. 4-1b).

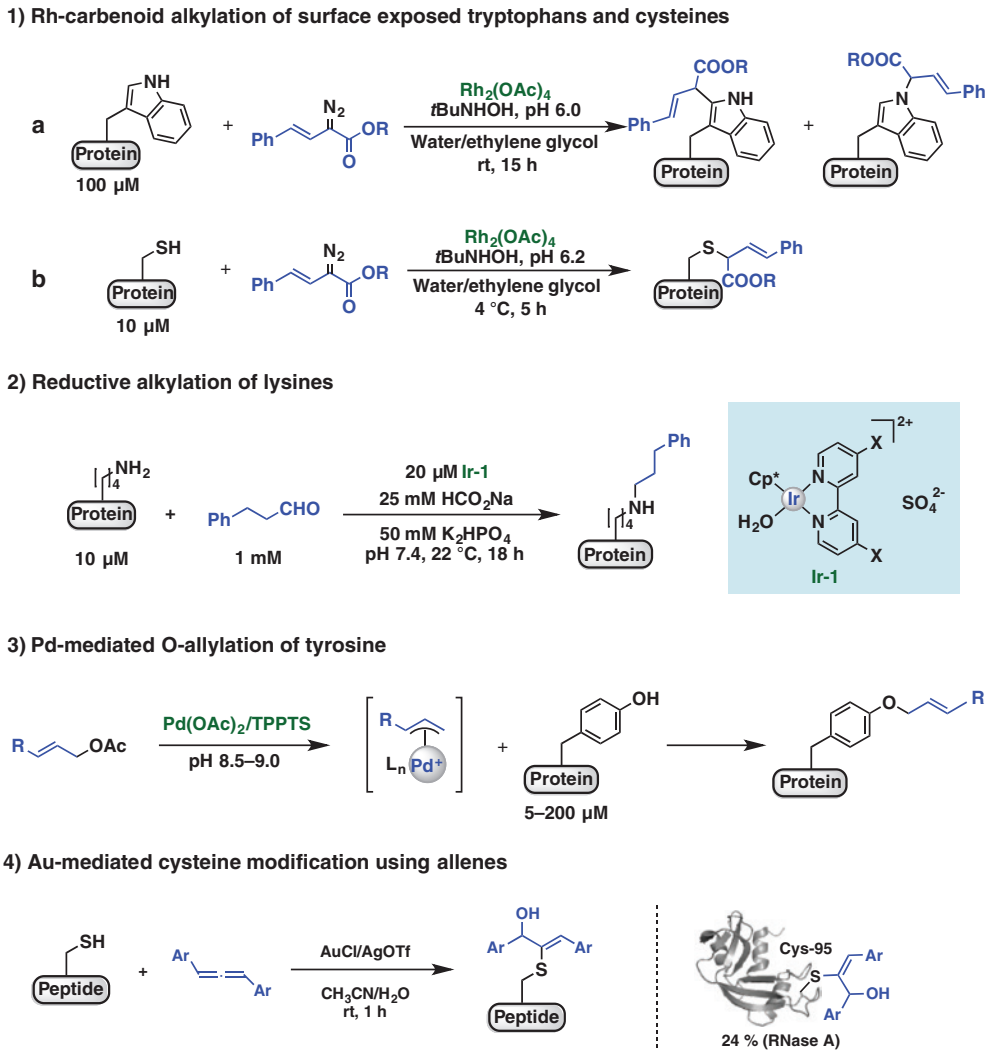

Fig. 4: Organometallic alkylation strategies for modification of natural amino acids.

In another instance, McFarland and Francis [77] applied iridium catalysis for reductive alkylation of lysine residues under physiologically relevant conditions (22–37 °C and pH 7.4) using sodium formate as the hydride source (Fig. 4-2). It was shown that a highly active iridium(III) catalyst could be obtained by simply tuning the electronic properties of the bipyridyl ligand in the water-soluble iridium complex **Ir-1** [78]. This demonstrates that selecting the appropriate metal/ligand combinations holds great potential for the development of new organometallic bioconjugation transformations.

Inspired by the breadth of transformations involving π-allylpalladium species in organic synthesis, Francis and coworkers pursued the alkylation of yet another understudied amino acid handle – tyrosine (Fig. 4-3). The authors used pre-formed π-allylpalladium(II) reagents to achieve selective tyrosine O-allylation in proteins in the presence of cysteine residues, even at pH 9.0 [79]. Only small amounts of diarylated tyrosine species were observed. On the contrary, traditional methods for tyrosine bioconjugation lead to functionalization of the carbon *ortho*- to the phenolic group, which can result in the formation of polyfunctionalized products [80]. The ability to change the leaving group in the π-allyl chemistry introduced an additional level of control over the payload, which could be transferred to tyrosine residues. Design of a more hydrophilic leaving group allowed for the enhanced water solubility of hydrophobic substrates [79], and can potentially be used in the future for tuning of other key substrate properties (e.g. cell-permeability and subcellular localization).

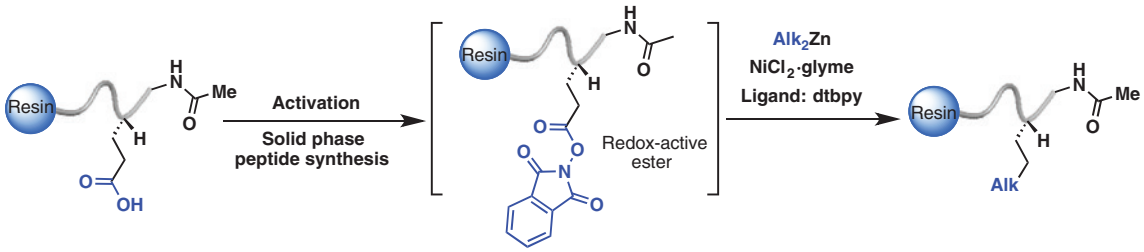

Fig. 5: Nickel-catalyzed decarboxylative alkylation of carboxyl group containing amino acids in SPPS [82].

Gold mediated processes are less common in the metal-mediated bioconjugation toolbox. Che and coworkers [81] showed that cysteine-containing peptides undergo 1,2-addition to allenes in the presence of gold(I) and/or silver(I) salts (AuCl, AgOTf) producing unusual hydroxyl substituted vinyl thioethers as products (Fig. 4-4). The reactions with short peptides produced the corresponding bioconjugates in high yields with exclusive selectivity for cysteine modification over other nucleophilic residues (e.g. lysine, serine, arginine, unprotected *N*-terminus). On the contrary, protein reactivity was significantly diminished, which might be explained by the coordination of gold to the protein backbone. Indeed, using pre-ligated gold species Au(TPP)Cl (H_2TPP = *meso*-tetraphenylporphyrin) led to diminished yields in reactions with peptides.

More recently, Baran and coworkers [82] described a nickel-catalyzed method for decarboxylative alkylation of glutamic acid and other amino acids containing free carboxyl groups (Fig. 5). The two-step approach involved formation of redox-sensitive esters followed by nickel-catalyzed decarboxylative cross-coupling with dialkylzinc reagents. While the current conditions are not suitable for functionalization of complex biomolecules, the method can find immediate use as a straightforward route for structure diversification during solid phase peptide synthesis (SPPS).

Metal-mediated alkylation of dehydroalanine

Dehydroalanine is a naturally occurring post-translationally modified amino acid first detected in the polycyclic antibacterial peptide nisin [83]. It is found in peptides isolated primarily from bacteria, which possess antibiotic, antifungal, and antitumour activities. Dehydroalanine is the most simple naturally occurring α,β–unsaturated amino acid, which lacks substituents at the β–position of the double bond. Recognizing its potential as a bioconjugation handle, a number of elegant synthetic methods have been developed for its production in a laboratory setting from cysteine, selenocysteine, and phosphoserine within peptides and proteins [84]. However, until recently little attention has been paid to modification of this amino acid beyond reactions with thiol nucleophiles to produce alkyl cysteine derivatives [85]. In 2016, two groups independently reported elegant organometallic biomolecule alkylation strategies using dehydroalanine as the reactive handle (Fig. 6) [86, 87]. This approach allowed for an overall chemical mutagenesis of phosphoserine and cysteine to a plethora of natural and unnatural amino acid residues, including ones not currently accessible for installation using standard genetic approaches [88, 89]. For example, methylated analogues of lysine, arginine and glutamine, which remain challenging to standard recombinant methods due to their structural resemblance to natural amino acids, could all be synthesized using the new metal-catalyzed processes. These new powerful methods introduce significant diversity into the structure of protein side chains and provide new opportunities for mechanism elucidation of a number of previously cryptic biological processes (e.g. protein methylation). It is worth noting that the question of diastereoselectivity in these transformations and its effect on the biological properties of the resulting proteins still remains open. Introduction of dehydroalanine leads to the loss of the corresponding α-stereocenter, which can only be re-installed in the reported radical processes through substrate control. In fact, currently available data on the enantioselectivity of these transformations suggests that the resulting mutated amino acids are racemates [87]. While the protein mutants could still be recognized by the corresponding antibodies and remained functionally active [86], the change in even one residue in the structure of proteins from L- to D- can result in drastic changes in

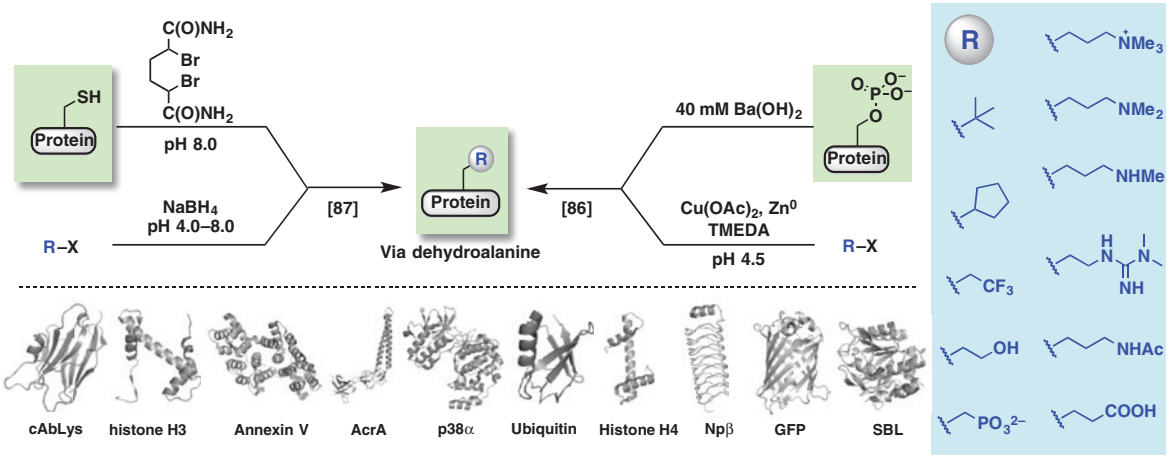

Fig. 6: Metal-mediated alkylation of dehydroalanine [86, 87]: general scheme, protein substrates and select R-groups described in the published reports.

protein folding, stability, and activity [90]. Enantioselective rhodium(I)-catalyzed 1,4-addition to dehydroalanine could serve as a better alternative to these methods. To this end, this approach has been investigated only briefly on short protected peptides and aryl boronic acid nucleophiles [91, 92], and still remains largely unsolved a decade after these initial findings.

Organometallic approaches to biomolecule arylation and alkynylation

Another major direction in the development of organometallic bioconjugation reactions involves the transfer of aryl groups to biomolecules of various complexities (including peptides, proteins, antibodies, and DNA molecules). A number of arylative bioconjugation technologies have been introduced over the years, which build upon existing knowledge of established cross-coupling methodologies in organometallic chemistry. To this end, reactive handles for arylation reactions typically include genetically or chemically incorporated aryl halides, arylboronic acids, and terminal alkynes, as well as naturally occurring nucleophilic amino acids (e.g. cysteine or lysine). The large range of these transformations, as well as the inherent differences of the resulting X–C(sp^2) from the commonly available X–C(sp^3) bonds diversifies the currently accessible biochemical space by providing new opportunities for manipulation of function, stability and pharmacokinetic properties of biomolecules of various complexity.

Arylation of unnatural amino acids: progress in the development and applications of Sonogashira and Suzuki-Miyaura reactions

Since the first reports in the late 1970s, palladium-catalyzed cross-coupling reactions and their application to organic synthesis have been extensively studied and optimized. These reactions have shown extraordinary functional group tolerance, mild reaction conditions and broad substrate scope. The initial proof of concept applications of palladium-catalyzed (**Pd-1a, 1b**, Fig. 7) Sonogashira, Heck, and Suzuki-Miyaura cross-coupling reactions for functionalization of peptide and protein substrates appeared in the late 1990s-early 2000s. These reports were instrumental for framing the desired transformation and the challenges for the organometallic community, as the reactions suffered from harsh conditions, organic solvents, and low bioconjugation yields [93–97]. Finally, almost 10 years after the initial report on the Sonogashira coupling of peptides in aqueous media, Davis and coworkers reported the first Suzuki-Miyaura cross-coupling reaction, which could be successfully applied to protein substrates in aqueous solvents under mild conditions [98]. The authors employed palladium(II) salts in combination with the 2-amino-4,6-dihydroxypyrimidine ligand

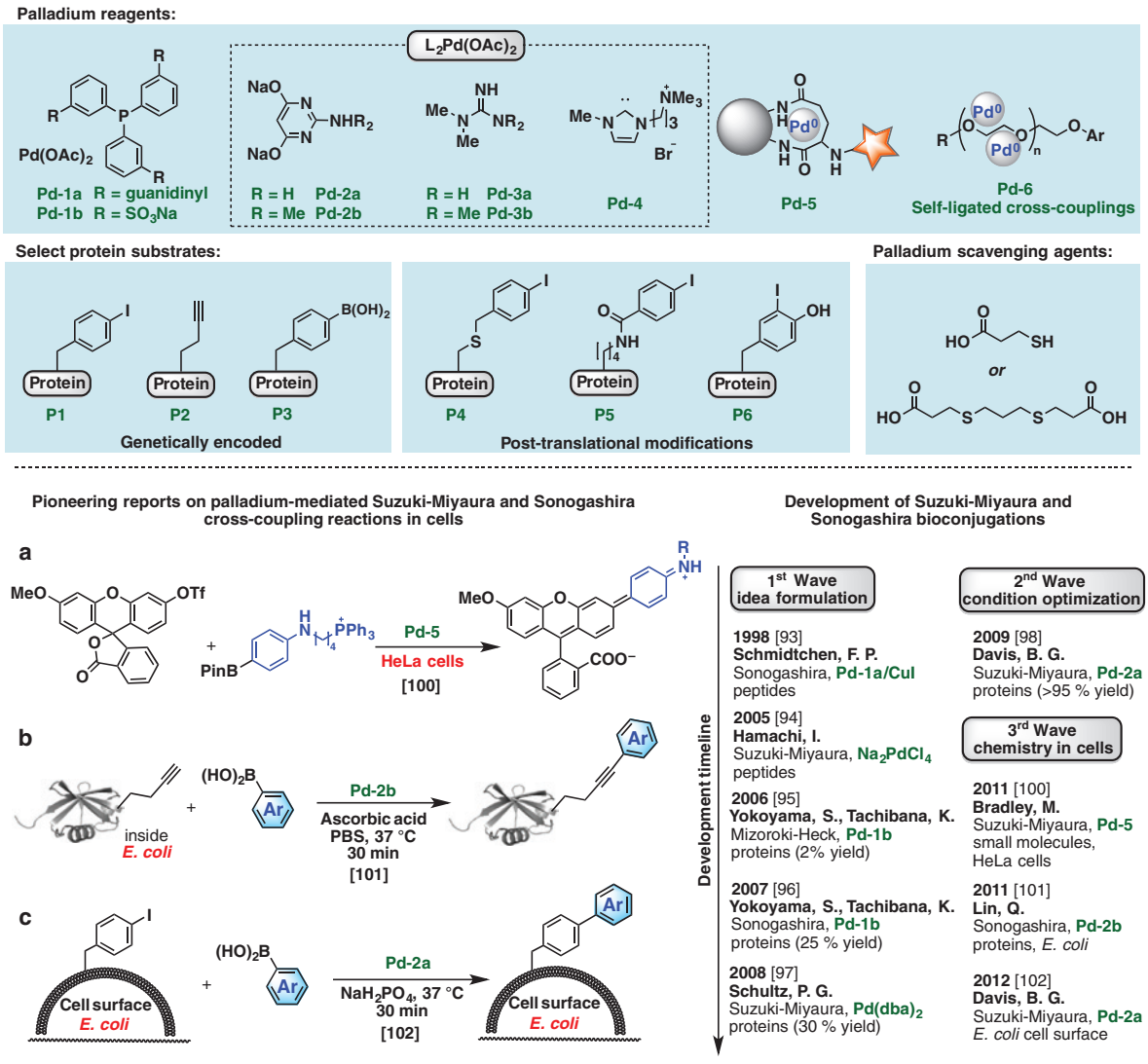

Fig. 7: Arylation strategies for functionalization of unnatural amino acids. Top: Palladium reagents, select protein substrates and palladium scavenging reagents used in biomolecule arylation reactions. Bottom, left panel: Select pioneering reports on palladium-mediated Suzuki-Miyaura and Sonogashira cross-coupling reactions in cells. Bottom, right panel: Development timeline for Suzuki-Miyaura and Sonogashira bioconjugations.

(**Pd-2a**, Fig. 7) to achieve the desired Suzuki-Miyaura coupling in aqueous media on proteins containing 4-iodobenzyl cysteine (**P4**). Later the group adapted these reaction conditions for chemical modification of genetically encoded 4-iodophenylalanine (**P1**) [99]. The couplings proceeded to completion at 37 °C in 30 min, however, a large excess of the palladium reagent and ligand were required for rapid coupling. The authors made an important observation that palladium(II) coordination to the functional groups of proteins made further analysis (and potentially palladium removal) more challenging. A number of palladium scavengers were evaluated, including EDTA (ethylenediaminetetraacetic acid) and DTT (dithiothreitol), however only 3-mercaptopropionic acid and its dimer connected through a propyl linker proved to be efficient (Fig. 7, palladium scavenging agents).

In 2011, Bradley and coworkers utilized the bioorthogonal nature of this transformation to achieve the first metal-catalyzed Suzuki-Miyaura cross-coupling reaction within HeLa cells by using fluorescently labeled Pd(0) microspheres as catalysts (**Pd-5**) (Fig. 7a) [100]. The same year, Lin and coworkers showed that a modified dimethylated amino-pyrimidine ligand (**Pd-2b**) could be used for successful copper-free Sonogashira reactions on isolated proteins and in *E. coli* cells (Fig. 7b) [101]. Shortly after, Spicer et al. [102] published a

method for site-selective cell-surface functionalization of *E. coli* using palladium-mediated Suzuki-Miyaura cross-coupling reaction (Fig. 7c).

Over the years, a broad set of bioorthogonal reaction conditions and metal catalysts has been developed for Suzuki-Miyaura protein arylation [103–105], hydroarylation of alkynes [106], and Heck-type [107] cross-couplings, including the use of simple guanidine ligands (**Pd-3a,b**) [103], NHC-ligands (**Pd-4**, NHC – *N*-heterocyclic carbene) [104], and self-ligating PEG-substrates (**Pd-6**, PEG – polyethylene glycol) [103, 105]. The reaction has been applied for rewriting the bacterial glycocalyx [108], generation of ^{18}F-labeled protein PET-tracers [109], and synthesis of drug molecules within cells [110]. Importantly, multiple research groups demonstrated negligible toxicity of different palladium species in cells [102, 105, 111, 112].

The conditions developed for palladium-catalyzed protein functionalization have been successfully applied to the arylation (Suzuki-Miyaura) and alkynylation (Sonogashira) of unnatural nucleic acids. This area has been recently reviewed in detail and won't be covered in more detail within the scope of this review [113].

Metal-catalyzed C–C, C–S, and C–N cross-coupling reactions on natural amino acids

More recent developments in the field have highlighted the possibility for arylation of native amino acids in peptides and proteins using transition metal mediated processes. To this end, metal-catalyzed C–H [114] and X–H (X = S, N) [115] arylation strategies of naturally occurring nucleophilic amino acids have been explored with the latter approach applied to more complex biomolecules.

Metal-catalyzed tryptophan C–H arylation and alkynylation

Since the initial reports on C–H arylation of tryptophan in 2010 (Fig. 8a) [116], a number of different types of electrophiles have been used for the transfer of aryl groups including aryl iodides [116], aryl bromides [117], diaryl iodonium salts [118], and aryl boronic acids [119]. Furthermore, limited success has been achieved for the development of gold-catalyzed tryptophan alkynylation strategies using hypervalent iodine reagents developed by Waser (Fig. 8b) [120, 121]. Unfortunately, current state of the art tryptophan C–H functionalization reaction conditions remain relatively harsh, requiring the use of organic solvents, low pH and/or high temperatures. While there is a lot of room for further optimization in order to apply metal-catalyzed

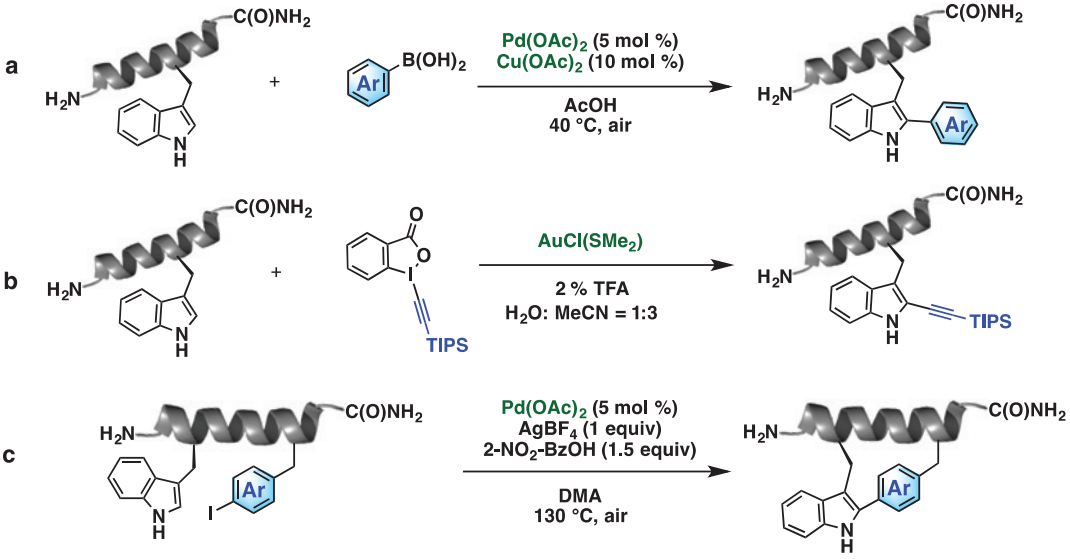

Fig. 8: Metal-catalyzed C–H arylation and alkynylation of peptides using (a) aryl boronic acids, (b) hypervalent iodine compounds and (c) aryl halides as coupling reagents [119–124].

C–H functionalization to more complex biomolecules (e.g. proteins and antibodies), it can nevertheless find immediate application in the synthesis of cyclic peptides (Fig. 8c), which have recently gained tremendous interest from the standpoint of new biotherapeutics development [122–124].

Copper-catalyzed selenocysteine arylation

Selenocysteine is a low abundant highly nucleophilic naturally occurring amino acid, which makes it a potentially attractive bioconjugation handle. However, the ease of its oxidation to the diselenide or selenic acid as well as the increased potential for elimination to form dehydroalanine has hampered the progress in the development of useful approaches for selenocysteine bioconjugation. Buchwald and coworkers [125] circumvented the problem of selenocysteine oxidation by starting their synthesis with a protected amino acid using 2-thiopyridine as a capping group. This modification allowed the group to develop a copper-catalyzed umpolung approach for the arylation of selenocysteine with a broad range of aryl boronic acid nucleophiles (Fig. 9). While it might be challenging to find a direct translation of this approach to functionalization of more complex biomolecules, it nevertheless highlights the unique potential of organometallic chemistry for the development of novel approaches to bioconjugation of natural amino acids, where a nucleophilic amino acid can be converted to an "electrophile" through oxidative addition of the organometallic catalyst into the S–Se or potentially the S–S bond.

Metal-catalyzed C(sp^2)-S bond-formation: cysteine arylation and vinylation strategies

Cysteine is one of the most utilized natural amino acid bioconjugation handles due to its increased nucleophilic properties, low abundance, and relative stability toward oxidation. For a long time, very little diversity had been introduced into existing bioconjugation tools for cysteine functionalization, with the major emphasis directed towards different alkylation strategies (e.g. S_N2 substitution of activated alkyl halides and Michael addition to α,β–unsaturated compounds). Cysteine arylation is a more challenging task due to the decreased reactivity of the *ipso*-carbon in S_NAr reactions with different nucleophiles, and has therefore been restricted to a limited selection of activated aromatic substrates containing strong electron-withdrawing groups in their structure. An organometallic approach to cysteine arylation offers the potential for expanding the scope of available aryl-cysteine bioconjugates with generally enhanced stabilities and tunable pharmacokinetic properties. Recently, both gold- [126] and palladium-mediated [127] bioconjugation approaches have been reported. Kung et al. [126] used cyclometallated gold(III) complexes to transfer aryl groups onto short peptides and BSA protein (Fig. 10a). In this work, the challenging reductive elimination step required prolonged incubation of the proteins in the presence of excess of gold reagents, and the system was limited to cyclometalated gold(III) complexes, which imposed a number of limitations on the potential aryl groups that could be transferred. Around the same time, Buchwald and coworkers [127] introduced their organometallic solution for cysteine-selective peptide and protein arylation by using air stable arylpalladium(II) triflate and chloride reagents (Fig. 10b). While the presence of free thiols has been long considered disadvantageous for palladium-catalyzed processes due to Pd-catalyst decomposition [128], Buchwald and coworkers showed that palladium(II) reagents supported by biarylphosphine

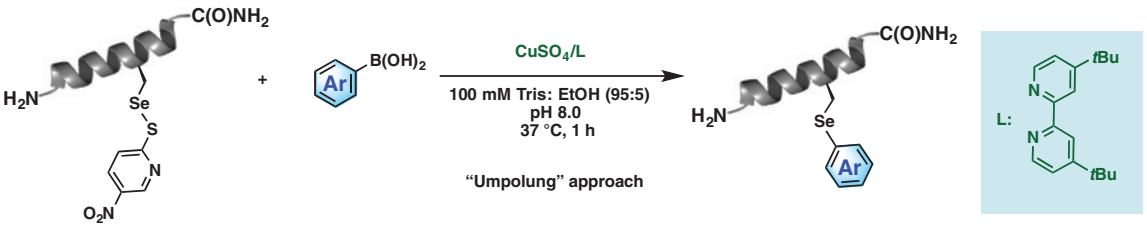

Fig. 9: Copper-catalyzed umpolung approach to selenocysteine arylation [125].

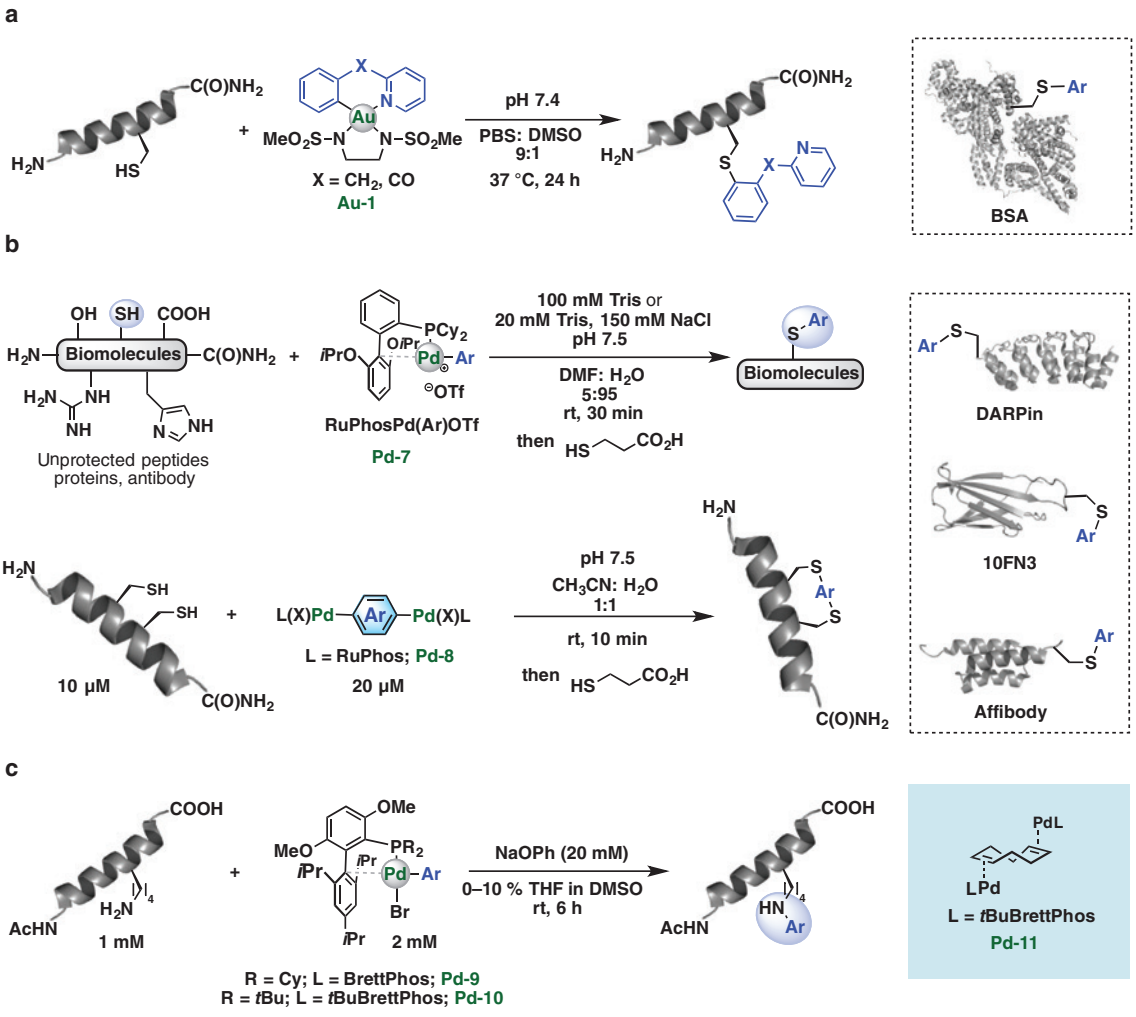

Fig. 10: Organometallic cysteine and lysine arylation strategies [126, 127, 131, 134]. (a) Cysteine arylation using cyclometallated Au(III) complexes [126]. (b) Cysteine arylation using palladium reagents [127, 131]. (c) Lysine arylation in unprotected peptides using palladium reagents [134].

ligands (e.g. RuPhos, Fig. 10b) can indeed provide a highly diverse and efficient solution to the cysteine S-arylation challenge. The reaction conditions are particularly mild and versatile, with full conversions of peptide substrates achieved within minutes at a range of temperatures, buffers, pH, and organic co-solvents (5 %), which could further be completely eliminated by the use of water soluble sulfonated biarylphosphines [129]. Following the report by Spicer and Davis [99], the authors used buffered thiopropionic acid for peptide and antibody purification from the remaining palladium species [127]. This new method allowed for the synthesis of a broad range of arylated biomolecules including representative examples of two important and highly pursued classes of biotherapeutics – an antibody drug conjugate and several stapled peptides [130]. The two-component peptide stapling provided an opportunity for straightforward high-throughput screening of structurally diverse peptide staples and their effect on the biological activity and pharmacokinetic properties of the stapled peptides [131]. The resulting conjugates were shown to be more stable than commonly used maleimide and acetamide adducts under a variety of conditions. Consequently, Al-Shuaeeb et al. showed that the reaction could be performed catalytically using palladium(II) aminobiphenyl precatalysts [132] with XanthPhos as the supporting ligand [133]. It is important to note, however, that the use of aminobiphenyl precatalysts can lead to the transfer of the aminobiphenyl group to the biomolecule, which is highly undesirable in the synthesis of more complex biotherapeutics (e.g. antibody drug conjugates), especially when higher amounts of the catalysts are necessary for the reaction.

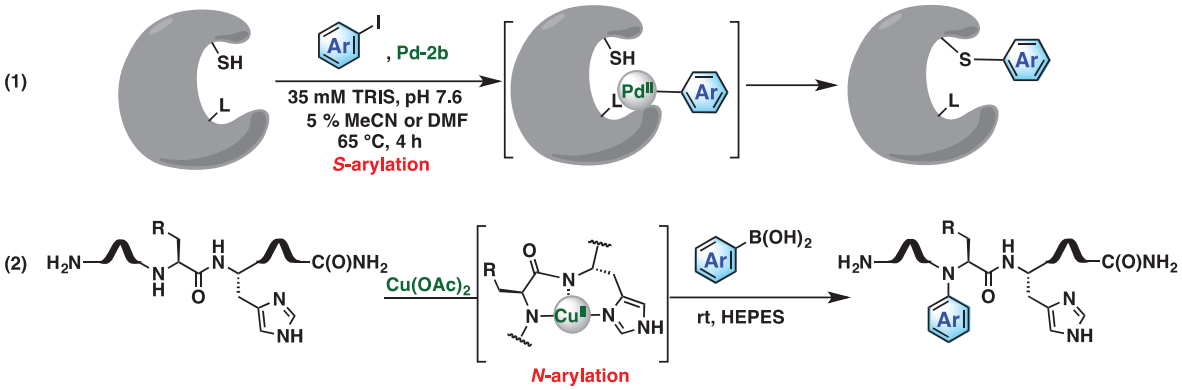

Fig. 11: Backbone-directed arylation of peptides and proteins [135, 136].

Recently, Buchwald and coworkers [134] employed palladium(II) complexes supported by biarylphosphine ligands to achieve lysine arylation in unprotected peptides under relatively mild conditions (6 h, room temperature, sodium phenoxide base) (Fig. 10c). The choice of the supporting ligand was found to be crucial for reactivity; for example, bulkier *t*BuBrettPhos and BrettPhos (Fig. 10c) were necessary to achieve high levels of conversion of peptide substrates to the corresponding bioconjugates. Palladium(II) complexes could be isolated and stored as bench-stable reagents [127] or formed in situ from palladium(0) precursor **Pd-11** and the corresponding aryl halides. While the method was not compatible with cysteines, selective lysine monoarylation was observed in the presence of serine, tyrosine, methionine, histidine, and tryptophan residues. Furthermore, other amine functional groups (unprotected N-terminus, asparagine, and arginine residues) could also be present in the peptide structure, albeit low to moderate levels of polyarylation were observed in these cases.

Achieving high levels of site-specificity of these transformations in the presence of multiple repeats of the same nucleophilic amino acid can be challenging even in the case of low abundant amino acids such as cysteine and tryptophan. However, organometallic reagents offer multiple opportunities for manipulation of reactivity and site-specificity of bioconjugation reactions, including variation of steric and electronic properties of the supporting ligands, the use of affinity recognition elements incorporated into the structure of ligands, leaving groups or target cargos, as well as metal coordination to protein side-chains. Recently, Davis and coworkers [135] showed that palladium-mediated S-arylation preferentially occurs at the endogenous metal binding sites of metalloenzymes when non-preligated palladium(II) catalysts were used (Fig. 11-1). While the use of long reaction times and high temperatures (4 h at 65 °C) raises a question of biocompatibility and enzyme stability under these relatively harsh conditions and warrants additional optimization, the concept of achieving side-chain coordination guided bioconjugations is very attractive. In another instance, Ball and coworkers [136] recently showed that histidine can direct the reactivity of copper(II) catalysts in Chan-Lam cross-coupling reactions with aryl and vinyl boronic acids towards neighboring amide bonds in the peptide backbone as well as lysozyme (Fig. 11-2). These reports begin addressing the challenge of site-specific modification of a particular subset of residues by using endogenous coordinating amino acids as directing groups, thereby opening an exciting area for future exploration. It is, however, important to be aware while designing novel directed metal-mediated modification approaches, that similar coordination to methionine and cysteine has been previously used for metal-catalyzed proteolysis [29], albeit at much lower pH.

Metal-catalyzed deprotection chemistries (chemical decaging)

Another exciting area of the emerging field of organometallic chemical biology is the application of organometallic complexes for chemical decaging of proteins, small molecule drugs and molecular probes in the cellular environment using π-allyl chemistry [137, 138]. While the alkylation and arylation strategies described

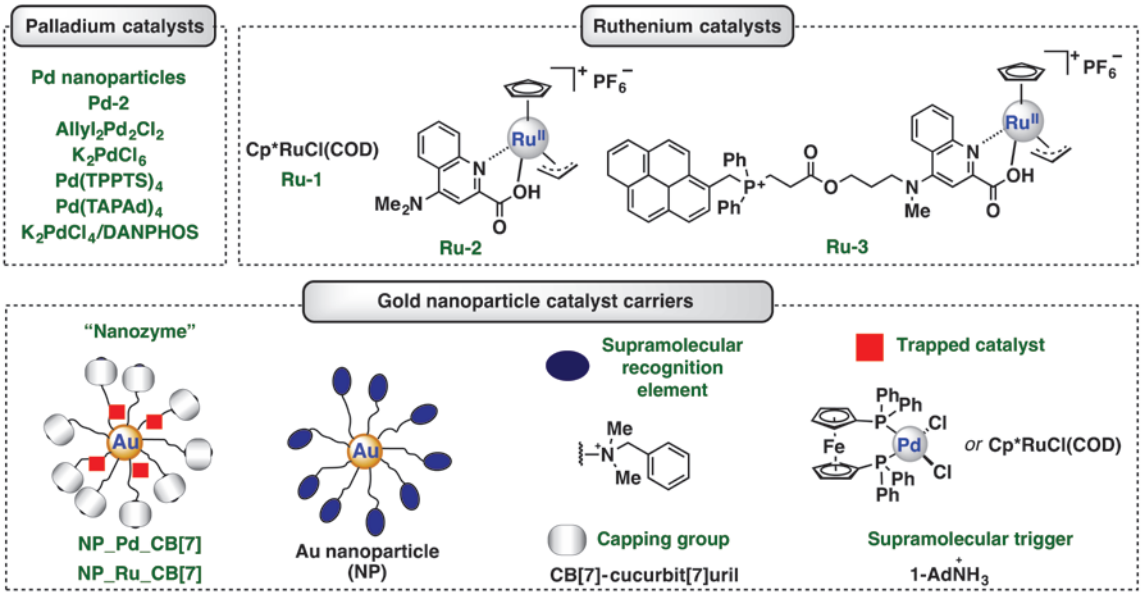

Fig. 12: Transition metal-mediated deprotection strategies in chemical biology: common catalysts for organometallic protein decaging. COD, 1,5-Cyclooctadiene; TPPTS, tris(3-sulfophenyl) phosphine trisodium salt; TAPAd, 1,3,5-triaza-7-phosphaadamantane; dba, dibenzylideneacetone; *o*-DANPHOS, bis(2-trifluoromethylphenyl)(3-sulfonatophenyl)phosphine, sodium salt.

above can prove useful for peptide bioconjugation and in vitro reactions on proteins for generating new types of biotherapeutics, chemical decaging approach has so far been primarily aimed at the ability of modulating the activity of enzymes and small molecule pro-drugs in cells and live subjects.

Pioneering reports by the groups of Meggers and Bradley developed ruthenium(II) [139, 140] and palladium(0) catalysts [100] for rhodamine dye decaging in live cells for potential molecular imaging applications (Figs. 12, 13a-1). Ahn and Shin [141] used a similar approach for visualization of residual palladium in zebrafish (Fig. 13a-2). Later, Unciti-Broceta and coworkers [142] applied this strategy for 5-fluoro-1-propargyl-uracil prodrug decaging (Fig. 12, **Pd-5**; Fig. 13a-3). In particular, the authors demonstrated that implanting Pd(0)-containing resins in the yolk sac of zebrafish embryos after fertilization did not cause any toxicity or developmental defects (e.g. change in phenotype). The catalyst retained its activity in vivo which was visualized via confocal imaging of Pd(0)-emplanted zebrafish embryos treated with *N,N′*-bis(propargyloxycarbonyl)-rhodamine. Later, Mascareñas and coworkers [143] performed spatially resolved decaging of protected rhodamine and dinitrophenol in live cells by preparing ruthenium(II) catalysts capable of selective localization within the mitochondria of mammalian cells (Fig. 12, **Ru-3**). These authors used a common approach for targeting mitochondria in living cells by incorporating a charged phosphonium substituent into the structure of ruthenium(II) catalysts introduced by Meggers and coworkers [140]. Recently, Rotello and coworkers [144] were able to achieve supramolecular regulation of small molecule chemical decaging by using ruthenium(II) catalysts trapped within gold nanoparticles containing charged ammonium end groups (Fig. 12, Fig. 13a-2, **NP_Ru_[CB7]**). The ammonium groups were capped with cucurbit[7]uril molecules via host-guest supramolecular interactions, which prevented immediate release of ruthenium catalysts in cells, while further use of 1-adamantyl amine allowed for the release of the active catalyst and subsequent decaging.

Subsequent reports on metal-triggered small molecule decaging in cells were focused on the application of this approach for activating proteins via decaging [145]. In their seminal work, Peng and coworkers used the ability to visualize the decaging progress with non-fluorescent caged rhodamine dye derivatives to develop conditions for the palladium-catalyzed lysine decaging (Fig. 13b, left). While both propargyl carbamate (Proc-Lys) and allyl carbamate (Aloc-Lys) lysine protecting groups were evaluated as potential substrates in the initial amino acid screen, it was shown that Aloc-Lys is much less reactive under the developed conditions.

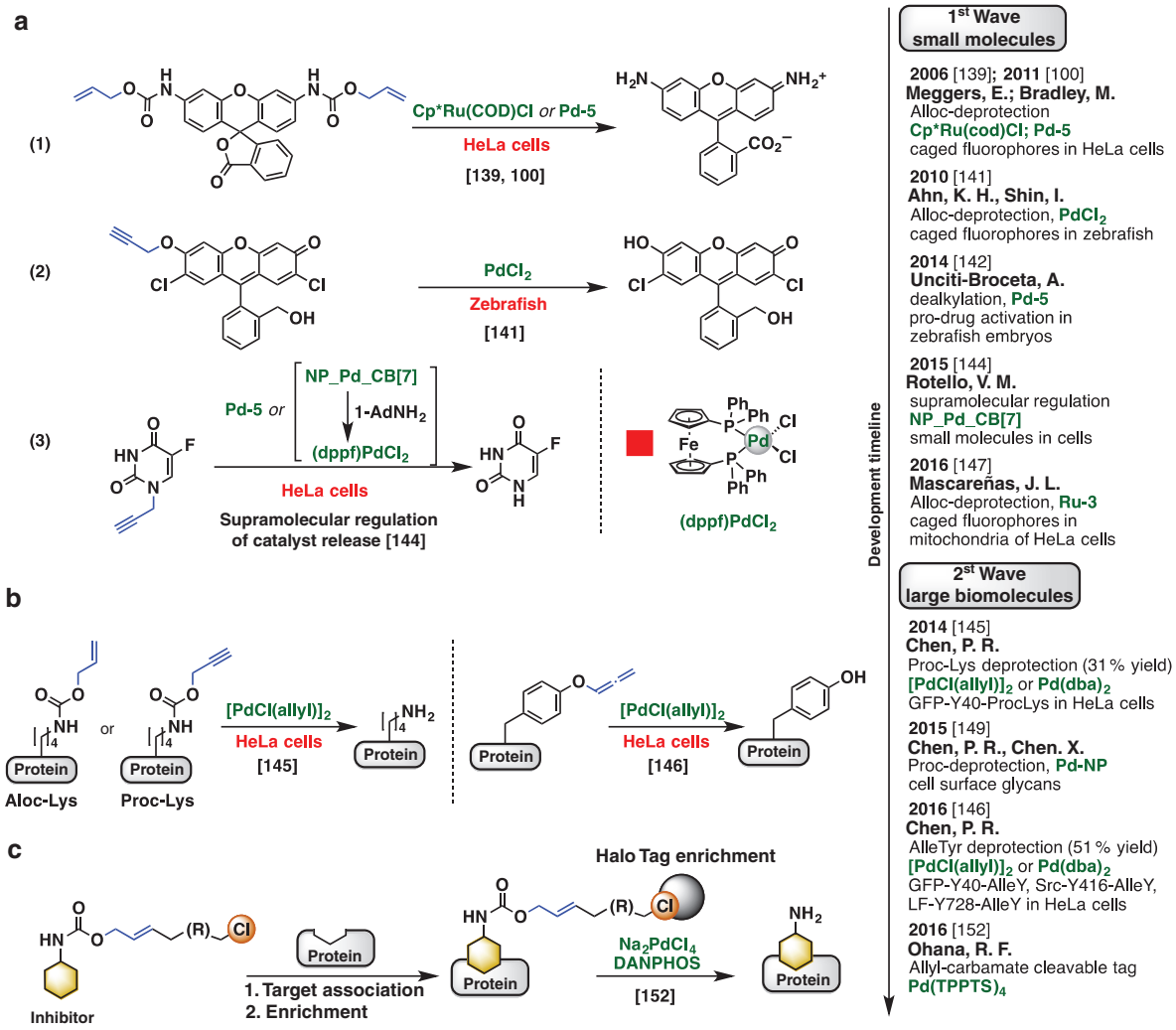

Fig. 13: Transition metal-mediated deprotection strategies in chemical biology: palladium- and ruthenium-catalyzed chemical decaging. (a) In situ chemical decaging of small-molecule drugs and probes. (b) Chemical decaging for protein activation. (c) Chemical decaging for cleavable linkers.

More recently, the same group expanded the protein decaging approach to allene-containing tyrosine amino acids [146] (Fig. 13b, right). The authors tested four different palladium-based catalytic systems for this transformation using a modified GFP protein as the model substrate – $[PdCl(allyl)]_2$, $Pd(dba)_2$, $Pd(TPPTS)_4$, and $Pd(TAPAd)_4$ (Fig. 12). The latter two catalysts were identified as the most active in reactions with isolated proteins. On the contrary, $[PdCl(allyl)]_2$ and $Pd(dba)_2$ were more efficient at producing decaged products within intact cells. The difference in the observed reactivities is likely due to higher cellular uptake of these palladium catalysts, which was corroborated by the authors using ICP-MS analysis. This approach has been used to achieve activation of tyrosine-dependent Src kinase and anthrax lethal toxin in live cells. Unfortunately, in both protein decaging studies by the group (Proc- and Allene-deprotection), generally decreased yields of the decaged products were observed in the cell-based studies (31–51 %) as opposed to experiments on isolated proteins (82–95 %).

Finally, it is important to note that allyl- and propargyl-containing carbamate groups, which have been studied extensively in the context of Pd- and Ru-catalyzed decaging strategies (Fig. 13b, left), are not truly bioorthogonal and can be deprotected in vivo through the reaction with various nucleophiles and digestive enzymes [143], which can potentially limit in vivo applications of these reagents. On the contrary, propargyl

or allyl derivatives of anilines and phenols, while less reactive, might be more suitable for in vivo applications of this pro-form activation approach.

To this end, a variety of applications have been developed using the organometallic decaging technology, including spatially resolved cellular imaging [143], design of metal-responsive pro-drugs with improved pharmacokinetic properties [147, 148], in situ regulation of enzymatic activities [149], expedited chemical protein synthesis [150, 151], and the use as a cleavable linker for HaloTag affinity enrichment [152] (Fig. 13c).

Aromatic azides have also been used as caged substrates for in-cell applications using iron catalysts for decaging [153]. However, aryl azides have been shown to be reduced in vivo in *C. elegans* and in zebrafish without the need for additional catalysts, which renders this approach non-bioorthogonal.

Olefin metathesis for bioconjugation

Another attractive example of bioorthogonal C–C bond forming metal-catalyzed transformation is olefin metathesis (Fig. 13) [154–156]. One of the earliest reports of ruthenium-catalyzed olefin metathesis on peptide substrates goes back to 1995, when Clark and Ghadiri [157] used first-generation olefin metathesis catalysts (**Ru-4a,b**) for cross-metathesis of homoallylglycine residues in two peptides under mild conditions ($CDCl_3$, room temperature, argon atmosphere). Over the years, this chemistry has been extended to other unnatural olefin-containing amino acids, including S-allyl cysteine and Se-allyl selenocysteine [158], as well as different length all-hydrocarbon linkers with terminal olefins (Fig. 14) [159]. In their seminal contribution, Grubbs and coworkers [160] applied olefin metathesis to cross-linking of *i*, *i*+4 *O*-allyl serine and homoserine residues in short peptides with the aim of alpha-helix stabilization (Fig. 14a). Since then, olefin metathesis has gained tremendous success in the synthesis of dicarba analogues of disulfide bridge containing cyclic peptides [161] and de novo design and synthesis of stapled peptides as new biotherapeutics [130]. The formation of the cross-link between the neighboring residues in peptides, or so-called peptide stapling, has been shown to stabilize the peptide alpha-helix secondary structure and confer advantageous properties on peptides, such as cell-permeability, proteolytic stability and higher binding affinities to the protein targets of potential peptide biotherapeutics. Systematic studies have been performed on the position of the cross-link, alpha-helix stability [162], and biological activity [130, 163] of stapled peptides synthesized via ruthenium-catalyzed metathesis.

Functional group tolerance of ruthenium-catalyzed olefin metathesis has been studied in great detail [163, 164]. In reactions with Hoveyda-Grubbs II catalyst (**Ru-5**), basic and/or coordinating amino acids, such as histidine and tryptophan, have been shown to be detrimental to reaction efficiency, while a number of

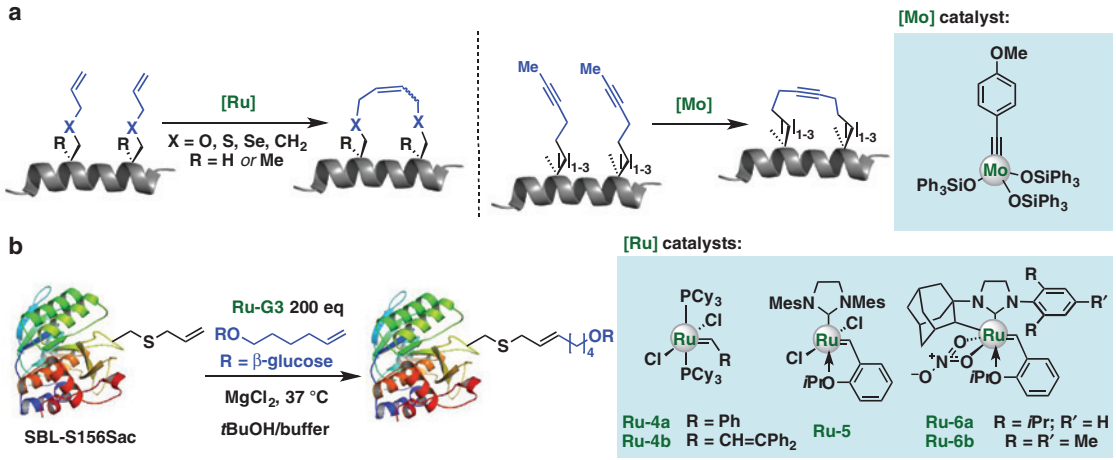

Fig. 14: Bioconjugation application of ruthenium and molybdenum catalyzed olefin and alkyne metathesis reactions. (a) Ring-closing metathesis. (b) Cross-metathesis.

other functional groups, including thioethers, were tolerated. Recently, Grubbs and coworkers [164] applied their new generation Z-selective ruthenium olefin metathesis catalysts (**Ru-6a,b**) to peptide stapling (*i*, *i*+4 and *i*, *i*+7) and cross-metathesis to produce Z-isomers of cyclic peptides with high stereoselectivity. These catalysts were less tolerant of peptide side chain functional groups and diminished yields and/or selectivities were observed in the presence of sterically hindered side chains (e.g. valine, isoleucine or amino acids bearing bulky protecting groups), amino acids bearing carboxylate and thiol groups. Histidine, glutamine, asparagine and arginine amino acids were not tolerated overall. Coupling of less sterically hindered homoallylic substrates is typically preferred over their allylic analogues [164]. As a result of these limitations, most of currently used olefin metathesis reactions on peptidic substrates are performed as part of the solid phase peptide synthesis with protected peptides. Furthermore, while a number of "designer" water-soluble ruthenium catalysts containing PEG [165] and ammonium groups [166] have been developed, first-generation Grubbs catalysts (**Ru-4a,b**) and Hoveyda-Grubbs II catalyst (**Ru-5**) still remain the most widely used.

While a number of limitations have been encountered in the development of ruthenium-catalyzed alkene metathesis reactions for peptide stapling, Davis and co-workers [167, 168] have been able to apply Ru-catalyzed olefin metathesis to protein functionalization by identifying allyl sulfides as privileged scaffolds for this transformation (Fig. 14b). This is another example where the organometallic catalysts (in this case, ruthenium-based) were considered incompatible with thiols prior to this report; however the fast kinetics of the transformation allowed the authors to outcompete catalyst degradation under aqueous conditions. The group used the standard Hoveyda-Grubbs II catalyst (**Ru-5**) for these transformations and magnesium chloride as a Lewis acid additive to prevent non-specific catalyst coordination of nucleophilic side chains. Under these conditions allyl sulfides were shown to be significantly more reactive than homoallyl glycine. The benefit of having a coordinating atom in the substrate for the pre-coordination and catalyst stabilization was further explored in the case of allyl selenocysteine amino acid, which was shown to be even more reactive in this transformation [167]. The authors further exploited the allyl-selenol leaving group ability to develop a "write"/"erase" cycle for histone modification, where a modification installed through olefin cross-metathesis could be easily "erased" through base-catalyzed dehydroalanine formation [169].

Molybdenum catalysts are generally more air and moisture sensitive, and have therefore found less success in terms of their application towards olefin metathesis reactions on biomolecules. In contrast, Cromm et al. [170] recently used molybdenum-catalyzed alkyne metathesis in conjunction with solid-phase peptide synthesis to afford alkyne-containing peptide staples (Fig. 14a, right). While the molybdenum-catalyzed ring-closing alkyne metathesis reactions required argon atmosphere and the use of organic solvents (unlike some of the recent ruthenium-catalyzed olefin metathesis reactions), they nevertheless present a number of unique features for peptide stapling: (1) alkyne metathesis is orthogonal to ruthenium-catalyzed olefin metathesis, (2) the resulting rigid alkynes can be used as functional handles for further diversification of peptide structures. Such characteristics make this approach highly attractive for future development of new macrocyclic peptides for various therapeutic applications.

Organometallic complexes and transformations for small molecule detection in chemical biology

Chang and coworkers [111] showed that cyclopalladated species based on borondipyrromethene difluoride (BODIPY) can serve as turn-on fluorescent probes; CO detection was achieved with high selectivity over other biologically relevant reactive small molecules within HEK293T cells using $[Ru(CO)_3Cl(glycinate)]$ (CORM-1) as the CO source (Fig. 15a). CO, among a selection of other small molecules (e.g. H_2S, NO, H_2O_2), has been shown to be involved in human physiology and pathology [171–173]; however, methods for its real-time detection and analysis on a cellular level are still limited. The authors hypothesized that palladium in the cyclometallated complex would quench BODIPY fluorescence, which could be restored upon CO-insertion and subsequent reductive elimination. Indeed, a 10-fold fluorescence enhancement was observed in aqueous buffers containing

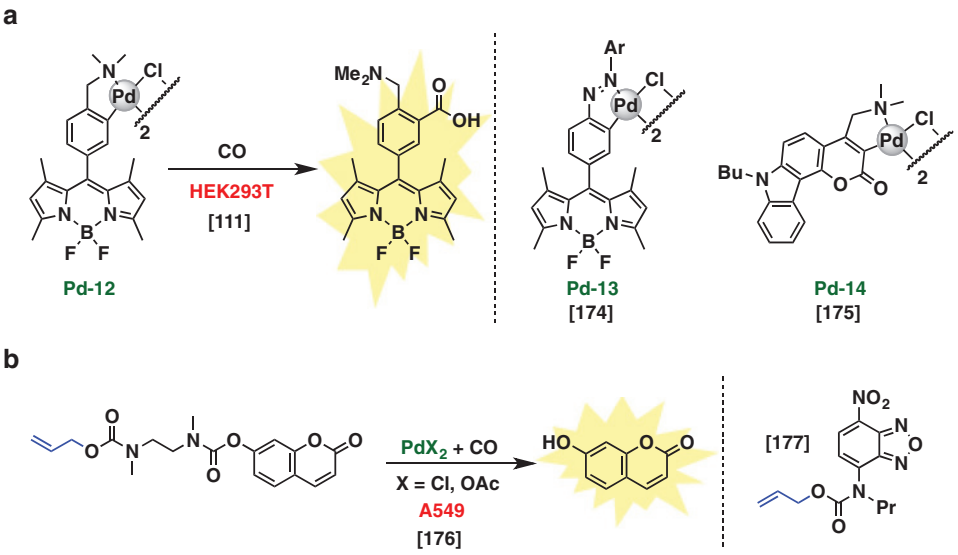

Fig. 15: Organometallic approaches for CO detection. (a) Palladium reagents for CO detection. (b) Palladium-catalyzed Tsuji-Trost reaction for CO detection.

Pd-12 in the presence of CORM-1 and low picomolar to high nanomolar levels of this CO-releasing molecule could be detected in cells. This approach has been used for imaging of endogenous carbon monoxide in cells under hypoxia conditions (**Pd-13**) [174], and in tissues (**Pd-14**) [175]. In another conceptually different approach, Dhara and coworkers designed a reaction sequence for CO detection, which involved reduction of Pd(II)-salts with endogenous CO in live cells followed by the Pd(0)-catalyzed Tsuji-Trost reaction with a caged fluorescent probe (Fig. 15b) [176]. More recently, Zhang and coworkers [177] designed a colorimetric and fluorescent probe based on the caged 4-amino-7-nitro-2,1,3-benzoxadiazole for carbon monoxide detection in HeLa cells.

To this extent, pre-ligated palladium complexes have been used in chemical biology applications for CO detection [111], C–O alkylation of tyrosine [79], C–S arylation of cysteine [127], Heck-type transformations [107], and alkyne hydroarylation reactions [106]. The diversity of reported applications in chemical biology applications coupled with the wide success of palladium catalysts in organic synthesis highlights the potential of future research in the area of palladium-mediated bioconjugation reactions.

Conclusions and outlook

Organometallic chemical biology has evolved significantly over the past several decades owing to the extensive work done by numerous pioneers in the field. Reactions that used to be only accessible to organic chemists, have now been applied to modification of unprotected peptides, proteins, and nucleic acids. To this end, key insights into stability, reactivity and toxicity of organometallic reagents in bioconjugation reactions have been gained, which will guide future developments in the field. A number of challenges, however, still remain; these include: (i) the expansion of the scope of mild, chemoselective, and site-specific transformations on complex biomolecules, (ii) the spatiotemporal resolution of reactions performed in cells, and (iii) issues with potential metal toxicity and removal. It is important to note, that the purpose of this review is not to separate organometallic chemical biology from the rest of the disciplines, but, on the contrary, to acknowledge the significance of the existing body of work, highlighting the potential impact of this rapidly developing field and urge scientists from multiple disciplines to merge their efforts. It is important that further evolution of this developing field is guided hand-in-hand by specialists from the areas of biology, biochemistry, chemical biology and chemistry in order to answer the most relevant and pressing questions, and ultimately generate powerful, general and widely accepted solutions to these problems.

Acknowledgements: I am truly grateful to Profs. Stephen L. Buchwald and Bradley L. Pentelute for their continuous support during my graduate studies at the Massachusetts Institute of Technology and for assembling an outstanding group of coworkers providing a stimulating scientific environment. I would also like to thank Prof. Benjamin F. Cravatt for opening the doors of his lab for my post-doctoral training, his encouragement and insightful discussions. Prof. Alex M. Spokoyny, Dr. Sean Reed, Prof. Simon Meek are acknowledged for their critical input during the preparation of this manuscript. E. V. V. is currently the recipient of the Life Sciences Research Foundation Fellowship sponsored by Pfizer. The content of this manuscript is solely the responsibility of the author and does not represent the official views of LSRF.

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