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Transition Metal-mediated Bioorthogonal Protein Chemistry in Living Cells

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Considerable attention has been focused on improving the biocompatibility of Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC), a hallmark of bioorthogonal reaction, for living cells. Besides creating copper-free versions of click chemistry such as strain promoted azide–alkyne cycloaddition (SPAAC), a central effort has also been paid to develop various Cu(I) ligands that can prevent the cytotoxicity of Cu(I) ions while accelerating the CuAAC reaction. Meanwhile, additional transition metals such as palladium have been explored as alternative sources to promote a bioorthognal conjugation reaction on cell surface, as well as within an intracellular environment. Furthermore, transition metal mediated chemical conversions beyond conjugation have also been utilized to manipulate protein activity within living systems. We highlight these emerging examples that significantly enriched our protein chemistry toolkit, which will likely expand our view on the definition and applications of bioorthogonal chemistry.

1. Introduction

Within the last decade, Bioorthogonal reactions have become a highly powerful tool for selective modification of biomolecules in living systems.[1-3] Such reactions typically require the partition components to be mutually reactive while remain inert to other surrounding molecules within a cellular environment. Majority of chemical

reactions are not able to meet these two requirements simultaneously, rendering the development of bioorthogonal reactions an extremely challenging task. However, due to the huge impact that might be generated by an effective bioorthogonal reaction on the study of diverse biological processes, this area is rapidly growing and many exciting applications have been reported in recent years.[4-10] A hallmark of bioorthogonal chemistry is the Cu(I)-catalyzed azide–alkyne cycloaddition (CuAAC) reaction that has been independently developed by Sharpless et al[11] and Meldal et al[12] in 2002. CuAAC represents a prototypical "click" reaction, coined by Sharpless and co-workers,[13] that have been widely adopted in synthetic chemistry, material sciences as well as in pharmaceutical industry.[14-17] In particular, its applications as a bioorthogonal reaction in living systems revolutionized our ability to label and manipulate intact biomolecules.[3,18,19] The success of CuAAC reaction is mainly because of the following reasons: (i) the azide and alkyne reaction groups are highly specific towards each other but remain inert to other chemically active molecules; (ii) the reaction can proceed with a high rate and efficiency under conditions resembling those met in living species (e.g. aqueous and near neutral medium, room temperature, and highly reduced environment with diverse active chemical groups); (iii) the reaction product triazole is regioselective (1,4-isomer only), stable and unreactive. These advantages made CuAAC a highly powerful bioorthogonal reaction with diverse applications in life sciences.[20-23]

The cytotoxicity of Cu(I) ions, however, hindered the direct utilization of CuAAC inside living cells. To circumvent this issue, strain-promoted azide–alkyne cycloaddition (SPAAC) reaction has been developed by Bertozzi and coworkers as a "copper-free" version of click reaction that has found many exciting applications, particularly on cell surface labeling.[24] However, the bioorthogonality and specificity of SPAAC reactions within an intracellular environment remain a concern. For example, the cyclooctyne type of molecules used in SPAAC such as difluorinated cyclooctyne (DIFO) may undergo side reactions with cellular nucleophiles (e.g. thiols).[25] In particular, molecules located in the highly reduced and fragile cytoplasmic space represent attracting yet challenging targets, and currently the

labeling of cytosolic components has been mainly conducted on fixed cells or in homogenates. In addition, although the inverse-electron-demand Diels-Alder (IEDDA) reaction features in higher reaction rate than SPAAC,[26,27] the complicated procedures for synthesizing tetrazine and trans-cyclooctene containing reagents prevent their broad utility at the current stage. As an alternatively strategy to overcome Cu(I)'s toxicity, scientists have also been looking for synthetic ligands to prevent the harmful effects of Cu(I) ions or additional transition metals to substitute the Cu(I) catalysts. Such efforts, initially started also on cell surface labeling, are rapidly moving towards the intracellular space with improved biocompatibility. In this review article, we present the recent progress of transition metal-catalyzed bioorthogonal reactions within living cells. We will focus on efforts towards developing and applying such reactions on proteins, the most abundant biomolecule with fundamental roles in virtually all biological processes.

2. Evolving bioorthogonal handles for intracellular protein chemistry

A prerequisite for conducting bioorthogonal reactions on a protein of interest (POI) is to incorporate a bioorthogonal function group into POI that can react with a tagging molecule bearing the complementary functionality. A variety of strategies have been developed for introducing bioorthogonal handles either in a site-specific or residue-specific fashion into a POI, which has been reviewed in great details previously.[7,28,29] For the current review, we will briefly describe a panel of recently reported, bioorthogonal handle-bearing unnatural amino acids (UAAs) that can be site-specifically incorporated into proteins by the Pyrrolysine-based genetic code expansion system. Pyrrolysine (Pyl, 1, **Fig. 1**) is the 22nd naturally occurring amino acid that can be genetically encoded by an amber codon (UAG) in the presence of Pyrrolysyl-tRNA synthetase (PylRS) and its cognate-$tRNA^{Pyl}_{CUA}$. The genetic code expansion strategy using this PylRS-$tRNA^{Pyl}_{CUA}$ pair has been developed by many groups including our laboratory to encode over a hundred Pyl analogues containing various unnatural functionalities into proteins so far.[30,31] Furthermore, this Pyl-based

genetic code expansion system has been successfully applied to bacteria, yeast, mammalian cells, and even in multicellular organisms,[32-36] which rapidly emerged as a "one-stop shop" for introducing diverse chemical functionalities into proteins in a broad range of living species.

Pyl analogues bearing the CuAAC reaction handles **2**, **3** and **4 (Fig. 1)** were first developed and incorporated into target proteins in 2009.[37,38] Since then, a variety of azide- and alkyne-containing UAAs have been successfully encoded by different labs, including the aliphatic alkyne-containing Pyl analogues **5-7**,[39-41] as well as an azide-containing, cyclic Pyl analogue (**8)**.[42] The cyclooctyne containing UAAs **9-11** for SPAAC have also been successfully incorporated into target proteins.[43,44] Among them, **9** and **11** could also undergo the IEDDA-mediated cycloaddition reaction with a tetrazine group.[44,45] Meanwhile, Pyl analogues **12-15** contain strain promoted alkene, which have been developed for site-specific protein labeling using IEDDA reaction.[44,45] A series of aliphatic alkene bearing Pyl analogues (**16-18**) with different side chain length have also been reported, allowing the site-specific labeling of proteins via the thiol-ene reaction under a biocompatible condition.[46] Further, the alkene-containing Pyl analogues suitable for "photoclick" reaction (**19-21**) were incorporated into proteins which could react with tetrazole molecules upon UV irradiation.[47-49] Interestingly, proteins containing **20** could undergo multiple reactions such as 1,4-addition, radical polymerization, as well as 1,3-dipolar cycloaddition with high efficiency.[50] In addition, the Pyl system has also been employed for genetic incorporation of twelve *meta*-substituted phenylalanine derivatives containing bioorthognal handles including alkyne，azide and ketone groups.[51] More recently, several UAAs containing conjugated rings have been incorporated into proteins by the Pyl-based system,[52] which further expand the repertoire of bioorthogonal handles that can be recognized by the PylRS- $tRNA^{Pyl}_{CUA}$ pair. Another group of genetically incorporated UAAs worth mentioning are the 1, 2-aminothiol containing Pyl analogues (**22-24)** which could undergo cyanobenzothiazole (CBT) condensation reaction, permitting the modification of target proteins with cyanobenzothiazole

containing probes.[53,54] Finally, an idol-benzene containing Pyl analogue **25** has been encoded which serves as a site-specific handle for palladium catalyzed Sonogashira cross coupling reaction on proteins with an alkyne modified fluorophore.[39]

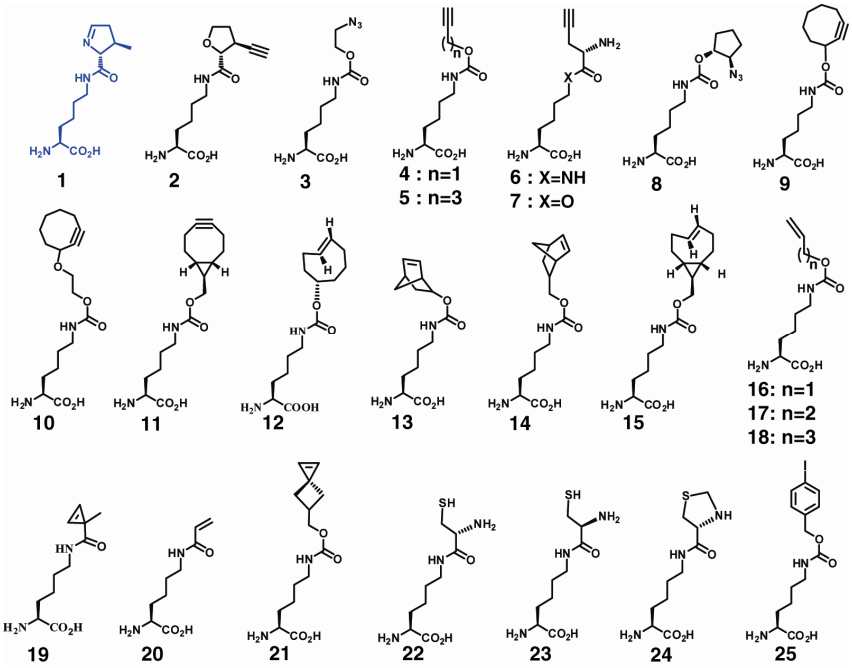

Figure 1. Structures of genetically encoded Pyrrolysine analogues bearing different functional groups for bioorthogonal or biocompatible reactions.

3 Copper(I)-mediated protein labeling

3.1 Mechanism of CuAAC reaction

Although the usage of Cu(I) ions in the CuAAC reaction has been successfully avoided by the SPAAC reaction, the cyclooctyne type of molecules react with azide in a much slower rate than the reaction between terminal alkyne and azide catalzyed by Cu(I).[55] Therefore, there is a growing interest in searching for suitable Cu(I) ligands to minimize the cytotoxicity while maintaining or even further increasing the rate of CuAAC. Before introducing these assisting ligands, we first highlight recent progresses in illustration of the mechanism underlying the CuAAC reaction which still remains controversial.

Since Cu(I) species can accelerate the azide-alkyne cycloaddition reaction with an over 7 orders of magnitude rate compared to the reaction without metal catalysts

(the activation barriers for uncatalyzed and copper-catalyzed cycloaddtion reactions between terminal alkyne and azide are 25.7 kcal/mol and 14.9 kcal/mol, respectively),[56] its role in catalyzing the triazole formation has been the subject of intensive investigations. Based on DFT (density functional theory) calculation, Sharpless and coworkers proposed a stepwise mechanism involving a mononuclear Cu-acetylide intermediate.[56] Although their calculation brought insights into the catalytic cycle, the kinetic study indicated that more than one Cu atom is involved in the transition state of the cycle, which is inconsistent with the proposed mechanism.[57] A recent study by Fokin and coworkers provided direct evidence supporting the presence of a dinuclear copper intermediate during the CuAAC reaction (**Fig. 2**).[58] By using a heat-flow reaction calorimetry to monitor the cycloaddition process in real-time, the authors found that the monomeric copper acetylide complex were not reactive unless an exogenous copper catalyst was added. The TOF-MS study further revealed a two copper centers as well as the stepwise nature of the carbon-nitrogen bond formation in the cycloaddition process.

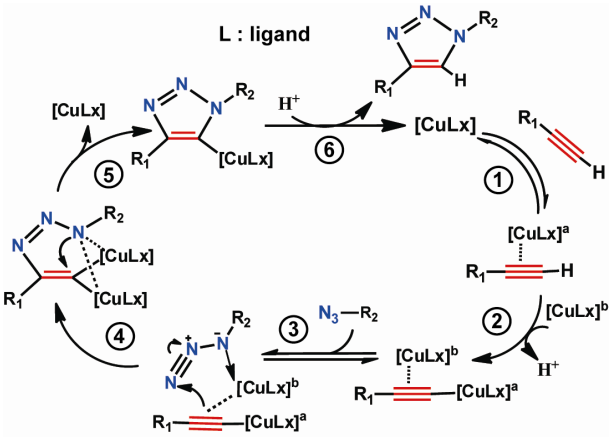

Figure 2. A recently proposed mechanism for CuAAC reaction with two copper centers.

3.2 Ligand-assisted CuAAC reaction

Although ligands are not required for Cu(I) catalysts in the triazole formation, they are frequently employed to enhance the reaction rate and protect Cu(I) from oxidation. The effect of ligands on catalysis can be due to the direct influence on the

stabilization of the catalytic complex, that is, the ligand is coordinated to Cu(I) during the reaction cycle. Here we will introduce some of the mostly used ligands for CuAAC-mediated bioconjugation.

The bipyridine type compounds have been shown by Finn and coworkers as effective accelerating ligands.[59] By screening a number of bipyridine type compounds, they discovered that the most active catalyst was Cu(I)-bathophenanthrolinedisulfonic acid sodium salt (BPS, **Fig. 3**). Kinetic measurements revealed a second order rate constant peak for BPS:Cu(I) at a ratio of 2:1, suggesting that two ligands were bound to one copper atom. Further study showed that more than two equivalents of this ligand could suppress the catalytic effect and thus inhibited the reaction. Furthermore, the same group used Cu(I)-BPS for attachment of sugars, peptides as well as fluorophores onto cowpea mosaic virus (CPMV) particle and obtained a high labeling yield.[60] However, the drawback of this system is that it is quite sensitive to oxygen and the reaction needs to be carried out under inert environment.

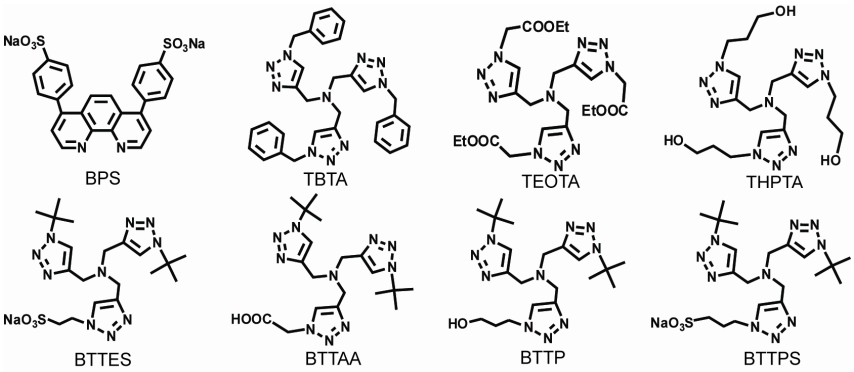

Figure 3. **Accelerating ligands for CuAAC reaction. BPS**=bathophenanthroline disulfonic acid, disodium salt; **TBTA**=tris-[(1-benzyl-1H-1,2,3-triazol-4-yl)methyl] amine; **TEOTA**=tris[(1-(2-ethoxy-2-oxoethyl)-1H-1,2,3-triazol-4-yl)methyl]amine; **THPTA**=tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl) methyl] amine; **BTTES**= 2-[4-{(bis [(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl] amino) methyl}-1H-1,2,3-triazol- 1-yl] ethyl hydrogen sulfate, **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. **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; **BTTPS**=3-[4-({bis[(1-tert-butyl-1H-1,2,3-triazol-4-yl)methyl]amino} methyl)-1H- 1,2,3-triazol-1-yl]propyl hydrogen sulfate .

Interestingly, Pezacki and coworkers used natural amino acid L-histidine (His) as

the Cu(I) ligand and they found that Cu(I)-$(His)_2$ could effectively catalyze the labeling of alkyne containing glycans on the surface of mammalian cells.[61] The toxicity of this copper complex were first analyzed on four human cell lines by measuring mitochondrial activity based on the metabolism of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). Next, they employed inductively coupled plasma mass spectrometry (ICP-MS) to study the cellular uptake of copper ions, and utilized coherent anti-Stokes Raman scattering (CARS) microscopy to study the potential effects on lipid metabolism after the labeling reaction. Together, the Cu(I)-$(His)_2$ complex was found to exhibit no apparent toxicity on all the cell lines they tested and the authors thus suggested the utilization of such simple ligands for execution of CuAAC reaction inside living cells.

Another important group of accelerating ligands was based on tris(triazolylmethyl)amine, including TBTA and its derivatives. Fokin and coworkers found that during the synthesis of polytriazole compound, the reaction rate was higher than usual and appeared to be autocatalytic.[62] They hypothesized that the polytriazole products themselves could potentially serve as the accelerating ligand for CuAAC. Their subsequent study indicated that these oligotriazole ligands were effective in protecting Cu(I) ions from aerobic aqueous conditions and thus promote the CuAAC reaction rate. Further investigations by Finn revealed the possible mechanism: the central nitrogen donor provides electron density at the copper atom; the three arms, carrying relatively weak coordinating ligands, bind to the copper atom with sufficient strength while providing access to necessary coordination sites.[63] Using a tri-triazole analogue TEOTA as the Cu(I) ligand, Fahrni and coworkers developed a fluorogenic probe for CuAAC reaction which reached completion in less than 5 min.[64] Interestingly, the differential multi-site protein chemical modifications were achieved through the combination of glycomethanethiosulphonate (glycol-MTS) glycosylation reaction with the TEOTA-assisted CuAAC reaction.[65] This strategy provided an *in vitro* platform for creating multiple post-translational modifications (PTMs) on the same protein that may ultimately mimic different protein PTM patterns.

By using TBTA as the accelerating ligand, Tirrell and coworkers performed

CuAAC-mediated labeling of the outer membrane protein (OmpC) on the surface of living *E. coli* cells.[66] The azide-containing alanine analogue-azidohomoalanine (AHA) was incorporated into recombinant OmpC before being labeled by an alkyne-biotin probe via TBTA-accelerated CuAAC. Upon staining with a fluorescent avidin molecule, *E. coli* cells harboring this AHA-bearing OmpC protein could be readily distinguished from control cells by flow cytometry. However, the Cu(I)-TBTA complex was still found to be toxic which rendered *E. coli* cells unable to divide after being transferred into rich medium.[66,67] Moreover, the poor water solubility of TBTA further hindered its application in aqueous medium.

To circumvent this problem, Finn and coworkers developed a more water soluble ligand-THPTA (**Fig. 3**) for assisting the CuAAC-mediated labeling of CPMV particles.[68] An important feature of THPTA is that it can also serve as a sacrificial reductant for oxidative species that are produced in the coordination sphere of the metal. Finn and coworkers also used the THPTA-assisted CuAAC reaction to label azide-containing glycans with an alkyne-fluorophore on three different cell lines (HeLa, CHO and Jurkat cells), which all yielded significant fluorescence signal.[20] Furthermore, they showed that THPTA prevented the toxic effect of Cu(I) in a dose dependent manner. A 5:1 ratio of THPTA:Cu was found to preserve the cell viability at copper concentrations ranging from 10 to 50 μM, and this study represents the first ligand-assisted CuAAC reaction on mammalian cell surface.[20]

Recently, Wu and coworkers reported two new TBTA analogues, BTTES and BTTAA, for CuAAC reaction (**Fig. 3**).[69,70] Employing these ligands, they conducted CuAAC-mediated labeling and imaging of fucosylated glycans on cell surface as well as on glycans during early embryogenesis of Zebrafish. Notably, a detailed and side-by-side comparison of ligand-assisted CuAAC in the presence of TBTA, THPTA, BTTAA or BTTES demonstrated that these two newly developed TBTA analogues accelerated the reaction more efficiently than the other ligands. Further, the authors demonstrated that these two ligands could effectively suppress Cu(I)-produced toxicity and 50 μM of Cu(I)-BTTAA showed negligible influence on cell viability. In contrast, cells treated with 50 μM Cu(I)-TBTA showed a slower rate of proliferation.

Therefore, these Cu(I)-BTTAA and Cu(I)-BTTES complexes exhibited improved biocompatibility for CuAAC-mediated cell surface labeling.

3.3 Moving Ligand-assisted CuAAC inside living cells

Despite the variety of choices on ligands to assist CuAAC, such reactions are mainly restricted to cell surface labeling (i.e. glycans and membrane proteins on mammalian and *E. coli* cells). Direct labeling of intracellular biomolecules still remains largely unexplored. Tirrell and coworkers first reported the utility of CuAAC reaction for labeling of newly synthesized proteins inside bacteria[71] (**Fig. 4**). *E. coli* cells were treated with a medium containing 19 natural amino acids and an alkyne functionalized UAA-homopropargylglycine (HPG) to produce the recombinant protein Bastar carrying alkyne handles. The bacterial cells were then subjected to TBTA-assisted CuAAC with an azide functionalized coumarin at 4°C for 14-15 h. After extensive washing, *E. coli* cells were excited at 395 nm and the emission was monitored from 450 nm to 500 nm. A significant fluorescence increase was observed only when cells were treated with HPG, azide-coumarin and Cu(I)-TBTA. Both confocal fluorescence microscopy and polyacrylamide gel electrophoresis confirmed the successful labeling of the Bastar protein inside *E. coli*. Furthermore, the labeling efficiency of two different Cu(I) sources, CuBr or $CuSO_4$ plus TCEP (tris(2-carboxyethyl)phosphine), were compared and the former catalyst was found to yield a slightly higher fluorescence enhancement.

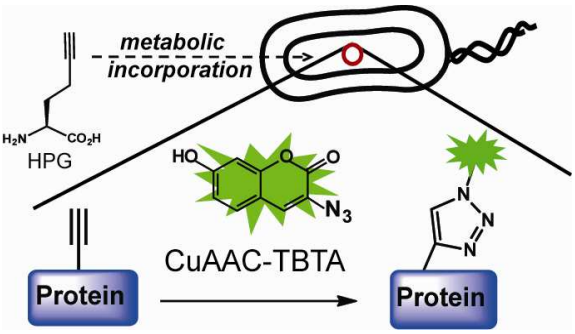

Figure 4. TBTA-assisted CuAAC labeling of newly synthesized proteins inside bacterial cells. A terminal alkyne containing Met analogue was metabolically incorporated into target proteins inside bacterial cells, followed by TBTA-assisted

CuAAC reaction with an azide modified coumarin fluorophore.

Recently, our group employed the BTTAA-assited CuAAC reaction for protein labeling inside bacterial cells.[72] Besides demonstrating the utility of BTTAA as a biocompatible Cu(I) ligand for live cell labeling, we chose an acid-responsive chaperone-HdeA as the template protein in order to develop a protein-based pH sensor. Enteric bacterial pathogens must pass through the highly acidic human stomach before reaching their primary infection sites in small intestine. Measuring this acidification process inside enteric bacteria is highly challenging due to the extremely acidic intracellular pH (pH<3) when these bacteria are surrounded by gastric acid from human stomach. The *E. coli* acid chaperone HdeA can sustain very low pH conditions (pH~2) and adopt a pH-dependent conformational change in a wide range (pH 2- pH 7). We first used PylRS- $tRNA^{Pyl}_{CUA}$ pair to genetically and site-specifically incorporate the azido-Pyl analogue **8** into HdeA expressed in *E. coli* periplasmic space. The BTTAA-assited CuAAC reaction was then applied between the azide-bearing HdeA and an alkyne-functionalized, environment-sensitive fluorophore (alk-4-DMN) under living conditions. The 4-DMN fluorophore can undergo a significant fluorescence increase upon switching from the hydrophilic to hydrophobic environment. Systematic survey of the attachment sites by fluorescence measurement led to the discovery of one position (Val 58) on HdeA that showed a strong fluorescence increase upon acidification, likely due to the conformation-change triggered local hydrophobicity increase that enhanced 4-DMN's fluorescence. This HdeA-based pH sensor, carrying the CuAAC-labeled alk-4-DMN at residue 58, was used to monitor the pH variation in *E. coli* periplasm during the acidification process. The pH-dependent fluorescence change has been observed by confocal fluorescence microscopy or flow cytometry when the pH dropped from neutral to pH 2.3, verifying that the pH sensor we developed could operate under a wide acidic pH range. Finally, we also showed in the same study that the BTTAA-assisted CuAAC reaction can operate inside *E. coli* cytosolic space without apparent toxicity. By removing the periplasmic signaling peptide, we constructed a cytosolic expressed version of HdeA

carrying the azido-handle at the same residue (58). CuAAC-mediated labeling of this cytosolic variant with alk-4-DMN in the presence of Cu(I)-BTTAA generated a pH-sensitive protein sensor located in *E. coli* cytoplasm.

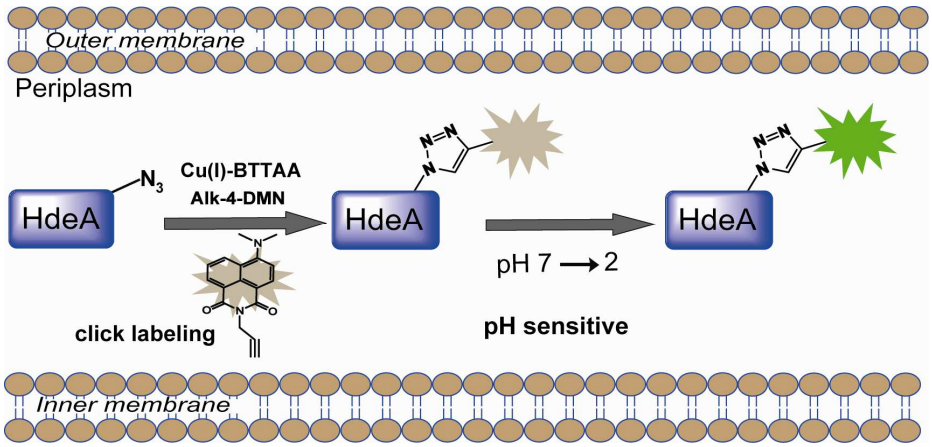

Figure 5. Ligand-assisted CuAAC labeling of proteins in bacteria periplasm. Azide-bearing HdeA protein was expressed in the periplasm, followed by BTTAA assisted CuAAC reaction with an alkyne modified fluorophore (Alk-4-DMN). 4-DMN=4-*N,N*-Dimethylamino- 1,8-naphthalimide.

More recently, two additional TBTA analogues, BTTP and BTTPS (**Fig. 3)**, have been reported by Wu and coworkers.[73] Both of these ligands could efficiently speed up the CuAAC reaction while further eliminating copper cytotoxicity. The negatively charged sulfate group on BTTPS may decrease the membrane permeability of the coordinated Cu(I) ions and thus reduce the toxicity, making it a better choice for labeling on live cell surface. Mechanistic study has showed that the pendant acid arms in the proper position could assist the reaction by accelerating the protiolysis step that cleaves the Cu-C bond of the Cu-triazolyl intermediate.[63] However, the negatively charged BTTPS may impose electrostatic repulsion with labeling probes, biomolecules or cell membrane that bears negative charges.

3.4 Chelation-assisted CuAAC reaction

The CuAAC reaction rate could be further accelerated in the presence of certain azide-containing substrates. Zhu and coworkers discovered that the azide substrate

containing an auxiliary ligand could effectively accelerate the CuAAC reaction, and was termed by the authors as "chelation-assisted CuAAC reaction".[74] Mechanistic study revealed that the catalytic copper center prefers to bind to the alkylated nitrogen **N-α** (**Fig. 6a**), thus increases the electrophilicity of the azide group and loweres the kinetic barrier to form the metallacycle intermediate upon nucleophilic attack by copper acetylide.[75] Moreover, the use of additional Cu(I) ligands could further increase the reaction rate. Based on this knowledge, Ting and coworkers[76] incorporated a picolyl azide-containing substrate (**Fig. 6b**) to the target protein using the Lipoic acid ligase (**LplA**) as the enzyme. By fusing the LplA acceptor peptide (LAP) with a POI, picolyl azide was able to be conjugated to the expressed target protein on cell surface with an engineered LplA mutant (Trp37-Val) enzyme. For proof-of-concept, the LAP-tagged cyan fluorescent protein (CFP) was fused to platelet-derived growth factor (PDGF) receptor and targeted to cell surface. After enzymatic conjugation with picolyl azide, cells were treated with an alkyne modified Alex647 fluorophore in the presence of Cu(I). Significant fluorescence signal, as measured by the ratio of fluorescence intensity between Alex647 and CFP, was detected only in the LAP-CFP transfected cells. To quantitatively demonstrate the labeling efficiency by this chelation-assisted CuAAC reaction, the authors further performed the cell surface labeling by varying the copper concentrations in the presence and absence of accelerating ligands THPTA or BTTAA. The results showed that the use of picolyl azide, in conjunction with THPTA or BTTAA, increased the protein labeling signal by as much as 25 folds compared to the non-chelating azide. Finally, to demonstrate the biocompatibility of this new reaction system, the authors applied their strategy on the surface of living hippocampal rat neurons. A postsynaptic transmembrane protein neuroligin-1 was fused with LAP tag, modified by picolyl azide and subjected to CuAAC reaction with alkyne-containing Alex 647 in the presence of THPTA and different concentrations of Cu(I) ions. The use of chelation-assisted CuAAC reaction effectively decreased the Cu(I) concentration from 100 to 10 μM, which showed negligible toxicity on neurons without sacrificing the labeling efficiency.

Recently，Wu and coworkers examined a small library of azides bearing an internal chelating motif to identify the probe with the best kinetic behavior. Interesting, they found that the picolyl azide with an electron donating group on the pyridine ring (**Fig**. **6c**) could further boost the efficiency of ligand assisted CuAAC reaction by 3-4 folds compared to the picolyl azide used by Zhu and Ting groups. By combining this picolyl azide and BTTPS-Cu(I), they observed a 20-38 folds increased labeling efficiency on alkyne-bearing glycans in living systems without apparent toxicity. Using this strategy, they successfully monitored the dynamic glycan biosynthesis in mammalian cells and in early embryogenesis of zebrafish.[77]

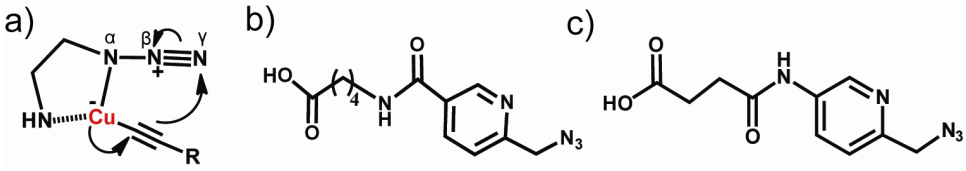

Figure 6. Chelation assisted CuAAC reaction. a) A simplified chelation model of the intermediate prior to the metallacycle formation en route to the 1,4-substituted triazole; **b,c**) Structures of electron-withdrawing picolyl azide (b) and electron-donating picolyl azide (c).

4. Protein bioorthogonal labeling by transition metals beyond copper

Despite a series of newly developed Cu(I)-stabilizing ligands that have now facilitated CuAAC inside living cells, labile copper(I) ions may still be harmful to various biomolecules. Many living species have evolved dedicated systems to deal with this toxic metal ion. For example, Gram-negative bacteria compartmentalize all their copper-dependent enzymes in the periplasmic space or the outer aspect of the cytoplasmic membrane, leaving almost no free copper ions in its cytosol.[78] Mammalian cells have evolved many copper chaperones or chelating proteins to sequester and transfer labile copper ions within their intracellular space.[79,80] These natural copper binding proteins can potentially compete with the exogenously delivered Cu(I)/ligand complex for copper ions, which may result in reduced CuAAC reaction rate or elevated toxicity due to the disruption of cellular copper homeostasis.

In contrast, the absence of many other transition metals, such as palladium and ruthenium, from all known biological systems provides an intriguing opportunity to test whether these metal ions can serve as alternative sources in catalyzing bioconjugation reactions on proteins *in vitro* and *in vivo*. Several emerging examples will be discussed below. However, although additional transition metals beyond copper ions have been utilized for many biological applications (e.g. hydrogenation of biological membranes), expansion of these metal mediated reactions to proteins, particularly inside living cells, is still in its infancy.

4.1 Palladium-triggered protein cross-coupling in aqueous buffer

Palladium-catalyzed chemical conversions had a major impact on modern organic synthesis.[81-83] Moving these fascinating chemical reactions from test tubes to living cells may find broad applications. However, even though people have used water soluble Pd compounds to study biological membranes through hydrogenation reaction in early 1990s,[84] Pd-triggered reactions on more complicated biomolecules such as proteins were rarely encountered. The fragile nature and complicated chemistry environment, as well as the lack of bioorthogonal reaction handles on protein substrates hindered the utilization of Pd chemistry. Inspired by the success of CuAAC reaction on proteins, people started to test the idea of extending diverse palladium mediated cross-coupling reactions to proteins. In fact, palladium mediated covalent protein modifications, especially protein-small molecule conjugation, has been tested back in early 2000.[85] However, these trials suffered from the harsh reaction condition, low reaction yield as well as the lack of bioorthogonality. In recent years, with a series of more biocompatible catalytic systems and the introduction of suitable bioorthogonal handles onto proteins, great progress has been made in this direction.

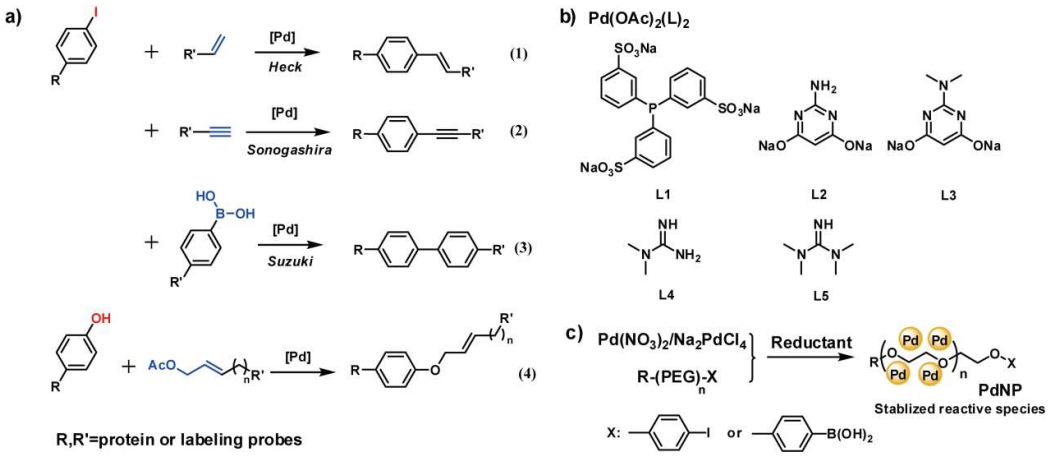

Figure 7. Palladium-mediated protein cross-coupling reactions. a) Reaction schemes of palladium mediated protein cross coupling reactions. Either R or R' represents a protein substrate, with the counterpart representing a labeling probe. **b**) Ligand-assisted palladium catalysis systems. **c**) Substrate-assisted palladium catalysis systems.

The idea of using palladium mediated cross coupling reactions to modify synthetic polypeptides was initially reported by Schmidtchen.[86] However, moving this method from peptides to more complicated and fragile protein substrates is not straightforward. In an effort to apply palladium-catalyzed reactions to modify biosynthetic proteins *in vitro*, Yokoyama and co-workers first reported the Heck (**Fig. 7a** (1)) and Sonogashira cross-coupling reactions (**Fig. 7a** (2)) between a terminal alkene or alkyne containing biotin probe and an iodophenyl-containing Ras protein in the aqueous medium containing approximately 10% DMSO (v/v).[85,87] However, low reaction yields (25% for Sonogashira coupling and 2% for Heck coupling) were observed when using $[Pd(OAc)_2(L1)_2]$ (**Fig. 7b**) as the catalyst after 80 min incubation at 6 ^{o}C under the N_2 protection condition. In addition, Cu(I) salt was used as a co-catalyst in Sonogashira coupling, which further limited its utility in cellular environment. Francis et al also reported a native protein labeling method by using the π-allyl palladium complex to label tyrosine residues[88] (**Fig. 7a** (4)), with an overall conversion rate around 30%. The first Suzuki cross-coupling reaction on biosynthetic proteins was reported by Schultz and coworkers[89] (**Fig. 7a** (3)). A boronate-containing amino acid was genetically incorporated into a cysteine-free T4 lysozyme, which

offered the reaction handle for Suzuki coupling. Due to the general applicability of the genetic code expansion strategy, this work represents a more general approach to perform Suzuki cross-coupling on proteins. However, the high reaction temperature (70^{o}C) and the low reaction yield (30%) hindered the wide application of such reactions on proteins.

Thanks to the discovery of an effective ADHP (2-amino-4.6-dihydroxypyrimidine, L2)-based catalyst system [$Pd(OAc)_2(L2)_2$] (**Fig. 7b**) by Davis and coworkers,[90] full conversion (>95% yield) was realized between an aryl iodide group chemically attached to the target protein via cysteine modification and a broad scope of aryl-/vinyl-boronic acid partners. This Suzuki–Miyaura protein cross-coupling reaction was subsequently applied to proteins bearing a site-specifically incorporated aryl halide-containing UAA (**Fig. 7a** (3)). Lin and coworkers also reported the discovery of an aminopyrimidine-palladium(II) complex [$Pd(OAc)_2(L3)_2$] (**Fig. 7b**) for copper-free Sonogashira cross-coupling that enables selective functionalization of a HPG-encoded ubiquitin protein in aqueous medium[91] (**Fig. 7a** (2)). Unlike the [$Pd(OAc)_2(L2)_2$] system that only works well in the Suzuki coupling, the [$Pd(OAc)_2(L3)_2$] system can be used in both Suzuki cross-coupling and Sonogashira cross-coupling reactions on proteins with satisfactory efficiency. The possible reason is that the dimethyl substituent in the amino group of L2 may alter the hydration shell of ADHP and thus fine tune the electron density of the pyrimidine ring. In a more recent work, Davis suggested that it is the central guanidine moiety that coordinates Pd atom in the system.[92] Based on this analysis, they designed “minimal” ligand structures, dimethylguanidine (L3) and tetramethylguanidine (L4), in an attempt to find more efficient ligands.[92] Indeed, their detailed study found that all three ADHP ligand variants, L2, L3, and L4, afforded increased catalytic activity. LC-MS analysis was routinely conducted on intact proteins after the bioorthogonal conjugation to confirm that the labeling reaction only occurred through the desired residue containing the reaction handle. Interestingly, no ion peaks were detected by the mass spectrometry after the Pd-mediated protein labeling, likely due to the none-specific absorption of palladium ions on protein surface.[93] To circumvent this

issue, the authors showed that 3-mercaptopropionic acid worked as a scavenger to remove these palladium ions and thus allowed the LC-MS analysis on labeled proteins.

All these reactions introduced above are ligand-assisted catalysis systems. Davis and co-workers recently reported a self-liganded Suzuki cross-coupling system, allowing the site-sepecific conjugation of polyethylene glycol (PEG) and proteins using a water-soluble Pd salt without the addition of ligands[94] (**Fig. 7c**). Their strategy relied on the stabilizing effect of PEG, which was previously observed in the Pd Nano particle (NP)-based catalysts. Two model proteins were chosen for demonstration: subtilisin from *Bacillus lentus* (SBL) and all-β-helix protein from *Nostoc punctiforme* (Npb). The SBL protein was chemically modified with p-iodobenzylcysteine whereas p-iodophenylalanine(pIPhe) was site-specifically incorporated into Npb via genetic code expansion. The two modified proteins were then allowed to react with the phenyl-boronic acid containing PEG reagents (2 KDa) in the presence of K_2PdCl_4. This Pd-mediated protein PEGylation reaction under the ligand-free conditions reached a ~70% yield as analyzed by SDS-PAGE and MALDI-TOF, which were more efficient than the previous ligand-assisted system using ligands L1 to L4. Therefore, the water soluble palladium salts can be mixed with PEG-containing labeling probes beforehand, which, upon the addition of reducing agents, can generate PEG-stabilized Pd NPs that largely improved the reactivity (**Fig. 7c**). We refer this process as a substrate-assisted Pd-mediated labeling system. In fact, Hamachi and coworkers have previously shown similar reaction mechanisms on Suzuki reaction for modifying the synthetic polypeptides[95] where Na_2PdCl_4 was used as the palladium source while glycerol was used as a stabilizer.[95] Finally, another substrate-assisted system was focused on the synthesis of stable arylpalladium(II) reagents that directly linked to the labeling probe, which mimicked the generation of active products in the first oxidative addition step during the cross coupling reaction. The cycled arylpalladium(II) compound seemed to serve as a more convenient storage reagent for effective and selective functionalization of the alkene or alkyne-bearing proteins under biological conditions.[96,97]

Notably, by taking advantage of the highly efficient, chemoselective and biocompatible palladium catalysts, the palladium triggered cross-coupling reactions have found broad applications in modifying various biomolecules in aqueous medium.[92,94,98] In comparison with the conventional protein modification methods, the "tag-and-modify" approach on proteins using the Suzuki−Miyaura coupling reaction possesses distinct advantages. For example, the C-C bond created between proteins and PEG molecules by this strategy is more stable than the conventional PEG-attachment linkers during protein PEGylation, which may provide a more stable PEGylated protein for therapeutic applications. Another example came from radiolabeling where the Suzuki−Miyaura coupling reactions could be effectively carried out at low substrate concentrations than the regular modification methods. The excess usage of labeling reagents is generally avoided in protein radiolabeling and therefore highly efficient ligation reactions are required.

4.2 Palladium-mediated protein cross coupling in living systems

Although many reaction systems discussed above are compatible with intact proteins, only a few of them have been shown to work under living conditions. The low reaction yields, harsh reaction conditions and high loadings of palladium compounds made most of these reactions incompatible with live cells. Here we introduce three successful cases of palladium-mediated protein cross coupling reactions in living systems.

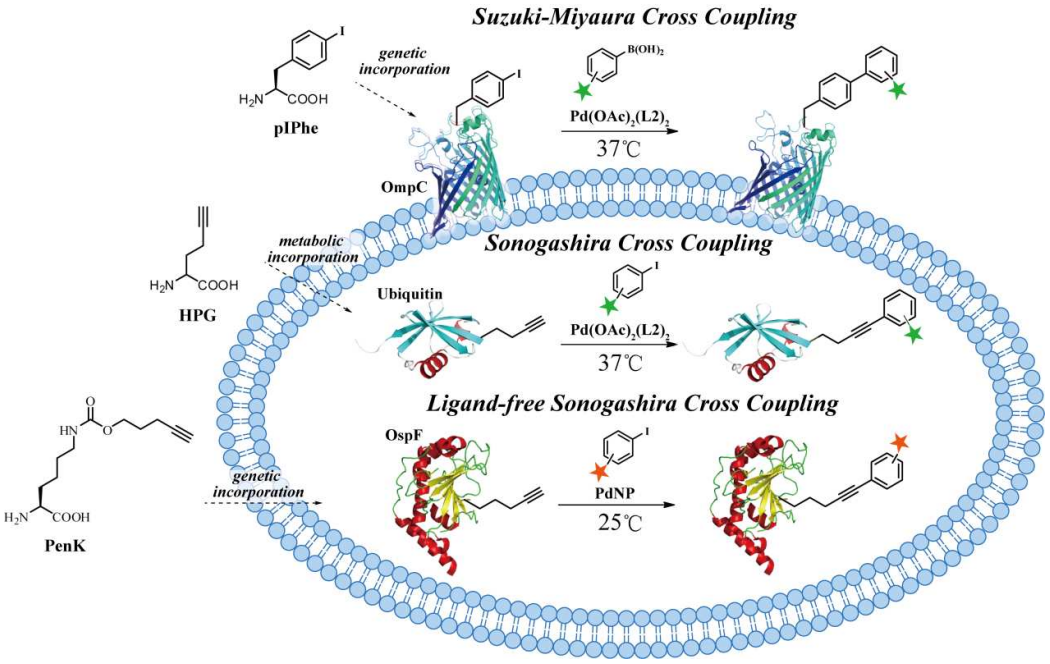

Figure 8. Reaction schemes of palladium mediated protein cross coupling in living Gram-negative bacterial cells. Suzuki cross coupling was conducted in pIPhe containing OmpC protein on a simplified *E. coli* cell membrane. Sonohashira cross coupling was conducted on HPG functionalized ubiquitin inside *E.coli* cells. Ligand free Sonohashira cross coupling was employed inside *Shigella* cells on PenK incorporated OspF protein.

Davis and co-workers first applied Pd-mediated Suzuki coupling reactions, in conjunction with their "tag and modify" strategy, to label an outer membrane protein on *E. coli* surface[99] (**Fig. 8**). Four "tag" sites with good accessibility on separate loops of OmpC were chosen for incorporating an aryl-halide-containing UAA (pIPhe) via the genetic-code expansion strategy, which allowed the site-specific labeling of a given protein rather than the whole cellular proteins by the metabolic incorporation. After obtaining bacterial strains containing the prepared four"tagged" versions of OmpC, they labeled this membrane protein by boronic acid-functionalized fluorescein (BAF) to test the efficiency of their $[Pd(OAC)_2(L2)_2]$ catalyst in promoting the Suzuki–Muyari cross-coupling reaction on live bacterial cells. The promising results promoted them further to optimize this reaction by altering the "tagging" position, temperature, reaction time, as well as the concentrations of BAF and Pd catalyst used. Notably, the dosage effects of the boronic acid and the Pd catalyst were found to be completely different: a sigmoidal logarithmic dose-response was observed in the case

of boronic acid, while a threshold effect was observed when raising the loadings of Pd catalysts. No detectable labeling was observed at Pd loadings lower than 300 μM while a dramatic increase of labeling efficiency was observed when Pd concentration equaled to or beyond 330 μM. This threshold effect indirectly indicated the catalytic activity of palladium. In addition, to further validate that the Suziki-Muyari cross coupling reaction on proteins was biocompatible, the authors probed the toxicity of Pd catalyst used in their system. Gratifyingly, a low concentration of Pd (<0.5 mM) in the ligands bound form of an activated catalytic species showed no obvious cell toxicity, while the labeling efficiency was satisfactory. In summary, this work demonstrated that the Pd-mediated coupling reaction can be carried out on the surface of living bacteria cells, and the same palladium complex was also employed to conjugate the boronic acid-modified carbohydrates on *E. coli* surface. After that, they also rewrote the bacterial glycocalyx using Suzuki–Miyaura cross-coupling.[100] A panel of carbohydrate-based boronic acids were site-specifically attached to *E. coli* cell surface via OmpC bearing a genetically encoded pIPhe handle, which allowed modulation of interactions with biomolecular partners via prokaryotic O-glycosylation mimics.

Lin and coworkers employed their water-soluble $[Pd(OAc)_2(L3)_2]$ complex to catalyze the copper-free Sonogashira cross-coupling reaction on proteins inside *E. coli*[101] (**Fig. 8**). After optimization of the labeling conditions for protein substrates metabolically incorporated with HPG in aqueous buffer, they transferred this reaction system to live *E. coli* M15A cells expressing a ubiquitin (Ub) protein bearing the metabolic-incorporated HPG residues (HPG-Ub). After reaction at 37℃ for 4 h, the collected bacteria gave an obvious green fluorescence upon 365-nm excitation compared to the cells without HPG-Ub protein. Furthermore, the in-gel fluorescence analysis on the Ni-NTA purified HPG-Ub protein confirmed the intracellular labeling of HPG-Ub in the presence of $[Pd(OAc)_2(L3)_2]$. However, this metabolic-incorporation method introduced HPG handles into virtually all internal proteins of a bacterial cell and the high loading of palladium compounds could be toxic. Indeed, when studying the cytotoxicity of this process, their results indicated

that the palladium complex and the fluorescein iodide were toxic when added separately, whereas the pre-activated mixture of palladium complex and fluorescein iodide showed much lower toxicity. This interesting finding suggested that detailed optimizations (both efficiency and toxicity) are necessary when applying this system for intracellular protein labeling.

Our group recently developed a ligand-free system with the water soluble $Pd(NO_3)_2$ salt as the palladium source and sodium ascorbate as the reductant for Pd-mediated cross-coupling reactions on proteins.[39] The short PEG linker between the iodo-benzene group and the labeling fluorophore was found to stabilize the active palladium species. We applied this efficient ligand-free Sonogashira cross-coupling reaction for intracellular protein labeling within bacterial cells[39] (**Fig. 8**). We found that Pd NPs generated from $Pd(NO_3)_2$ was sufficient to catalyze the cross-coupling reaction inside E. coli bacteria without additional ligands. The biocompatibility of our system was characterized in details *in vitro* and inside cells. Potential denaturing effects from Pd catalysts on proteins was monitored by the intrinsic fluorescence of GFP as well as the enzymatic activity of a more fragile protein Luciferase, both of which demonstrated the excellent compatibility of our system with protein substrates. To further characterize the compatibility of our reaction system within living bacteria, the viability and the proliferation rate of *E. coli* cells after $Pd(NO_3)_2$ treatment were also measured. All these experiments indicated that our reaction system had negligible toxicity or damaging effects to bacterial cells. Moreover, to confirm that our palladium species can enter bacterial cells, we assessed cellular uptake of the palladium complex by ICP-MS. Taken together, we showed that bacterial cells were able to uptake the palladium species with no apparent toxicity, consistent with the previous studies on Pd NPs. To further demonstrate this system for site-specific protein labeling inside *E. coli* cells, GFP bearing a site-specifically incorporated alkyne handle was first expressed in *E. coli* cells using evolved PylRS- $tRNA^{Pyl}_{CUA}$ pair, which were then treated with $Pd(NO_3)_2$ and Rhodamine-conjugated phenyl iodide at room temperature. In-gel fluorescence and western blot analysis confirmed the

specificity of our Pd-mediated Sonogashira cross-coupling on intracellular POIs. Finally, we took advantage of this labeling strategy and the Pyl-based genetic code expansion strategy to label internal proteins from additional types of Gram-negative bacterial pathogens such as *Shigella*. The alkyne-bearing Pyl analogue **5** was first site-specifically incorporated into a Shigella Type-III secretion (T3S) effector OspF. Pd-mediated Sonogashira labeling of this azide-bearing toxin protein was then conducted with Iph-FL525 inside living *Shigella* cells, which was confirmed both by in-gel fluorescence assay and confocal fluorescence microscopy.

4.3 Other transition metals for protein labeling in aqueous buffer

Besides Cu and Pd, other transition metals have also long been used for protein modifications in aqueous medium. However, many of these reactions are not compatible with living conditions and thus further applications within live cells are still difficult at the current stage. Here we mainly discuss the Rhodium carbenoids-mediated selective modification on intact proteins via tryptophan residues, which were originally developed only for purified proteins but now can be performed in cell lysate.

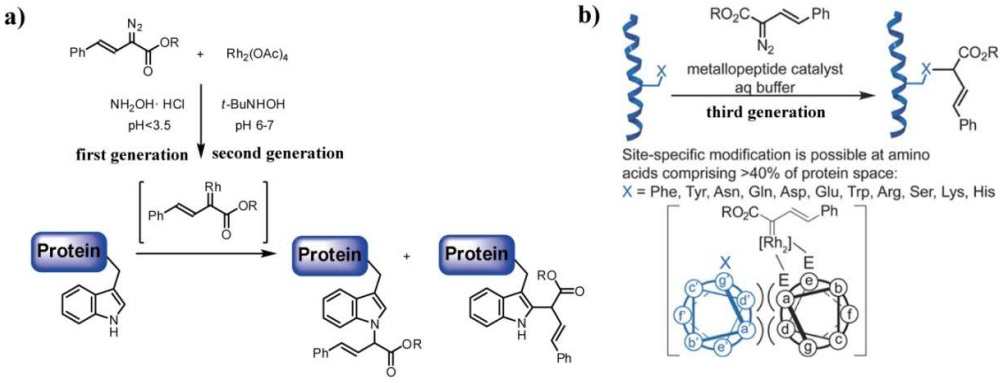

Figure 9. Rhodium carbenoids-mediated native protein modification. a) Scheme of rhodium-mediated selective modification on intact proteins via tryptophan residues. **b**) The peptide-based molecular recognition and a rhodium metallopeptide catalyst capable of modifying a wide range of amino acid side chains *in vitro* as well as in cell lysates. Reproduced with permission from ref.104. Copyright 2013, American Chemical Society.

Classical native protein modifications often chose nucleophilic residues such as

cysteine and lysine that can react with electrophilic reagents. Other nucleophilic residues, tryptophan for example, can also be modified with electrophilic organometallic species. In this case, substrates show no reactivity toward native protein until activated by rhodium catalysts, which would improve the chemoselectivity and avoid cross reactivity with other nucleophilic residues. The first report of this kind chemistry on proteins came from Francis group in 2004.[102] The reactive rhodium carbenoids was generated *in situ* through the catalytic degradation of diazo compound in the presence of $Rh_2(OAc)_4$ and $H_2NOH \cdot HCl$ (**Fig. 9a**). Two model proteins, horse heart myoglobin (contains two tryptophans) and subtilisin Carlsberg (contains one tryptophan), were chosen as substrates for proof-of-concept. Approximately 60% conversion was obtained for this reaction as analyzed by ESI-MS. The follow up proteolysis revealed that the modifications were confined to tryptophan-containing fragments by MS-MS analysis. However, the low pH of the reaction environment (pH<3.5) limited further applications of this methodology in biocompatible context. In 2009, Francis and co-workers improved the biocompatibility of this rhodium carbenoids method by the utilization of N-(tert-butyl)-hydroxylamine (t-BuNHOH), which allowed this reaction to be carried out at near neutral pH.[103] A possible explanation is that, when pH is above 6, the oxygen of t-BuNHOH binds to $Rh_2(OAc)_4$ rather than the nitrogen atom from the original H_2NOH ligand.

More recently, Ball et al. reported a modified version of this rhodium chemistry for protein labeling.[104] Site-specific, proximity-driven protein modification can be carried out using a unique combination of peptide-based molecular recognition and a rhodium metallopeptide catalyst capable of modifying a wide range of amino acid side chains *in vitro* as well as in cell lysate (**Fig. 9b**). The compatibility of this catalyst may potentially be improved for protein labeling inside living cells.

Other transition metals, such as Iridium and Gold, have also been employed for biomolecule labeling.[105] These topics are not included here because the substrates were mainly peptides or oligosaccharides.[106]

5. Bioorthogonal protein chemistry beyond conjugation

Although conjugation reactions, as exampled by CuAAC and SPAAC, are the most extensively studied bioorthogonal chemistry that have found broad applications in labeling biomolecules including proteins, other types of chemical conversions that are compatible with living systems are rapidly emerging. The unique utility of such reactions significantly expanded the repertoire of biocompatible chemistry. In particular, transition metal ions are well known for catalyzing diverse chemical conversions beyond conjugations that are hardly feasible previously. Here we introduce two recently reported cases where a transition metal-mediated cleavage reaction and a cross-metathesis reaction have been employed to selectively activate a POI and to create reversible protein modifications, respectively.

5.1 Palladium-mediated cleavage reactions for protein activation

Transition metal-mediated cleavage reactions have been previously applied to unmask the chemically caged fluorophores inside living cells. For example, Meggers et al reported a ruthenium catalyst for the cleavage of allycarbamate on a caged Rhodamine 110 under living conditions.[107] This reaction was further utilized by Schultz and coworkers for in situ generation of a methyl-lysine residue from its caged precursor on intact proteins.[108] Similar Pd-mediated reactions have also been used to uncage fluorophores within living cells.[109,110] However, applying such reactions on intracellular proteins remains to be explored. Inspired by their excellent efficiency and biocompatibility, we recently employed a palladium-mediated deprotection reaction to specifically control the activation of an intracellular POI[111] (**Fig. 10**). Our strategy relied on the rational design in conjunction with catalyst screening for a biocompatible "protection-group/catalyst" pair that can be used to cage-and-uncage a specific lysine residue on a given protein *in vitro* and inside living cells. The propargyloxycarbonyl (Proc)-caged lysine (ProcK, **4**) was originally synthesized as a Pyl analogue for CuAAC labeling that can be site-specifically incorporated into a POI by using the PylRS-$tRNA^{Pyl}_{CUA}$ pair. Interestingly, we showed that the Proc moiety

could serve as an effective caging group to mask a selected lysine residue, thus rendered the lysine dependent target protein inactive. Our identified palladium catalysts provided a facile approach to liberate free lysine from its Proc-caged lysine analogue (ProcK, **4**) that was site-specifically incorporated into proteins via the PylRS-$tRNA^{Pyl}_{CUA}$ pair (**Fig. 10**). This method offered a unique and complimentary approach with the photocaging strategy to regenerate the activity of a specific amino acid residue (eg. Lysine) on its carried protein, and therefore allow the study of lysine dependent protein activity under living conditions.

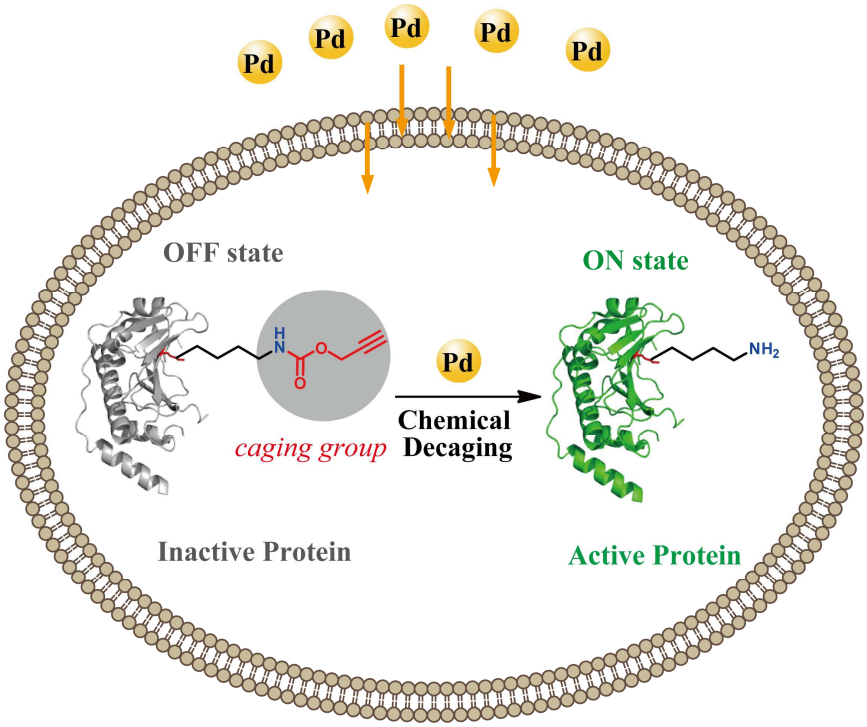

Figure 10. Scheme of palladium mediated elimination reactions for protein activation. The propargyloxycarbonyl (Proc)-caged lysine analogue (ProcK, **4**) can be genetically and site-specifically incorporated into a lysine-dependent protein to replace its essential lysine residue, generating an "OFF" state mutant. Biocompatible and efficient palladium catalysts were identified to cleave this Proc-protected lysine residue on intact proteins inside living cells, switching the protein activity back to the "ON" state.

Next, to show that our Pd-triggered lysine depropargylation strategy can effectively modulate the function of an enzyme containing a catalytic lysine residue, we focused on OspF, a bacterial secreted lysine dependent phosphothreonine lyase

that is known to enter host cells and modulate MAPK (Mitogen-activated protein kinase) signaling pathways through a *Shigella* Type III secretion (T3S) system. By blocking the catalytic lysine reside using Proc-Lys, we created a mutant OspF resembling the "proenzyme" form of OspF that relies on intracellular administration of a single-component palladium reagent for its activation. Because this Pd-mediated protein activation strategy was initiated from a completely "OFF" state background, it helped to reveal the detailed mechanism underlying OspF-mediated impairment of Erk and thus MAPK signaling pathway within host cells. This work extended the rapidly expanding toolkit of palladium-mediated intracellular cleavage reactions from small molecules to proteins, which may ignite more interests in exploiting transition metals for manipulation of protein functionalities, particularly in a gain-of-function fashion, under living conditions.

Site-directed mutagenesis has been frequently utilized to change the identity of a given residue, facilitating the structural and functional analysis of the target protein in a site-specific fashion. However, the traditional site-directed mutagenesis technique is irreversible: the mutated residue cannot be converted back to its wild-type residue *in situ*. Both the photocaging and our reported chemical caging strategies, by coupling with the genetic-code expansion technique, can offer a general method to perform reversible site-directed mutagenesis on proteins. For example, a specific lysine residue can be genetically replaced by a photocaged or chemically caged lysine analogue, which represents the mutant form of the protein lacking the specific lysine residue. The subsequent photolysis or transition-metal catalysis may unmask and convert this caged lysine precursor to the free lysine residue, resulting in the regeneration of wild-type proteins. These strategies are particularly useful for reversible manipulation of enzyme's activity. For example, the site-specific replacement of a key lysine residue by Proc-Lys can generate the "OFF" state mutant of a lysine-dependent enzyme. Pd-mediated cleavage and liberation of lysine from ProcK may reproduce wild-type enzyme with the activity switched "ON" (**Fig. 10**). In addition, the chemical uncaging strategy shown on lysine may be transferable to other amino acids that will become a complimentary method to reversible modulate protein

activity when the photo-induced toxicity becomes a concern or when deep penetration in tissue samples is needed.

5.2 Cross-metathesis for reversible chemical modification on proteins.

Cross metathesis reactions, typically catalyzed by transition metals such as Ru, is an emerging biocompatible reaction, but only a few specific substrates have been successfully carried out in aqueous solution. Davis and coworkers reported that allyl sulfides were privileged substrates for site-specific protein modification via cross metathesis.[112] Recently, they further developed a method to generate Se-allyl-selenocysteine (SeaC) on proteins as a reversible protein modification strategy by the sequential usage of cross-metathesis and elimination reactions.[113] A dehydroalanine (Dha) was first produced from a cysteine-containing protein[114] in the presence of 2,5-dibromohexanediamide, which, upon reacting with allyl selenocyanate, generated SeaC on a POI at the site that originally harbors the cystein residue (**Fig 11**, *step 1 and 2*). The SeaC containing protein could then undergo efficient cross metathesis with a variety of allyl-bearing molecules (eg. allyl GlcNAc, N-allyl acetamide) that were not possible for its sulfur counterpart (**Fig 11**, *step 3*). Moreover, by adopting a mild peroxide oxidation (H_2O_2) procedure, the previous cross metathesis-generated "marker" could be fully converted back to Dha (**Fig 11**, *step 4*), which can undertake another round of SeaC generation and cross-metathesis. To demonstrate the applications of this strategy, Davis and coworkers created a cysteine residue at the key epigenetic site 9 on Histone protein H3. The 2,5-dibromohexanediamide-catalyzed conversion of Cys to Dha and the subsequent allyl selenolate addition successfully generated H3 protein containing SeaC at residue 9 (H3-9SeaC). Cross metathesis between H3-9SeaC and N-allyl acetamide olefin led to a complete conversion to generate modified H3 as verified by mass spectrometry. The product was further analyzed by an antibody raised against natural epigenetic

marker N-acetyl lysine at position 9 of H3 (K9Ac), which showed that this antibody successfully recognized the post-translational modification at position 9 as an acetylated lysine (K9Ac). Finally, the K9Ac mimic can be further removed by Cope-type elimination reaction through the generation of labile selenoxide using mild peroxide oxidation that recreate Dha. Taken together, this approach, coined by the authors as a "write-read-erase-rewrite" cycle, demonstrated the impressive ability of organometallic chemistry for reversible appending and removing modification groups on specific residue(s) on target proteins (**Fig. 11**). Although this strategy can only work on purified proteins at this moment, the idea of reversible chemical modification and/or manipulation of proteins inside living cells may become possible in the future.

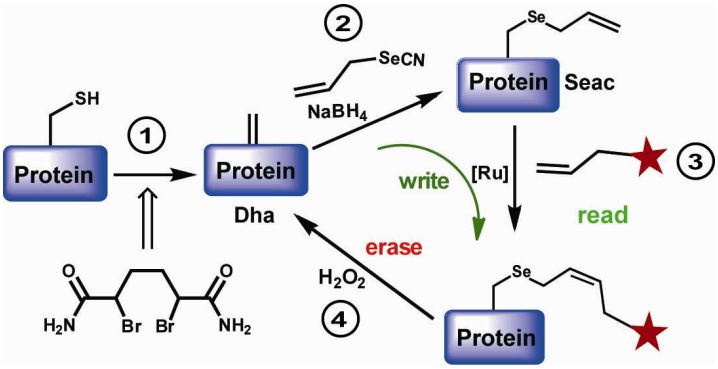

Figure 11. "write-read-erase-rewrite" cycle for protein reversible modifications. The process was accomplished via a sequential combination of ligation and elimination reactions, including conversion of cysteine to Dha (step-1), conjugation with allyl selenocynate (step-2), ruthenium catalyzed cross metathesis (step-3) and Cope-type selenoxide elimination (step-4) that regenerates Dha.

Conclusion and Outlook

In summary, transition metals represent a powerful toolkit for selective construction and destruction of chemical bonds, offering diverse chemical transformations that were hardly feasible without these catalysts. The ligand-assisted CuAAC reactions and palladium-mediated cross-coupling reactions have been recently developed to circumvent the cytotoxicity from the original CuAAC reaction for labeling of intracellular proteins, with more progress being made inside prokaryotic cells. Unlike the traditional chemical reactions carried out within

flasks or chemical reactors, reactions on protein substrates must be operated in mild conditions (e.g. aqueous media, neutral pH, ambient temperature) to preserve the integrity of target proteins. Moreover, the use of protein labeling chemistry within a native cellular context should further consider the biocompatibility of the reaction and the impact of the cellular chemical environment surrounded. To meet these criteria, the repertoire of transition metal catalysts have been increasingly explored for developing biocompatible reactions because of their remarkable catalytic activity and chemoselectivity. However, it has to be pointed out that none of these covalent reactions are truly "bioorthogonal" inside cells. Besides the aforementioned potential side reactions between cyclooctyne and free thiols, terminal alkyne has also been suggested to react with certain cysteine residues on proteins,[115] whereas azide can be converted to amine under highly reduced conditions. Moreover, both copper and palladium are thiol-philic metals and the uncomplexed metal ions may react with thiol-containing small molecule or biomolecules (e.g. GSH, cysteine). However, as long as live cells are tolerant to these exogenous functional groups and reagents, such reactions might be able to proceed without perturbing normal functions of living species. Future efforts should be focused on improving the biocompatibility while enhance the catalytic property of these powerful metal catalysts, particularly within eukaryotic cells.

Beyond bioorthogonal conjugation reactions, transition metal-mediated protein chemistry has been rapidly expanding to other types of transformations under living conditions. In particular, as a key transition metal in advancing modern organic synthesis, palladium's capability in triggering diverse chemical conversions including cleavage reactions have started to be appreciated for biological applications. Our recently reported palladium-mediated chemical uncaging strategy represents a new approach to activate a target protein within its native cellular context. This method, when coupled with the genetic-code expansion strategy, offers a chemically-enabled, reversible mutagenesis method on desired proteins such as enzymes. Meanwhile, reversible protein modifications, coined "write-read-erase-rewrite" cycle, mediated by a sequential combination of ligation and cleavage reactions, offers another elegant

example in which transition metals played more diverse and multifunctional roles in manipulating proteins than without such metal ions. These two emerging strategies permit intact proteins to undergo multiple rounds of alterations *in situ*, which may find broad applications in biomedical research. Embarking on these exciting recent achievements that significantly expanded our bioorthogonal protein chemistry repertoire, more interests will be ignited in exploiting transition metals for manipulation of various protein properties under living conditions. Look ahead, these new reaction types beyond simple bioconjugation will likely expand our view on the definition and applications of bioorthogonal chemistry.

Acknowledgement

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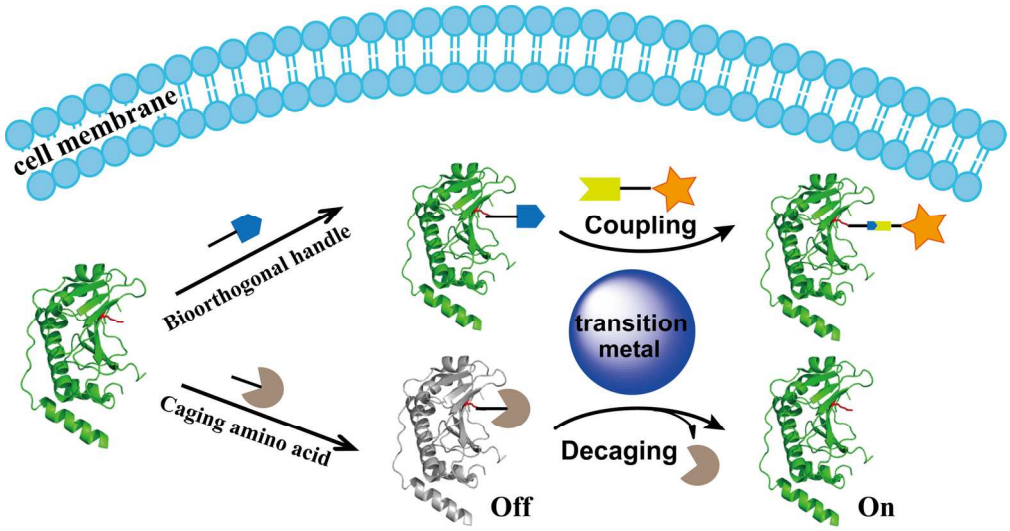
Footnote

† These authors contributed equally to this work.

One sentence for TOC :

Transition metal-mediated chemical conversions have rapidly expanded our intracellular protein chemistry toolbox, which would broaden our view on bioorthogonal chemistry.



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Dr. Peng Chen obtained his BS degree in Chemistry from Peking University in 2002 and Ph.D degree with Prof. Chuan He in Chemistry at The University of Chicago in 2007. After a postdoctoral training with Prof. Peter Schultz at The Scripps Research Institute between 2007 and 2009, he started his independent career as an Investigator in Chemical Biology at Peking University in July 2009. His Lab is interested in developing and applying novel chemistry tools to investigate protein-based interactions and activities in living cells. He received NSFC Distinguished Young Scholar Award in 2012 and China Young Scientists Award in 2013.

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