Catalysis in Protein Bioconjugation

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Abstract: Advancement of catalytic transformations in traditional synthetic organic chemistry have made significant impacts on development of novel bioconjugation technologies. While a wide range of applications have become possible through catalytic protein bioconjugation approaches, there has been a lack of literature collectively reviewing advances of chemical modification of proteins through the lens of catalysis. This review article is focused on design principles and chemical strategies of nonenzymatic catalysis for targeting natural protein substrates by identifying seven catalysis patterns as organizing topics: Electrocatalysis, photocatalysis, metal catalysis, acid catalysis, organocatalysis, supramolecular catalysis, and heterogeneous catalysis. Many literature examples demonstrated possibility of simple translation of small molecule-based catalysis into protein bioconjugation methodologies whereas others demonstrated unique approaches such as dual catalytic systems and polypeptide structure-specific catalysis design. With a series of successful examples, the survey of catalytic approaches for protein bioconjugation also highlighted the remaining challenges and potential future directions of the area of catalytic bioconjugation.

1. Introduction

As the growth of synthetic organic chemistry fields was tremendously enabled by various forms of catalytic principles, modern protein bioconjugation strategies also rely heavily on the power of catalysis for fundamental development and various applications. Catalytic transformations have revolutionized industries of small molecule synthesis over the century. Because there have been growing interests in chemically modified proteins as renewable resources and materials (e.g., batteries,[1] supercapacitors,[2] anti-bacterial materials,[3] and enzymes[4,5]),

catalytic chemical modification of proteins would hold great promise for industrial production of such useful bioconjugates to address the global challenges regarding energy and environments.[6] In addition to the potential improvement of production efficacy of bioconjugates, catalytic platforms can also be leveraged for site-specific modification of proteins for production of therapeutically important agents such as antibody–drug conjugates.[7] Furthermore, a range of chemical tools in biochemistry and chemical biology fields is predicated on catalytic systems enabling target-specific labeling in living systems.[8] Those protein modification applications have been achieved by addressing challenges of protein-targeting catalysis such as aqueous environments, low reaction concentrations (often nM to µM), catalyst deactivation by protein functional groups, and mild reaction conditions (e.g., 37 °C or rt). Despite the substantial development of protein bioconjugation through catalysis, systematic analysis of catalytic bioconjugation has been simply lacking to date, even though numerous reports reviewed the advances of protein bioconjugation either in broader fashions including non-catalytic systems[9–11] or with emphasis on specific catalysis types such as transition-metal catalysis.[12,13]

This review article is focused on impacts of design, selection, and applications of catalysts on protein bioconjugation purposes and categorized into seven types of catalytic transformation mechanisms. The scope of the review article is chemical modification of natural proteins comprised of 20 canonical amino acids, particularly methods without genetic engineering. Methods that can be applicable at a protein level are primarily discussed, and relevant work has been chosen based on arbitrary molecular weight cutoff of 5,000 Da. Importantly, the main focus is on catalytic strategies rather than residue-specific organization found in many existing protein modification review papers.[14–16] Nonetheless, recognizing the practical importance of a list of

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catalytic bioconjugation methods organized by target amino acid residues and other factors, we included an excel file containing lists of the discussed literature as Supporting Information, in which readers can sort and analyze the collection of papers with a parameter of interest. Among the diverse catalytic systems, seven catalytic mechanisms were used for organization of the article (Figure 1) as the catalysis types are serving as common organizing topics of major catalysis-focused journals such as *Nature Catalysis*, *ACS Catalysis*, and *ChemCatChem*. Although, strictly speaking, only catalytic amounts (i.e., less than the amounts of reactants) should be ideally used for a given catalysis system, bioconjugation processes that are mechanistically catalytic even with the necessary use of excess amounts of a catalyst have been included in this present review. Though bioconjugation often indicates bond formation for functionalization purposes, catalytic bond cleavage reactions were also discussed. Because the focal point of the article is nonenzymatic processes, we refer readers to enzymatic bioconjugation-specific reviews.[17,18]
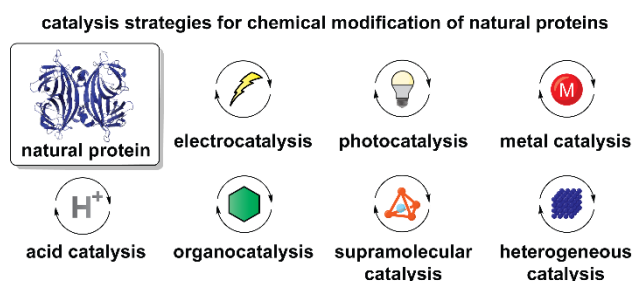
Figure 1. Seven catalysis types for protein bioconjugation discussed in this review article.
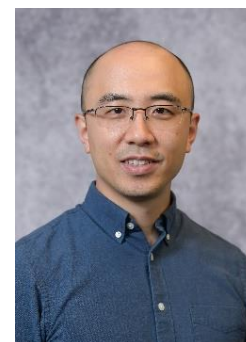
Kounosuke Oisaki was born in Tokushima, Japan, and received his Ph.D. from The University of Tokyo (UTokyo) in 2008 under the direction of Prof. Masakatsu Shibasaki. Then, he moved to the University of California-Los Angeles as a JSPS postdoctral fellow with Professor Omar M. Yaghi. In 2010, he joined Professor Motomu Kanai's group at UTokyo as an assistant professor, then promoted as a lecturer in 2016. He moved to National Institute of Advanced Industrial Science and Technology (AIST) in 2022, now he is working as a team leader. His interest is the development of novel synthetic methodologies focusing on organoradical-based chemoselective reagents/catalysis for and peptide/protein modifications.

Shinichi Sato obtained his Ph.D. from the University of Tokyo in 2011 under the supervision of Prof. Yuichi Hashimoto. He held a position as a Japan Society for the Promotion of Science (JSPS) Research Fellow for one year within the research group led by Prof. Carlos F. Barbas at Scripps Research Institute. Subsequently, he worked as an Assist. Prof. in Prof. Hiroyuki Nakamura's laboratory at both Gakushuin University and Tokyo Institute of Technology. Currently, he holds the position of Assoc. Prof. (Principal Investigator) at the Frontier Research Institute for Interdisciplinary Sciences, Tohoku University.

Seiya Ishizawa was born in Japan. He received his B.S. in Agriculture in 2019 and M.S. in Chemistry in 2021 from Tohoku University. For his bachelor's study, he worked on the total synthesis of a natural compound, and for his master's study, he performed research on oligonucleotide chemistry. He then moved to North Carolina State University in 2021 to pursue a Ph.D. degree in Chemistry with Prof. Ohata where he works on developing new biomolecule modification methods.

Jun Ohata was born and raised in Japan. He received his BSc and MSc from Osaka Prefecture University, where he worked with Prof. Hiroyuki Matsuzaka studying reactive carbon species on diruthenium complexes. He earned his PhD in the Zachary Ball group at Rice University, studying transition metal-catalyzed protein bioconjugation. He conducted his postdoctoral work with Prof. Chirstopher Chang at the University of California–Berkeley as a JSPS postdoctoral fellow, developing chemical probes for the detection of cellular metal ions. He then took up his current position at North Carolina State University as an assistant professor, developing novel strategies for bioconjugation.

Koki Fujimura was born in 1995 in Kagawa, Japan, and received his Ph.D. from Kyoto University in 2024 under the direction of Prof. Kiyosei Takasu. Then, he moved to National Institute of Advanced Industrial Science and Technology and currently works as a JSPS postdoctoral fellow collaborating with Dr. Kounosuke Oisaki. His current research interests are development of new methodology in organic synthesis, especially peptide synthesis and reaction selectivity.

2. Electrocatalysis

In this section, we categorize protein modification mediated by electrocatalysts into two types based on their reaction patterns: (1) protein modification through aromatic electrophilic substitution and (2) protein modification *via* radical addition.

Electrochemical synthesis, known for its low waste, high selectivity, and mild reaction conditions, has recently attracted attention as a clean approach for small molecule conversions.[19–21] Because of these advantages, electrochemical synthesis has been studied as a method for direct late-stage modification of complex compounds.[22] The potentials make it a promising technique for chemical modification of proteins, which often contain numerous reactive functional groups and can be challenging to purify from a reaction mixture with excess labeling reagents.

At a peptide level, electrochemical synthesis offers two methodologies for modification. The first method involves generating an active electron acceptor from a stable precursor *via* electrode oxidation, which then modifies residues in a peptide through electrophilic substitution reactions. The second method directly generates radical species by single-electron oxidation of a specific functional group in a peptide, which are subsequently captured under suitable reaction conditions to produce a modified peptide. In terms of application to protein modification, the first method is currently applicable to proteins or long-chain peptides,[23,24] while the second is limited to peptides with up to 10 residues.[25,26] Therefore, this review focuses only on the first method through electrode oxidation. However, it is worth noting that in this approach, the modified residues themselves may also undergo electrode oxidation as the reaction progresses.
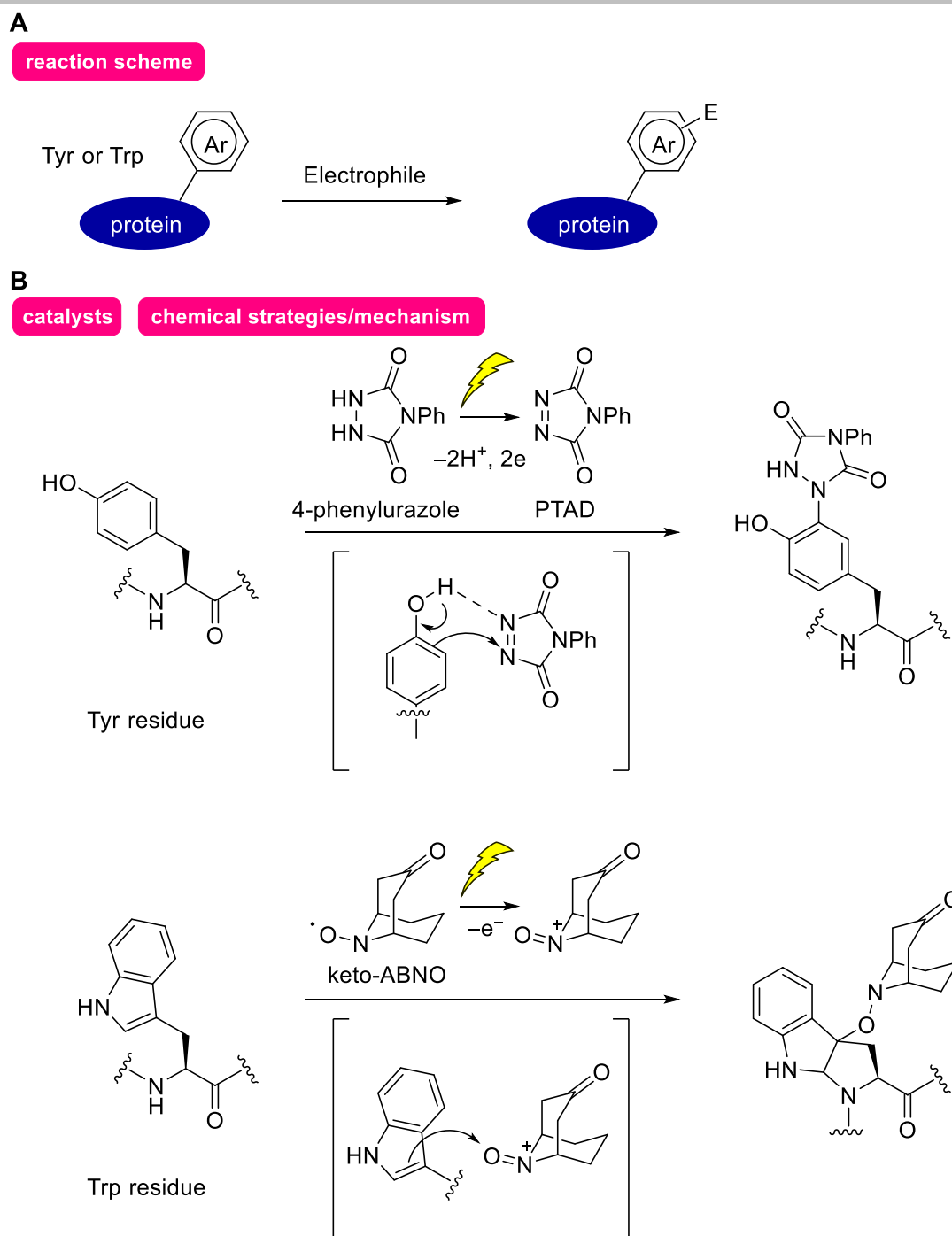
2.1. Protein modification by electrophilic aromatic substitution

In this subsection, we introduce a method for protein modification using electrophilic species generated by electrochemical reactions as reactive anchors (Scheme 1A). By applying an appropriate potential specific to the anchor precursor, active anchor sites are oxidatively generated in the reaction system, while preserving the functional groups of the protein. These electrophilic anchor sites modify proteins *via* electrophilic aromatic substitution reactions at Tyr and Trp residues.

The Tyr-ene reaction of 4-phenyl-3*H*-1,2,4-triazole-3,5(4*H*)-diones (PTAD) and Tyr residues has been extensively studied by Barbas and co-workers*,*[27–29] and is regarded as a promising approach for protein modification. However, the oxidative generation of PTAD from 4-phenylurazole in such systems is not orthogonal to the various functional groups of proteins. Furthermore, competing side reactions, such as hydrolysis of PTAD, restrict its applicability in a broad context. In 2018, Gouin and co-workers reported the first protein modification employing electrochemical techniques.[23] This method exploits the low redox potential of 4-phenylurazole (+0.36 V *vs.* SCE), enabling rapid Tyr-ene reactions while generating active PTAD species *in situ*, without oxidizing the aromatic rings of the protein (>0.45 V, Scheme 1B). Another example comes from Heptinstall and co-workers, who reported modifications of protein Tyr residues *via* iodination or nitration.[30–32] These modifications are achieved through the electrochemical oxidation of KI or $NaNO_2$, which activates the reagents.

Trp residues can also be modified by aromatic electrophilic substitution reactions under appropriate conditions (Scheme 1B).[24] In this approach, Trp residues are selectively modified by oxoammonium anchors generated by the polar oxidation of the stable organoradical (keto-ABNO). The redox potential of keto-ABNO is lowered when it complexes with Trp residues, enabling functional group tolerance and selective modification of Trp residues.

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A

reaction scheme

B

catalysts   chemical strategies/mechanism

Scheme 1. Electrochemical protein modification through electrophilic aromatic substitution (A) General reaction scheme. (B) Catalysis mechanism of Tyr-ene reaction of 4-phenyl-3H-1,2,4-triazole-3,5(4H)-diones (PTAD, top), and Trp modification by oxoammonium anchors (bottom).

2.2.   Protein modification by radical addition

In addition to the electrophilic reagents discussed in the previous subsection, electrochemical methods utilizing radical species as anchors have been explored for protein modification (Scheme 2A). In this approach, proteins are modified by radicals generated through one-electron oxidation of anchor precursors at the electrode surface. Radical reactions have been a powerful tool for protein modification because the approach can proceed efficiently in aqueous environments and exhibit low reactivity with polar functional groups.

The Tyr-ene reaction shown in the previous subsection can also proceed under one-electron transfer conditions when 1-methyl-4-phenylurazole or *N*-methyl phthalic hydrazide is used instead of 4-phenylurazole (Scheme 2B).[33–35] In 2020, Nakamura and co-workers reported that nitrogen-centered radicals generated by electrode oxidation of *N*-methyl phthalic hydrazide can modify Tyr residues on variety of proteins.[34] Similarly, Lei and co-workers reported that nitrogen-centered radicals generated by electrochemical oxidation of phenothiazine are also effective for modifying Tyr residues.[36]

In 2022, Weng and co-workers introduced a modification method targeting Trp residues using azidyl radicals ($N_3\cdot$, Scheme

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2B). These radicals add to indole side chain, enabling the modification of peptides with 20 or more residues.[37] Azidyl radicals are generated electrochemically from $Mn^{II}\text{-}N_3$, accompanied with C=N double bond formation. Weng and co-workers subsequently reported that trifluoromethyl radicals ($CF_3\cdot$) are also applicable to modify Trp residues.[38]

The methods described in the above two subsections are notable for specifically targeting Tyr or Trp residues for protein modification. These residues are appealing targets because of

their low surface exposure, the controlled nature of their modification reactions, and the minimal impact on post-modified protein structure. However, the conversion of phenol and indole side chains typically requires stringent reaction conditions. Electrochemical methods are noteworthy as the strategies enable efficient modification of Tyr and Trp residues under mild conditions, serving as a powerful tool for protein modification.
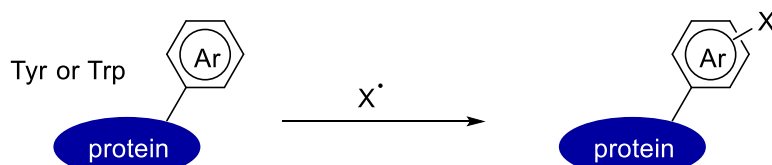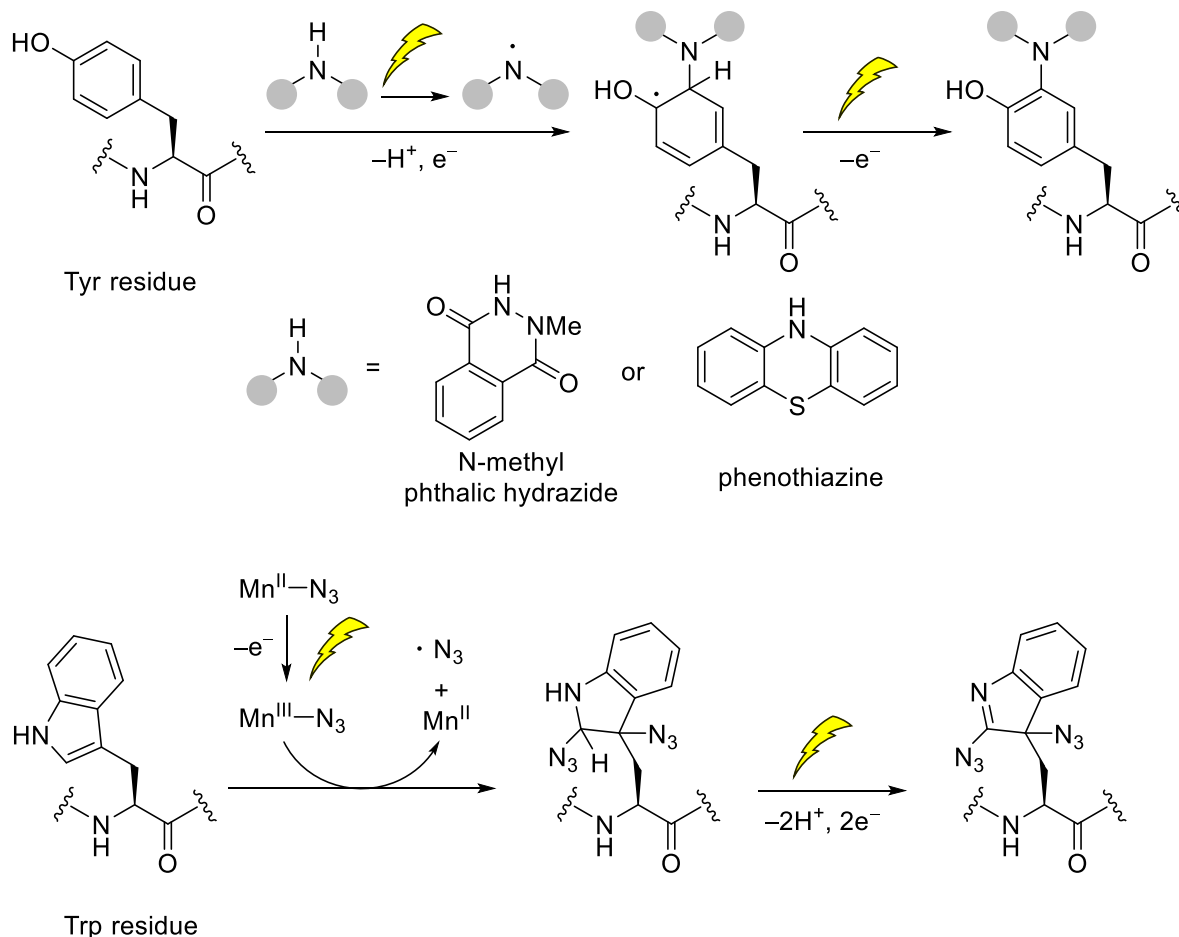

Scheme 2. Electrochemical protein modification through radical addition. (A) General reaction scheme. (B) Catalysis mechanism of Tyr-ene reaction in one-electron transfer conditions (top), and Trp modification by azidyl radicals.

3. Photocatalysis

In this section, we overview four categories of protein modification based on different activation patterns mediated by photocatalysts: (1) oxidative protein modification through electron transfer to a photocatalyst, (2) protein modification activated by the redox

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cycle of a photocatalyst, (3) protein modification via $^1O_2$ generation through energy transfer, and (4) protein modification through the activation of a labeling reagent by energy transfer. Photocatalysis, as a robust strategy for activating small molecules, has emerged at the forefront of organic chemistry, experiencing rapid development throughout the 2010s.[39–41] In these approaches, metal complexes and organic dyes engage in single-electron transfer (SET) reactions or energy transfer reactions with substrates, converting visible light into chemical energy. In traditional chemical reactions, the process generally proceeds in a "thermodynamically downhill" direction, making a reaction drive toward an energetically favorable direction through energy release. In contrast, photocatalytic reactions enable the introduction of light energy from an external source, raising the energy level of reactants to facilitate "thermodynamically uphill" reactions, which are typically more challenging to achieve.[39–41] Recently, an increasing number of studies have focused on protein modification through photocatalysis, reflecting a growing interest in this area. While transformations of biomacromolecules using UV light date back several decades including applications in photoaffinity labeling chemistry,[42] approaches utilizing photocatalysis present the advantage of achieving reaction control with visible light, which is considerably more biocompatible than UV light. Reviews have been published, focusing on various perspectives, such as classification based on structural characteristics of catalysts,[43] the application of photocatalytic chemistry in diverse fields,[44] and photochemistry on a broad range of proteins, including photo-click reactions, nucleic acid modifications, and photo-uncaging.[45] In this section, on the other hand, we will focus specifically on applications of protein modification based on different reaction/catalysis mechanisms.

3.1. Oxidative protein modification through electron transfer to a photocatalyst

This subsection introduces methods for oxidative protein labeling mediated by electron transfer reactions between photocatalysts and substrates (Scheme 3A, 3B). In this mechanism, the activated photocatalyst extracts an electron from the substrate, generating a radical species. Based on the oxidation potential of the photocatalyst, radicals can form on both labeling reagents and tyrosine (Tyr) residues of the protein. Due to high reactivities of these radical species in general, rapid bond formation often follows the excitation process. With the loss of an additional electron and proton, a reaction proceeds—in which, formally, two electrons and two protons are removed—resulting in the formation of either C-C or C-N bonds.

The precise mechanism determining whether the labeling reagent or the Tyr residue on the protein undergoes radicalization, as well as the specific reaction intermediates involved, is still not fully understood and remains open to further investigation. However, in tyrosine modifications using tyramide, the radical-radical recombination pathway has been shown to predominate over other pathways between neutral Tyr residue and radical.[46] Additionally, a report indicated that the reaction can proceed even under conditions where the radical species of the labeling reagent

is generated electrochemically at potentials that do not directly radicalize Tyr.[34]

Several reagents have been reported for Tyr modification, including tyramide, phenylenediamine, N-methylurazole, and phenoxazine, while pyrazole has been shown to label phenylalanine (Phe) residues. In the catalytic mechanism, for instance, Ru catalysts can be photoexcited, and with the presence of oxidants such as oxygen or ammonium persulfate (APS), transition to a Ru(III) state. This Ru(III) species subsequently oxidizes the substrate via SET as it returns to its ground Ru(II) state. The use of Ru-based photocatalysts has long been known for Tyr-Tyr cross-linking reactions.[47,48] More recently, it has become possible to label proteins by mimicking tyrosine residues with tyramide derivatives as labeling reagents. Tyramide conjugated with tags such as biotin (often referred to as "biotin phenol") enables visualization of labeled proteins and proteomics analysis through enrichment using avidin-beads. Additionally, methods have been explored for switching reactions using complexes with quencher molecules on DNA oligomers.[49]

Apart from tyramide, ethylenediamine-type labeling reagents have been utilized for radical modifications of tyrosine residues.[50–54] This labeling reagent is also capable of labeling not only Tyr but also Cys, when free Cys residues are located near the catalyst binding site.[55]

Additionally, N-methylurazole has been developed as a radical-based labeling reagent specifically for labeling tyrosine residues in proximity to Ru complexes.[33,56,57] The ligand structure of these catalysts has been examined from multiple perspectives, including the minimization of nonspecific adsorption to proteins,[53] as well as enhancement of ligand binding with proteins.[57] The importance of such proximity effects is described in the Supramolecular catalysis section as well.

Covalent bond formation between the catalyst and protein has also been reported, with the Ru(TAP)$_2$phen^{2+} complex (TAP = 1,4,5,8-tetraazaphenanthrene; phen = 1,10-phenanthroline) enabling tryptophan (Trp) labeling via a SET reaction.[58]

For photocatalysts such as flavin, acriflavine, and 2,4,6-triphenylpyrylium (TPT), it is postulated that the excited photocatalyst abstracts an electron from the substrate. Flavin, in particular, can accept two electrons and two protons to achieve its reduced H$_2$-Fl state, after which it regenerates its ground state by donating electrons to oxygen. Lumiflavin-based photocatalysts have been applied in selective Tyr modification of proteins using a phenoxazine dialdehyde tag.[59] Flavin-catalyzed Tyr modifications with tyramide have also found applications in analyzing cell-cell interactions by enabling controlled reactivity on cell membrane surfaces.[60,61]

Additionally, acriflavine, with its higher cell membrane permeability compared to organometallic complexes, has been adapted for controlling reactions within cells.[62] TPT, known for its high oxidation potential (+2.55 V vs saturated calomel electrode (SCE)),[63] has even been reported to facilitate modifications of Phe residues, which are typically challenging to activate.[64]
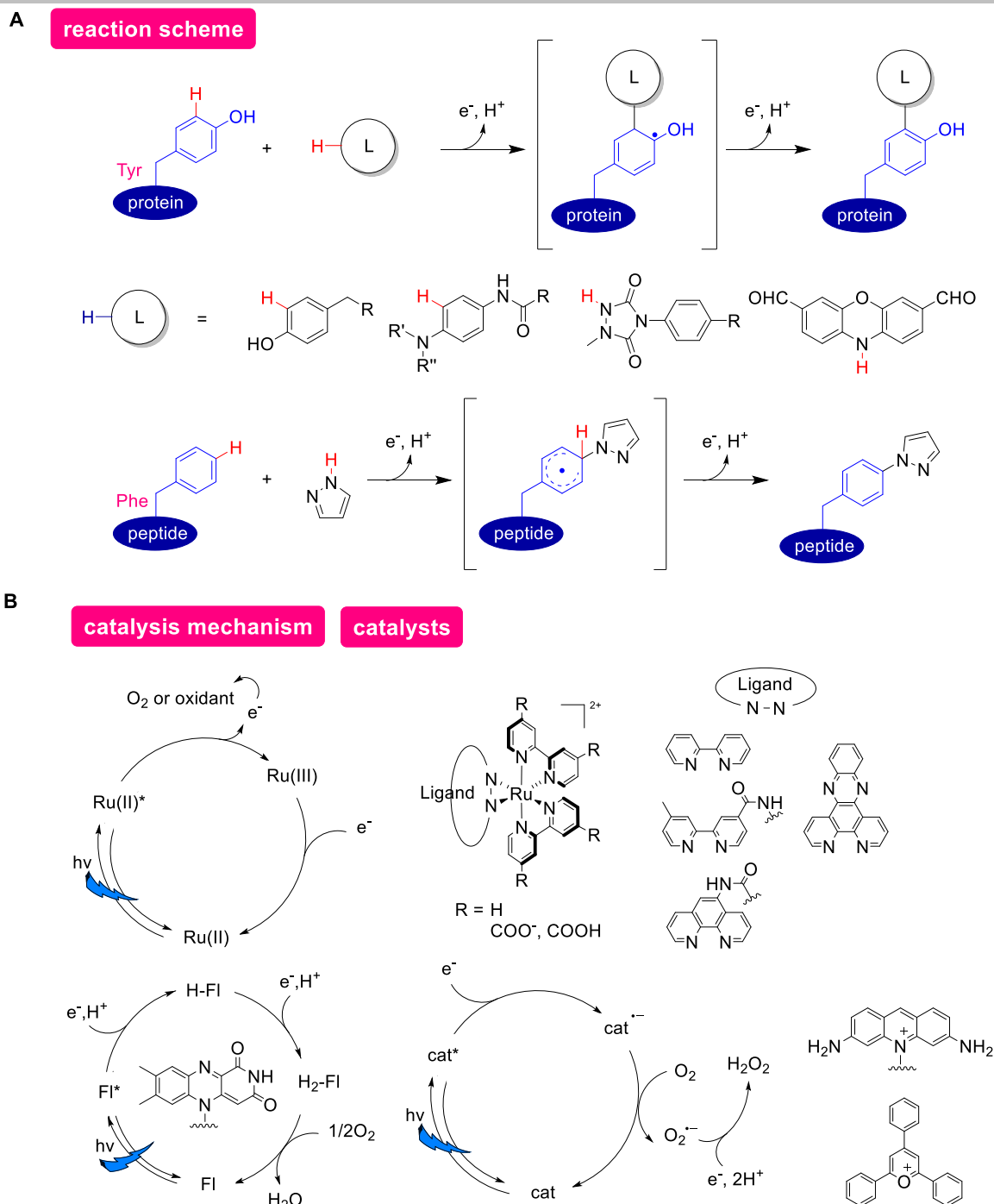
Scheme 3. Oxidative protein modification via photocatalysis. (A) Modification of Tyr and Phe through oxidation mediated by SET, along with the structure of the labeling reagents. (B) Structure of photocatalysts and catalytic cycle.

3.2. Protein modification activated by the redox cycle of a photocatalyst

In protein modification through redox processes by photocatalysts, an electron transfers from a substrate to an excited photocatalyst (Scheme 4A, 4B). This electron transfer results in oxidation of the substrate while the photocatalyst is reduced (cat⁻). Chemical modification of proteins can be achieved at the α-position of the C-terminus, the β-position of Trp residues, and the methyl group of Met residues as details are described in the following paragraphs. In these instances, it was proposed that radicals formed on the protein are captured by Michael acceptors. Namely, the radical intermediates receive an electron from the photocatalyst in its reduced state, forming bonds as the photocatalyst returns to its ground state.

The C-terminus can be preferentially reduced via a single-electron process at a lower potential compared to the carboxylates of aspartic acid (Asp) or glutamic acid (Glu) in proteins ($E_{1/2}^{red}$: ~1.25 V for Asp, Glu; ~0.95 V for C-terminus vs SCE). The carbon radicals generated from CO_2 loss can be captured by Michael acceptors such as diethyl

ethylidenemalonate and 3-methylene-2-norbornanone. Selective modification of the C-terminus using flavin photocatalysts in various peptides and insulin has been documented.[65] Furthermore, for peptide substrates, use of an iridium photocatalyst (Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$, $E_{1/2}^{red}$: 1.21 V vs SCE) alongside a polyaromatic photocatalyst (4CzIPN, $E_{1/2}^{red}$: 1.35 V vs SCE) for C-terminal alkynylation with ethynylbenziodoxolone (EBX) reagents,[66] and conversion of the C-terminus to N,O-acetals for electrophilic activation[67] have been reported. Unique to Trp modification is that labeling the β-position of the Trp side chain using Ir[dF(CF$_3$)ppy]$_2$(dtbbpy)PF$_6$ with Michael acceptor modification has been accomplished.[68] In a report of Met modification, lumiflavin ($E_{1/2}^{red}$ = 1.5 V vs SCE), that is capable of accepting an electron from Met (E_{pa} = 1.36 V vs SCE), was utilized, as reduced lumiflavin (HLF$^{\cdot}$) facilitates proton transfer from the Met radical cation (pKa = ~3.5), acting as a base (pKa of HLF$^{\cdot}$ = 8.5) and catalyzing the transfer of electrons and protons.[69] The extension of these photocatalysts' redox cycles for peptides substrates to protein bioconjugation is based on similar mechanisms, necessitating thorough evaluation of reaction conditions to ensure orthogonality and mitigate side reactions with Michael acceptors and residues such as Cys and Lys.

An exceptional example of photocatalytic protein bioconjugation involves using a quinolinone chromophore-based photocatalyst to accelerate the thiol-ene reaction between Cys and terminal olefins through hydrogen-atom transfer catalysis.[70] Activation of electrophilic species employing SET and combinations of SET and hydrogen atom transfer (HAT) for Cys modification[71] and histidine (His) modification[72] are also noteworthy.
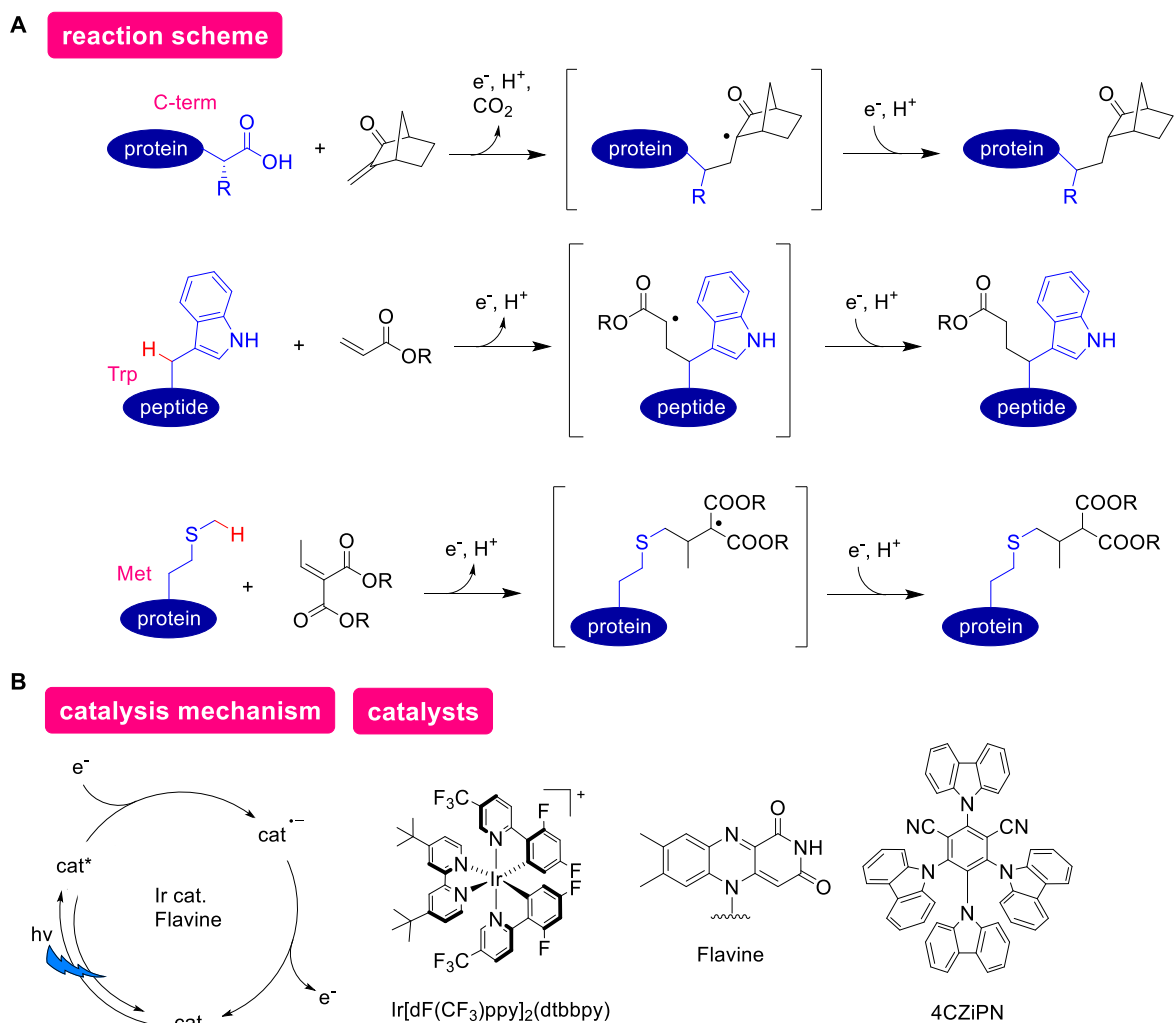
Scheme 4. Protein modification via the photoredox catalysis. (A) Reaction mechanism involving the generation of radicals on the protein structure and their capture by labeling reagents. (B) The redox cycle mediated by photocatalysts and the structure of the photocatalysts.

3.3. Photocatalytic protein modification via $^1O_2$ generation through energy transfer

Distinct from photocatalytic mechanisms that involve a SET, various protein modification techniques have utilized energy transfer mechanisms from an excited catalyst to the substrate.

Numerous photo-responsive molecules known as photosensitizers activate molecular oxygen to generate highly reactive singlet oxygen, $^1O_2$ (Scheme 5A). $^1O_2$, which has a lifespan of only microseconds in water and limited diffusion,[73] leads to oxidation reactions in close proximity to the photosensitizer. Histidine residues are primary targets for $^1O_2$-induced oxidation, undergoing Diels-Alder additions that form reactive endoperoxide intermediates on the imidazole rings.

Mechanistic studies have shown that these reactions do not proceed through stepwise oxidation and nucleophilic addition (Scheme 5B),[74,75] indicating that the formed reactive species are electrophilic. There was a demand for nucleophiles that can efficiently capture these active species, as such nucleophiles enable various tagging applications. According to a few studies comparing different nucleophiles, 3-ethynylaniline demonstrated higher reactivity compared to other anilines, amines, and phenylhydrazines.[75] Additionally, 1-methyl-4-arylurazole (MAUra), with a pKa of 4.7,[76] predominantly exists in its anionic (N^-) form at neutral pH, enhancing its nucleophilicity and thereby facilitating the efficient capture of oxidized histidine.[74]

This strategy for protein modification, known as proximity labeling (PL), exploits the proximity-dependent nature of photocatalysis and the brief lifespan of $^1O_2$. By generating $^1O_2$ and capturing oxidative intermediates nucleophilically (Scheme 5C), it facilitates a wide range of applications. These include identifying RNA-binding proteins,[77,78] site-selective modification of antibodies,[74,79] controlling reactivity within cells for subcellular proteomic mapping,[80,81] analyzing histidine on aggregated proteins after catalytic photo-oxygenation,[82] examining metal-binding histidines,[75] controlling surface reactions for cell-cell interaction studies,[83,84] and interactome analyses in live mouse brains.[85]

While this review primarily focuses on protein bioconjugation, it is notable that the concept of using photocatalysts to generate reactive oxygen species, thereby oxidizing and degrading proteins, has been well-established in techniques such as chromophore/fluorophore-assisted laser inactivation (CALI/FALI).[86–88]
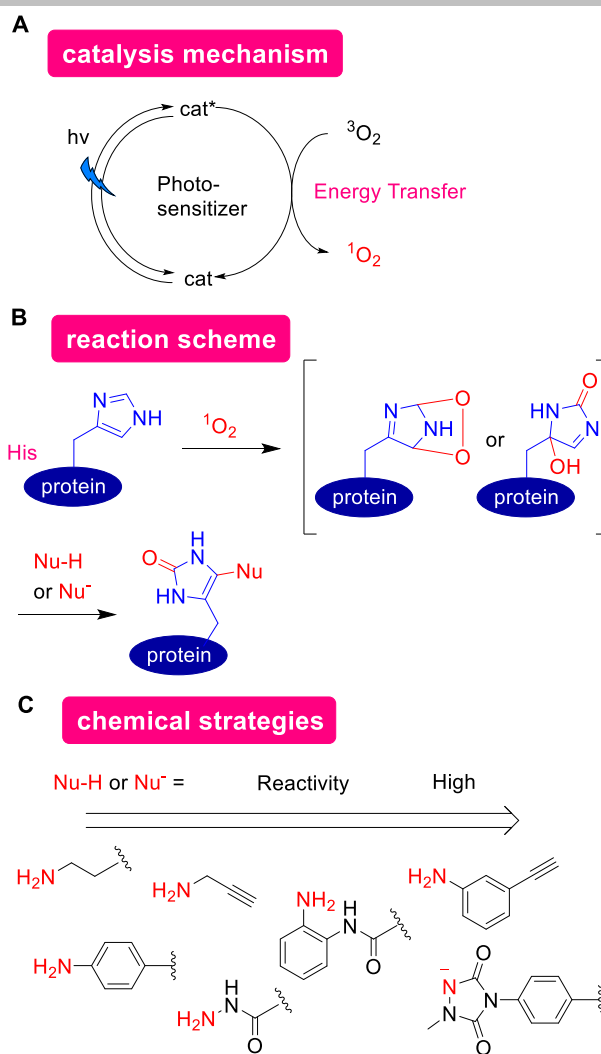
Scheme 5. Protein modification utilizing $^1O_2$ generation from a photocatalyst. (A) Generation of $^1O_2$ through energy transfer reactions between an excited photosensitizer and oxygen molecules. (B) A technique involving the capture of electrophilic intermediates produced by Diels-Alder reactions between His residues and $^1O_2$ using nucleophiles. (C) The structure and reactivity of the nucleophiles used in this method.

3.4. Protein modification through the photoactivation of a labeling reagent by energy transfer.

Developments have also been made in methods that transfer energy and activate labeling reagents (Scheme 6A). Such activation could proceed through Dexter energy transfer between an excited Ir-photocatalyst and a diazirine-based labeling reagent, for instance (Scheme 6B, 6C). This activation of diazirine in the proximity of the catalyst generates carbene, a highly reactive chemical species with a half-life of 2 ns.[89] The photoactivation allows for precise control of protein modification reactions within a tightly restricted area less than 4 nm around the catalyst.[90] The first-generation Ir photocatalyst based on Ir[dF(CF$_3$)ppy]$_2$(dtbbpy) (Ir-G1 cat) not only produces carbene but also activates arylazide, generating nitrene.[91] This catalytic activity has been applied to analyze protein-protein interactions (PPI) on cell membrane surfaces,[90] ligand binding site mapping,[92] control labeling radius by altering labeling reagents,[91] study binding proteins of sialylated glycoproteins,[93] and dynamic analysis of phagocytic surfaces.[94] Additionally, the second-generation Ir photocatalyst (Ir-G2 cat),

which addresses cell membrane permeability issues of the first generation catalysts, has been employed for small molecular compound target identification,[95] analysis of PPI in chromatin proteins,[96] and application to stress granule components in cells.[97]

Activation of arylazide has been further explored using organic dyes such as Acridine Orange, fuorescein, rhodamine 123,[98] a red light-activated osmium photocatalyst,[99] and Sn[IV] chlorin e6 catalyst.[100] These catalysts function through a SET mechanism rather than energy transfer, involving the reduction of arylazide in the presence of NADH through a stepwise reduction–dissociation–oxidation pathway.

As an alternative method, photocatalytic conversion of arylazide to aniline has been utilized in photo-uncaging techniques. This approach also produces *o*-thioquinone methide, an electrophilic species used for protein modification and subcellular proteomic mapping.[101]
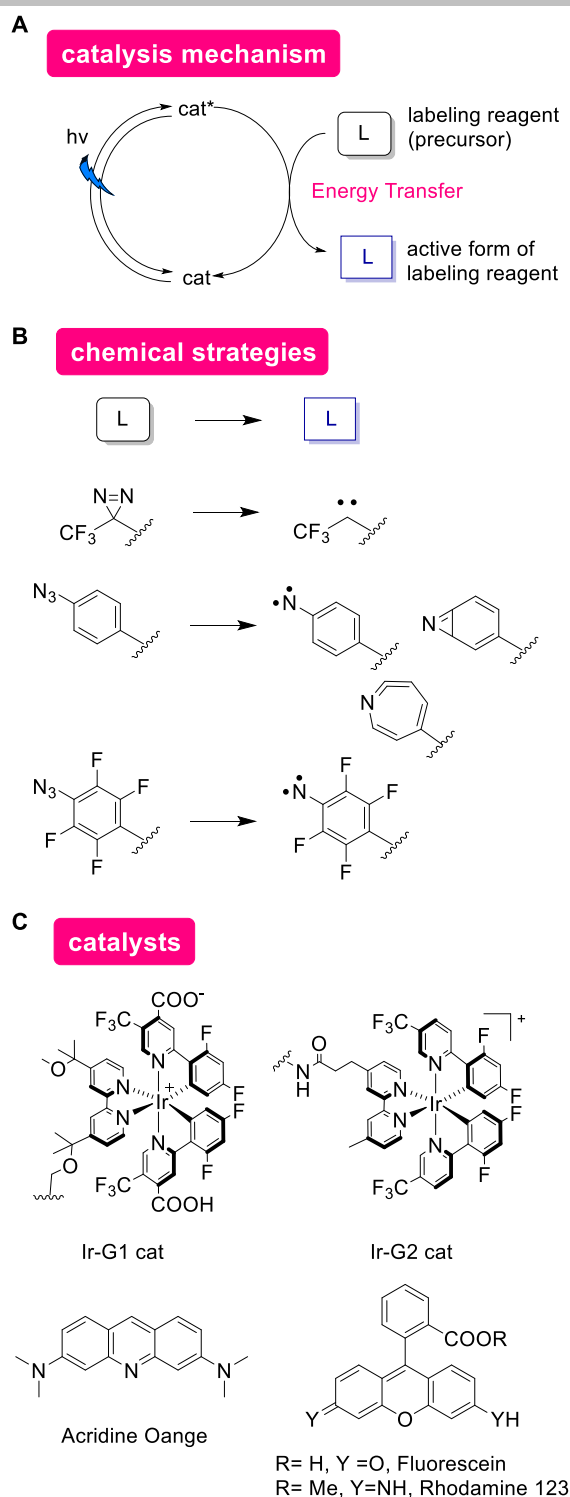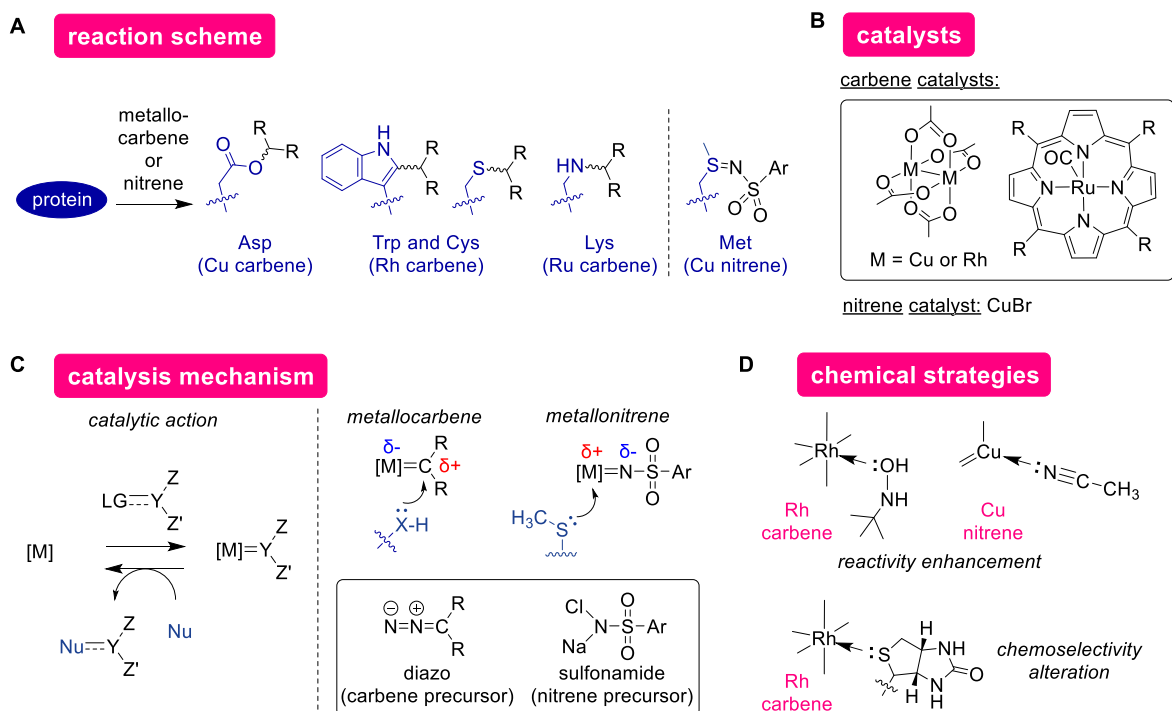

Scheme 6. Protein modification utilizing energy transfer from a photocatalyst. (A) Activation of modifiers through energy transfer from an excited photocatalyst. (B) Reactive species that can be generated by this method, capable of labeling various amino acid residues. (C) The structure of the photocatalysts used in this method.

4. Metal catalysis

Metal-catalyzed protein modification has been enabled through a series of chemical strategies such as bioorthogonal chemistry and design/utilization of protein-compatible transition-metal complexes. A number of metal-mediated protein modification were achieved by use of noncanonical amino acids. For instance, copper-catalyzed azide-alkyne cycloaddition—a quintessential "click" reaction— has been widely used by introducing an azide or alkyne handle onto proteins through methods such as chemical modification and genetic/metabolic incorporation.[102,103] Similarly, instillation of arylboronic acid or aryliodide allows for Suzuki-Miyaura coupling with a palladium catalyst.[104–106] Olefin metathesis with a Grubbs-type ruthenium complex is also possible by introduction of noncanonical alkene groups with a thioether moiety.[107–109] A large portion of metal-mediated modification of canonical amino acid residues, on the other hand, is often a non-catalytic system. Even for catalytic reactions of natural proteins, an excess amount of metal catalysts is necessary likely because of interaction of proteins with metal salts and because of challenges of catalysis in aqueous media. Indeed, there are many catalytic systems that uses organic solvents that are typically not compatible with protein substrates (but peptides).[110,111] Examples described below tackled the challenges of protein modification in aqueous solutions by modulation of reactivity of a metal catalyst such as use of a coordinatively saturated complex to avoid undesired interactions with proteins. As there have been multiple review articles about metal-based protein bioconjugation in the past,[112–115] this section will be focused on catalytic aspects of the processes. The metal catalysis leveraged by their Lewis acidic nature is discussed in the Acid catalysis section.

4.1. Metallocarbene/metallonitrene

Metallocarbene- and metallonitrene-based catalysis has been widely explored for chemical modification of natural proteins (Scheme 7A, 7B, 7C). The carbene and nitrene chemistry functions with the nucleophilic reactivity of amino acid side chains such as indole (Trp) and amine (Lys) through generation of electrophilic metal species from stable precursor molecules (e.g., diazo and sulfonamide compounds). Copper,[116,117] rhodium,[118] and ruthenium[119] complexes have been shown to be useful for targeting various amino acid residues through metallocarbene intermediates (Scheme 7B). The majority of the metallocarbene-based strategies relied on discrete complexes such as a paddle-wheel rhodium complex and ruthenium porphyrin complex, perhaps increasing the life time of the catalysts in aqueous solution in the presence of the nucleophilic biomolecules. Those metal complexes exhibits electrophilic nature, and the reactivity of the carbene complex can be enhanced through coordination of a buffer component to the metal center (Scheme 7D).[120] Such a ligand binding can also cause the alteration of chemoselectivity of dirhodium carbene reactivity toward tryptophan to cysteine by use of a reagent bearing thioether group (i.e., biotin group).[121] The rhodium carbenoid system can be coupled with a proximity-driven strategy to target many amino acid residues other than tryptophan (see the Supramolecular catalysis section).[122] More recently, copper nitrene complex was shown to act as a methionine-selective protein modification method, where copper bromide salt and sulfonamide are the precursors of the nitrene complex.[123] The unique chemoselectivity of the nitrene chemistry was ascribed to the thioether reactivity to the electrophilic metal center bound with acetonitrile ligands. As such, there has been a variety of carbene- and nitrene-based metal catalysis for protein modification since the initial report of the tryptophan-selective carbenoid method as one of the early examples of the modern protein bioconjugation study.[118] In addition to the carbene and nitrene catalysis, a copper-catalyzed azide transfer reaction using sulfonyl azide to alkyl amine groups of proteins should be mentioned here, as the transfer mechanism has similarity to the diazo reagent preparation.[124]

A — reaction scheme

Asp (Cu carbene) · Trp and Cys (Rh carbene) · Lys (Ru carbene) · Met (Cu nitrene)

B — catalysts

carbene catalysts: M = Cu or Rh

nitrene catalyst: CuBr

C — catalysis mechanism

catalytic action

metallocarbene · metallonitrene

diazo (carbene precursor) · sulfonamide (nitrene precursor)

D — chemical strategies

Rh carbene · Cu nitrene

reactivity enhancement

Rh carbene — chemoselectivity alteration

Scheme 7. Carbene and nitrene catalysis by copper, rhodium, and ruthenium complexes. (A) General reaction scheme. (B) Chemical structures of catalysts. (C) General depiction of mechanism of actions of catalysts. (D) Coordination of an additional ligand to a metal center as strategies to increase catalytic activity or alter chemoselectivity

4.2. Cross coupling

Cross-coupling reactions are another class of metal catalysis that has been one of the main focuses in the protein modification field (Scheme 8A, 8B). As various palladium cross-coupling methods emerged in the realm of synthetic organic chemistry during the past decades,[125–127] the bioconjugation field has also been extensively examining their capabilities and utilities. The fundamental catalytic actions and reaction mechanisms of such cross-coupling bioconjugation approaches follows the same principle as that of the small molecule chemistry (e.g., oxidative addition, transmetallation, and reductive elimination). Often, electrophilic nature of a metal center after oxidative addition has been leveraged for both catalytic and noncatalytic protein bioconjugation strategies.[128] A tyrosine-selective palladium-catalyzed approach (Tsuji-Trost coupling) is one of the earliest examples among the palladium-based methods, driven by deprotonation of the phenol group making it a favorable nucleophile at high pH over other amino acid side chains.[129] Another catalysis example is active-site cysteine selective through coordination of an auxiliary ligand to the palladium

center.[130] Other metal sources can be utilized for cross-coupling reactions of proteins including copper catalysis with use of boronate compounds as transmetallation reagents that modifies the amide backbone N-H group (Chan-Lam coupling).[131] The original report by Ball and co-workers made use of a copper binding motif with a histidine residue—that is akin to known metal-binding peptide sequence called amino terminal copper and nickel (ATCUN) motif— to activate the backbone N-H and to facilitate the reductive elimination between the amide and boronate-derived group.[132] Recently, use of a different solvent[133] and different amino acid binding patterns[134] have been shown to achieve similar chemistry without aids of the histidine residues as well. Gold would be another metal source that allows for cross-coupling reactions on proteins targeting a tryptophan residue in aqueous acetonitrile solution.[135] While this specific gold chemistry is the sole example of cross-coupling-based gold catalysis, several non-catalytic Au bioconjugation approaches have been reported to date,[136,137] and together with its relatively lower toxicity compared to other transition metals,[138,139] development of gold catalysis may be merely in a nascent state for potential growth.
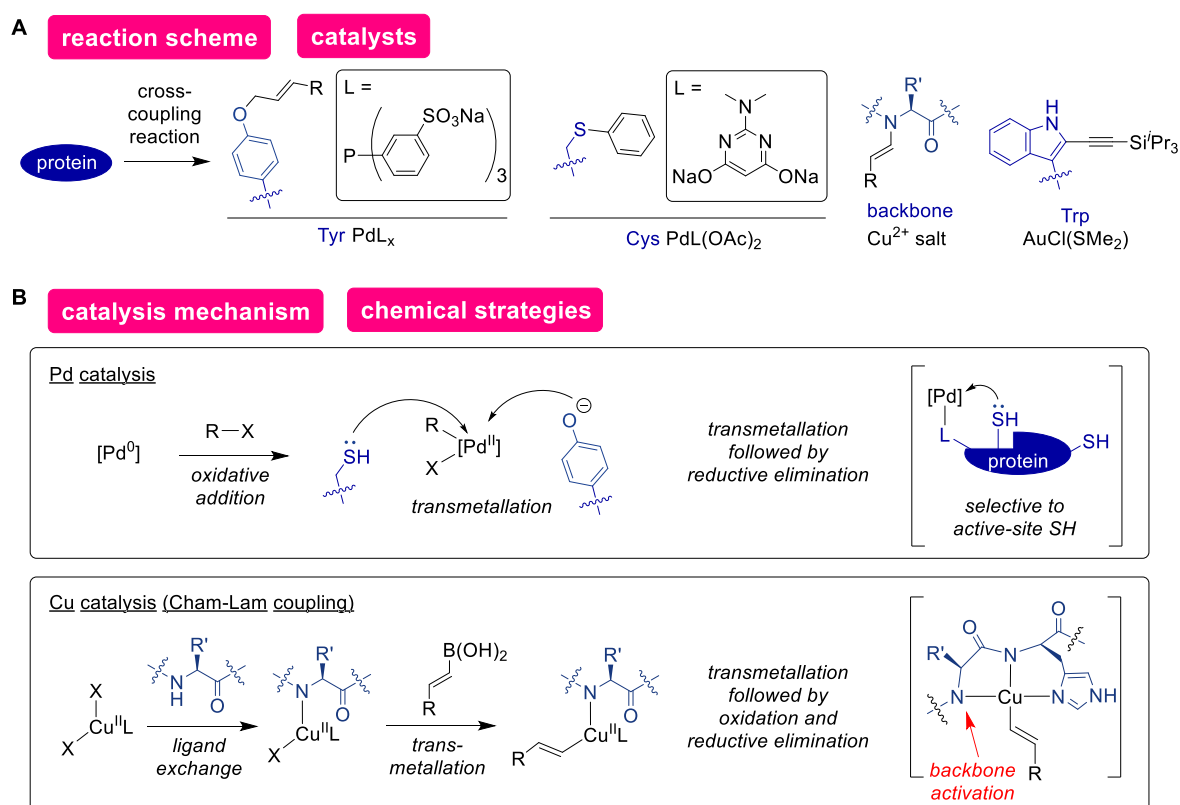
Scheme 8. Metal-catalyzed cross-cross coupling reactions. (A) Chemical structures of the reaction products on modified amino acid residues, catalysts, and ligands. (B) Mechanism of actions for the palladium and copper catalysis.

4.3. Metal hydride reduction

While redox processes are often utilized for protein modification processes, iridium hydride-based catalytic reductive alkylation

remains the sole example of the catalytic reduction for protein bioconjugation purposes in this metal catalysis section (Scheme 9A, 9B).[140] Analogous to the traditional protein modification strategy using aldehyde and cyanoborohydride reagents,[141] the iridium-based hydride reduction would occur through the Schiff

base formation from the protein amines followed by the reduction of the Schiff base to amine. The catalytic system reported by Francis and co-workers utilized activation of a pro-catalyst, water-soluble iridium Cp* complex through reduction with sodium formate (Scheme 9B). Electron-rich bipyridine ligands were found to be particularly effective, which perhaps is an indication of importance of nucleophilicity of the metal hydride species. While this iridium catalysis has been used in other reports, there has not been other metal-catalyzed reductive approaches developed for protein bioconjugation. The lack of the development may be due to the challenges of retention of reduction-sensitive S-S bonds in proteins, although mild and/or bulky reductants such as ascorbate[142,143] and triarylphosphine[144] have been successfully utilized in the protein labeling strategies. Reduction-based metal catalysis may grow dramatically for protein modification fields, as reductive metal catalysis (e.g., metal hydride chemistry) has been useful in many chemical biology applications.[145,146]
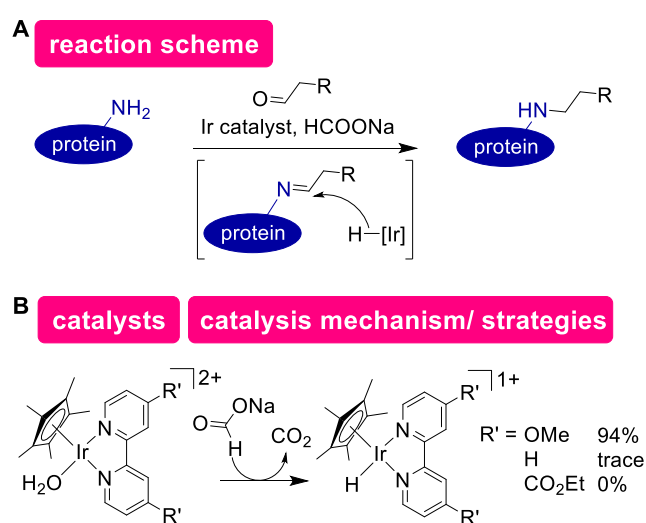

Scheme 9. Iridium-catalyzed reductive alkylation/amination. (A) General reaction scheme. (B) The structure of the procatalyst (left) and active catalyst after reduction with sodium formate (right).

4.4. Oxidative coupling through metal catalysis

Catalytic oxidation processes can be applied for protein modification through activation of amino acid side chains and labeling reagents (Scheme 10A, 10B). As oxidation of proteins is one of the fundamental processes in living systems, there is a range of oxidative reactions used for protein modification in non-catalytic manners.[147] The two major approaches for oxidative catalysis for protein modification occurs through activation of either amino acid side chains (e.g., thiyl radical generation) or labeling reagents (e.g., diazene generation). An example includes alkenylation of a cysteine residue by a gold- and silver-mediated system using allene-based labeling reagents.[148] The gold catalyst was proposed to be useful for not only the single electron oxidation of cysteine, but also activation of allene labeling reagent as Lewis acid (Scheme 10C). The necessity of silver triflate additive may limit the utility of this catalysis, as a silver salt is known to induce precipitation of proteins (e.g., common staining protocols for protein gel known as silver staining).[149]

An oxidation process can be utilized for catalytic activation of labeling reagents, as the hemin-catalyzed tyrosine modification takes advantage of such a mechanism.[150] Inspired by biological

oxidation processes of luminol in firefly chemiluminescence,[151,152] this approach proceeds by formation of tyrosine-reactive diazene reagent through oxidation of N-N bond to N=N bond. A previous approach of tyrosine-selective modification by Barbas and co-workers necessitated preparation of an unstable diazene reagent prior to protein modification processes,[27] and this catalytic oxidation strategy omits the technical challenges through in situ generation of the active species. It is interesting that the most effective reagent proved to possess *N*-methyl group that should not be able to form neutral N=N bond species as proposed (Scheme 10C). The high activity of the *N*-methyl reagent may be indicative of potential involvement of single-electron oxidation by the iron complex.[35]
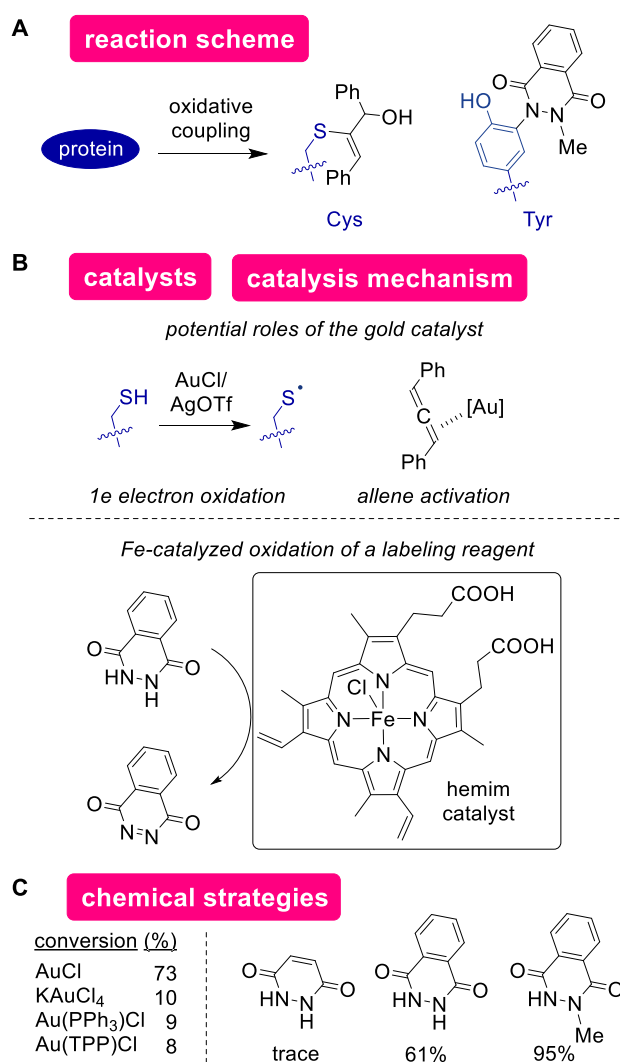
Scheme 10. Metal-catalyzed oxidative coupling. (A) General reaction scheme. (B) Depiction of reaction promotion through oxidation of amino acid (top) or labeling reagent (bottom). (C) Effects of catalyst types and labeling reagents on the modification efficiency.
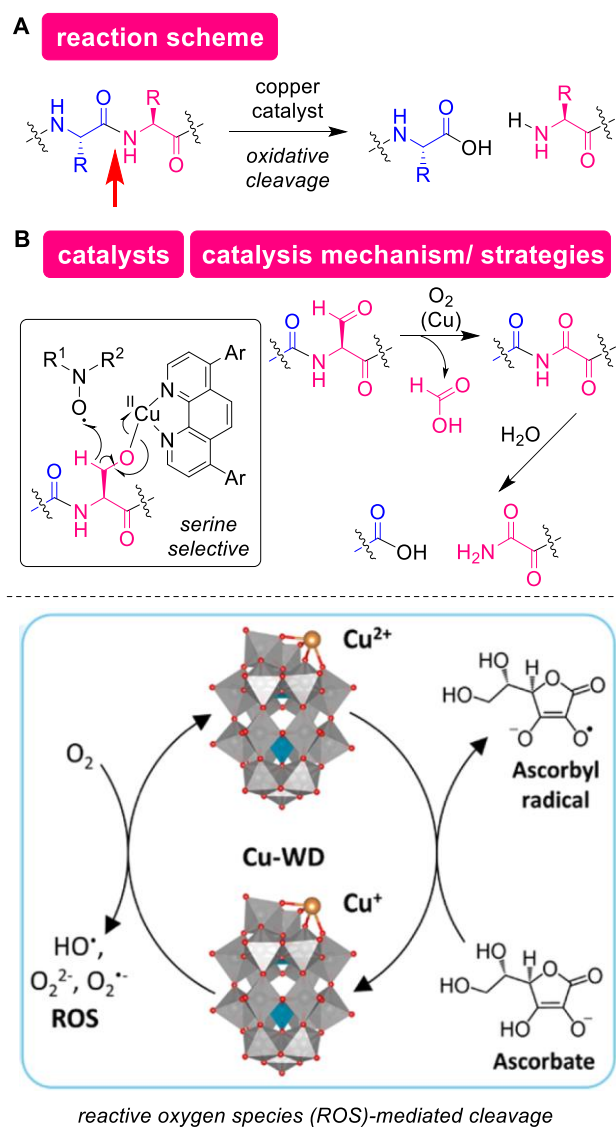
4.5. Catalytic oxidative cleavage

Oxidative cleavage of the peptide backbone can be induced through metal catalysis, mimicking enzymatic processes in natural systems (Scheme 11A). Reactive oxygen species (ROS) is important biological species both for physiological and pathological conditions, where protein oxidation play pivotal

13

roles.[153,154] Through sophisticated design of a catalytic system with judicious choice of reaction components (catalysts, ligands, and oxidants), Oisaki, Kanai, and co-workers developed copper-mediated backbone cleavage, selectively at serine residues (Scheme 11B).[155] Single-electron oxidation of the primary alkylalcohol of serine through copper(II)-phenathroline complex and *N*-oxide reagent initiates the catalysis, and subsequent oxidation of the generated aldehyde group produces a hydrolytically unstable imide intermediate that eventually undergoes the bond cleavage. Though minor reactions at threonine (secondary alkylalcohol) would occur, the serine-selective cleavage was achieved even for a small protein ubiquitin through this catalytic system. Another oxidative cleavage strategy is by a copper cluster that can generate ROS species with ascorbic acid to cause site-specific cleavage of lysozyme an enzyme (Scheme 11B).[156] The three-dimensional structure of the copper cluster was attributed to the observed site-specific cleavage of the enzyme through binding interaction between the cluster and enzyme. As those reports showed a single example of a protein substrate for the catalytic cleavage, the future directions of the field are likely to expand the generality and scope of the methods to achieve enzyme-like catalysis.

reactive oxygen species (ROS)-mediated cleavage

Scheme 11. Copper-catalyzed oxidative cleavage of protein backbone. (A) General reaction scheme. The cleavage site is highlighted with a red arrow. (B)

Serine-selective cleavage by a copper complex (top) and site-selective cleavage of lysozyme through reactive oxygen species (ROS) generation with a copper- and tungsten-based cluster (Cu-WD). The imide intermediate of the serine-selective cleavage can be hydrolyzed at both imide C=O groups (blue and pink), but only one of the two possible hydrolysis products is shown for the sake of simplicity. The image of ROS-mediated cleavage was reprinted with permission.[157] Copyright 2023, American Chemical Society (https://pubs.acs.org/doi/10.1021/jacsau.3c00011).

5. Acid catalysis

As acid-catalyzed processes are ubiquitous in enzyme active sites, numerous protein bioconjugation approaches have been also leveraged by a range of acid catalysts. In natural systems, enzymatic catalysis often depends on acid-mediated activation of weak electrophiles in proteins including proteolysis of amide backbones through interaction of acids to the carbonyl groups.[158] While such enzymatic catalysis could often be substrate-specific processes, acid-catalyzed chemical modification of proteins can offer broader substrate scope with a potentially unique reactivity and selectivity paradigm. One of the traditional approaches for acid-catalyzed protein modification is to employ strong Brønsted or Lewis acid (e.g., zirconium (IV) chloride-derived acid and perchloric acid) to enable reactions of weak electrophiles such as amides and carboxylic acids,[159–162] although such harsh conditions may not be compatible with many protein substrates. More recently, various chemical strategies (e.g., sophisticated ligand design, proximity-accelerated catalysis through reversible covalent bond formation, and nonaqueous systems) have been devised to overcome the challenges, as described in the following sections. It is noteworthy that the development of many of those acid-catalyzed protein bioconjugation methods has been driven by knowledge of synthetic organic chemistry including Lewis acid strengths,[163] metal affinity,[164–166] and unique solvent properties.[167] This section discuss catalysis by both Brønsted acid and Lewis acid including metal Lewis acid.
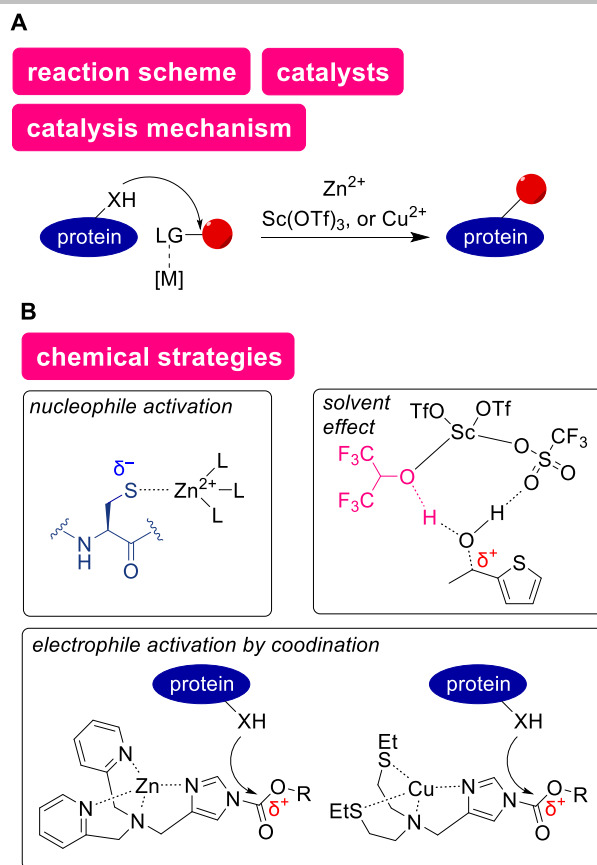
5.1. Acid-catalyzed substitution reaction

A zinc salt was employed as Lewis acid for lipidation of cysteine through the catalytic activation of both nucleophiles and electrophiles (Scheme 12A, 12B).[168] *S*-lipidation is a naturally occurring post-translational modification that is relevant to various cell signaling events including synaptic transmission and GPCR protein signaling.[169] In the report by Fairlie and co-workers achieving a chemical way for *S*-lipidation, zinc ions played catalytic roles in the S_N2 reaction between thiol groups on cysteine residues and alkyl halides that contain fatty acid moieties. The zinc catalyst was suggested to have dual functions: increase of nucleophilicity of the thiol group and increase of electrophilicity of the alkyl halide. In other words, the catalyst would not only interact with the halide leaving group to enhance the electrophilicity of the alkyl halide, the nucleophile of cysteine residue can also be activated by the catalyst interaction lowering pKa of SH to thiolate ion. This design was perhaps inspired by a similar phenomenon found in natural zinc-containing enzymes (e.g. zinc-dependent transferases[12] and zinc finger proteins[13]).[170,171] As the catalysis proceeds with other divalent ions such as Ni^{2+} and Cd^{2+}, the affinity of the metal ions to the thiol group might be playing key roles in the system.[164–166]

Hexafluoroisopropanol (HFIP) can facilitate electrophilic aromatic substitution of tryptophan through activation of thiophene-ethanol labeling reagent by a metal, Lewis acidic

catalyst (Scheme 12A, 12B).[172] HFIP, like other fluoroalcohols such as trifluoroethanol (TFE), is known to induce α-helical structures of polypeptide,[173–175] and protein substrates may not always tolerate such conditions.[176,177] The recent work by Ohata and co-workers demonstrated that increased protein compatibility of HFIP by ionic liquid additives.[172] For instance, an anti-HER2 antibody, trastuzumab was shown to lose its selective antigen-binding activity after treatment in HFIP, but the activity and selectivity was retained when the antibody was treated with HFIP containing ionic liquids. Because HFIP and other fluoroalcohols are increasingly used for biomolecule modification recently,[111] the potential compatibility of the solvents with proteins motivates their application for catalytic protein bioconjugation. The HFIP-based bioconjugation work by Ohata and co-workers took advantage of Lewis-acid-catalyzed dehydrative alkylation reaction (Friedel-Crafts type process)[178] of tryptophan residues.[172] Catalytic actions of the Lewis acid such as scandium ions were studied using density functional theory (DFT) calculations, suggesting that the acidity of HFIP is increased through the coordination of HFIP to scandium. The increased acidity of HFIP by the coordination was shown to cause protonation and liberation of the OH group of the labeling reagent (thiophene-ethanol) as shown in Scheme 12B. In other words, the preliminary computational study indicated HFIP as a proton donor for the dehydration process, and consistent with the observation, the same group also demonstrated use of Brønsted acid to catalyze the process as well.[179] Although aqueous media have been often considered a requirement for useful protein bioconjugation methods,[180] this example in addition to a carboxylic acid-based serine modification method described below (the acid-catalyzed hydrolysis and alcoholysis section) may suggest practical usefulness of nonaqueous approaches.

Acyl imidazole derivatives have been applied as activity-based sensing probes to detect intracellular metal ions by leveraging catalytic activity of the ions (Scheme 12A, 12B). Metal ions serve as chemical signals in cells,[181,182] and chemical probes that detect these ions are useful for understanding their biological functions.[183] In particular, reactivity-based sensing approaches offer advantages that traditional reversible binding sensors do not possess.[184] An acyl transfer-based approach originally developed by Hamachi and co-workers relies on coordination of tetradentate acyl imidazole derivatives to metal ions (Lewis acid), which makes the carbonyl group more electrophilic to trigger a substitution reaction of the complex with nucleophiles on intracellular proteins.[185] The original report developed a zinc-selective probe with dipicolylamine as the zinc-binding site. Although the binding affinity of the probe to Zn^{2+} was shown to be quite strong (0.7 nM) for this particular system, the approach could function as catalysis as long as there is a certain degree of dissociation process. Later, Chang and co-workers designed a copper-selective sensor through the incorporation of thioethers enabling detection of labile brain copper.[186] The same group recently applied the catalytic system to multiplex imaging of Cu(I) and Cu(II) by stimulated Raman scattering using isotopically labeled nitrile vibration tags.[187] The development of the zinc- and copper-targeting acyl imidazole sensors indicates that ligand design would be able to modulate the binding selectivity to produce new catalytic chemical probes toward different intracellular metal ions.

Scheme 12. Acid-catalyzed substitution reactions. (A) Schematic illustrations of metal acid-catalyzed substitution reaction. XH: Nucleophilic side chains of amino acids such as cysteine and lysine. LG: Leaving groups such as iodide, water, and imidazole derivatives. (B) Chemical strategies that enhance the catalytic substitution reactions. Top left: Activation of nucleophile by thiolate formation. Top right: Acid-catalyzed Friedel-Crafts reaction promoted by hexafluoroisopropanol (depicted in magenta). Bottom: Enhancement of electrophile reactivity through coordination to metal ions.
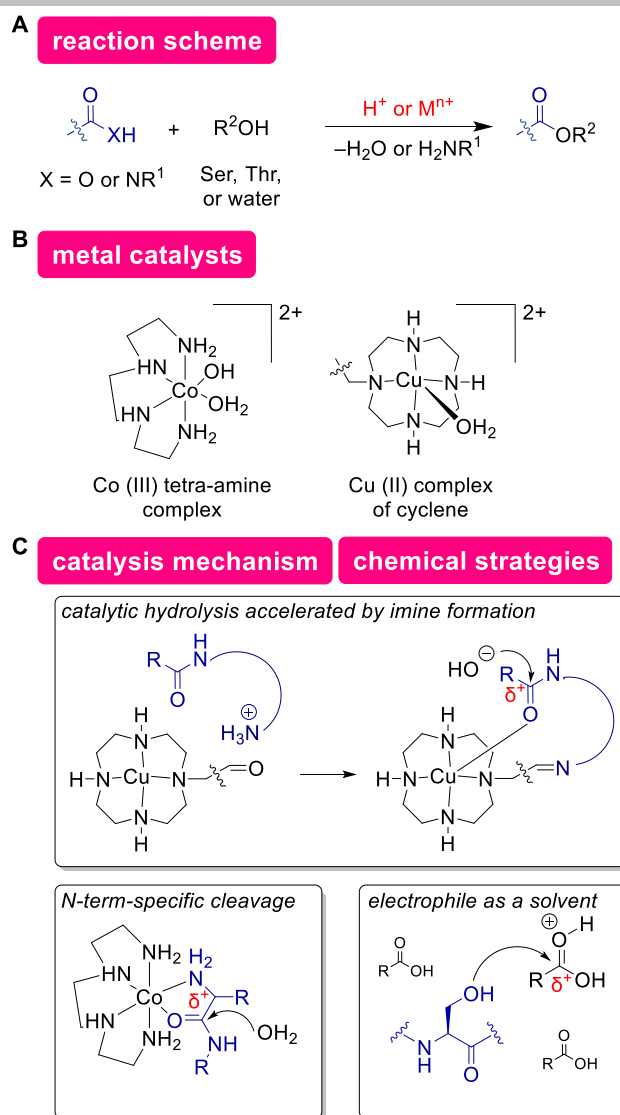
5.2. Acid-catalyzed hydrolysis and alcoholysis

Lewis acidic metal complexes can serve as a catalyst to cleave amide bonds in proteins at specific amino acid sequences (Scheme 13A, 13B). Proteases such as trypsin or pepsin are enzymes to catalyze proteolysis (i.e., hydrolysis of the peptide backbones) and are widely used for proteomic studies.[188] Although the proteolysis processes with such natural enzymes can proceed efficiently in mild conditions, their exclusive cleavage recognition patterns (e.g., Lys and Arg for trypsin) are not always compatible with a peptide or protein of interest, and development of non-natural, artificial proteolytic systems with alternative sequence recognition patterns has been actively studied.[189,190] Metal catalysts that displays Lewis acidic nature can be used as alternatives to natural proteases, for example. The metal center of such Lewis acid catalysts would facilitate the hydrolysis of the amide backbone by interaction with the carbonyl oxygen of the amide backbone making the carbonyl group more susceptible to the nucleophilic addition. An example includes a report by Kanai and co-workers showing scandium (III) triflate catalyst-mediated serine/threonine selective cleavage. Kostic and co-workers reported a different type of amide backbone cleavage with histidine and methionine selectivity using palladium-based catalysts.[191,192] In addition, N-terminal residue-selective cleavage

through the chelation of the α-amino group can be achieved with cobalt(III) complex as well (Scheme 13B, 13C).[193] In order to facilitate selective and efficient cleavage process by a metal complex, Suh and co-workers developed a system making use of reversible imine formation between aldehyde tethered to the metal-ligand and amine on proteins, which enabled specific cleavage at Gln(91)−Ser(92) and Ala(94)−Thr(95) of myoglobin protein (Scheme 13B, 13C).[194] Such a reversible bond-forming process can be also applicable to site-specific installation of functionality as well.[195,196] Instead of the reversible covalent-bond formation, supramolecular catalysis has been leveraged to induce the proximity-driven effects for peptide cleavage as well.[197] Those catalytic proteolysis examples demonstrated that choice of metals and ligand design can produce enzyme-like catalytic systems, and there have been many other reports of different metal systems as described in recent review articles.[198–201]

Serine residues in proteins can be catalytically modified in carboxylic acid-based non-aqueous media (Scheme 13C).[202] Although serine undergoes a variety of enzymatic modifications in living systems,[203] chemical modification of serine remains to be one of the challenging tasks owing to the modest nucleophilicity of the side chain and the abundance of the OH groups in aqueous media.[204] Encourage by the catalytic tryptophan labeling in nonaqueous medium (i.e., hexafluoroisopropanol or HFIP) as described above, Ohata and co-workers described that carboxylic acids could serve as a potentially protein-compatible reaction medium for serine-targeting modification,[202] which was motivated by the widespread use of many carboxylic acid-based compounds as biocompatible buffer components in biochemistry and protein science (e.g., acetate, glycine, and citrate buffers).[205] The chemical modification of hydroxyl groups of serine residue proceeds with an acid catalyst (e.g., trifluoroacetic acid and dysprosium(III) triflate) in carboxylic acid media where the excess carboxylic acid serves as an electrophile and reacts with the hydroxyl group (i.e., Fischer esterification-type reaction). The method was shown to be able to label protein substrates as well, including an intact antibody (trastuzumab), concanavalin A, and chymotrypsin. As chemoselective serine labeling strategies have been simply lacking, this acylation chemistry may be of indicative of the power of the nonaqueous, catalytic approach for protein modification.

Scheme 13. Acid-catalyzed hydrolysis and alcoholysis. (A) A general reaction scheme of acid-catalyzed hydrolysis/alcoholysis. (B) Structures of representative metal catalysts that mediate protein-backbone cleavage. (C) Chemical strategies that enhance the catalytic hydrolysis and alcoholysis. Top: Backbone hydrolysis accelerated by the copper center as Lewis acid and reversible formation of imine inducing proximity-driven effects. Bottom left: N-terminal specific cleavage by a CoIII catalyst. Bottom right: Catalytic acylation of alkylalcohols on proteins accelerated by an acid-activated carboxylic acid used as a solvent.

5.3. Acid-catalyzed enolate-based reactions

Aldol reactions using a dual catalytic system of copper and aldehyde catalysts enabled labeling of protein N-termini (Scheme 14A). Site-specific protein labeling methods can be advantageous for producing well-defined protein conjugate compared to chemoselective approaches and are useful for various applications such as single-molecule localization microscopy,[206] preparation of polyethylene glycol-tagged (PEGylated) therapeutic proteins,[207] and production of antibody-drug conjugates.[208] N-terminal amino groups can be attractive site-specific modification handles, as its decreased basicity compared to lysine amines can be utilized for N-terminal labeling through pH control.[209] While there is a repertoire of N-terminal selective methods to date,[209] Hanaya and co-workers reported a catalytic

variant by utilization of copper and aldehyde catalysts activating N-terminal α-proton.[210] The copper-catalyzed aldol reaction was proposed to occur through activation of an N-terminal amino acid by the copper and aldehyde catalysts, forming a nucleophilic Cu(II)-enolate intermediate (Scheme 14B). This nucleophilic activation of the protein would be followed by electrophilic activation of another aldehyde molecule, eventually leading to aldol-type reactions between the activated species to produce the product with a stable C–C bond. Interestingly, in contrast to other acid catalysis earlier,[172,202] other metal catalysts such as Sc(OTf)$_3$ did not function as effective catalysts for the aldol reaction, perhaps indicating the importance of the subtle control of Lewis acidity and affinity toward certain ligands in this catalytic system. The aldehyde catalyst/reagent (2-pyridinecarboxaldehyde) was previously reported to form a hydrolytically unstable imidazolidinone product on N-termini of proteins,[211,212] and the catalytic aldol process can be useful to produce more stable reaction products by alteration of the outcome of the aldehyde reaction with N-terminal amines by introduction of the copper catalyst (Scheme 14C). Whereas the hydrolytically unstable N-

terminal product can be of use for reversible elimination of the chemical modification in a certain context,[212] this catalytic example represents the importance to develop an alternative approach for production of protein conjugate with a different property. The same research group recently reported another copper(II)-mediated N-terminal modification by leveraging the hydrolytically stable intermediate (Scheme 14A).[213] Kanemoto and co-workers reported copper-catalyzed [3+2] cycloaddition between metalated azomethine ylide (bidentate) on the glycine N-terminus of peptides and maleimides in organic solvents.[214] Hanaya and co-workers expanded the scope of this approach to proteins by the use of pyridyl-aldehydes, allowing reactions in the aqueous buffer through formation of a more stable intermediate, tridentate azomethine ylide (Scheme 14B, 14C). The reaction was applicable to peptides and proteins with various N-terminal amino acid residues. Site-specific modification of an antibody, trastuzumab with this modification method prepared antibody–drug conjugates with uniform drug–antibody ratio (DAR), which was applied for mice cancer models.
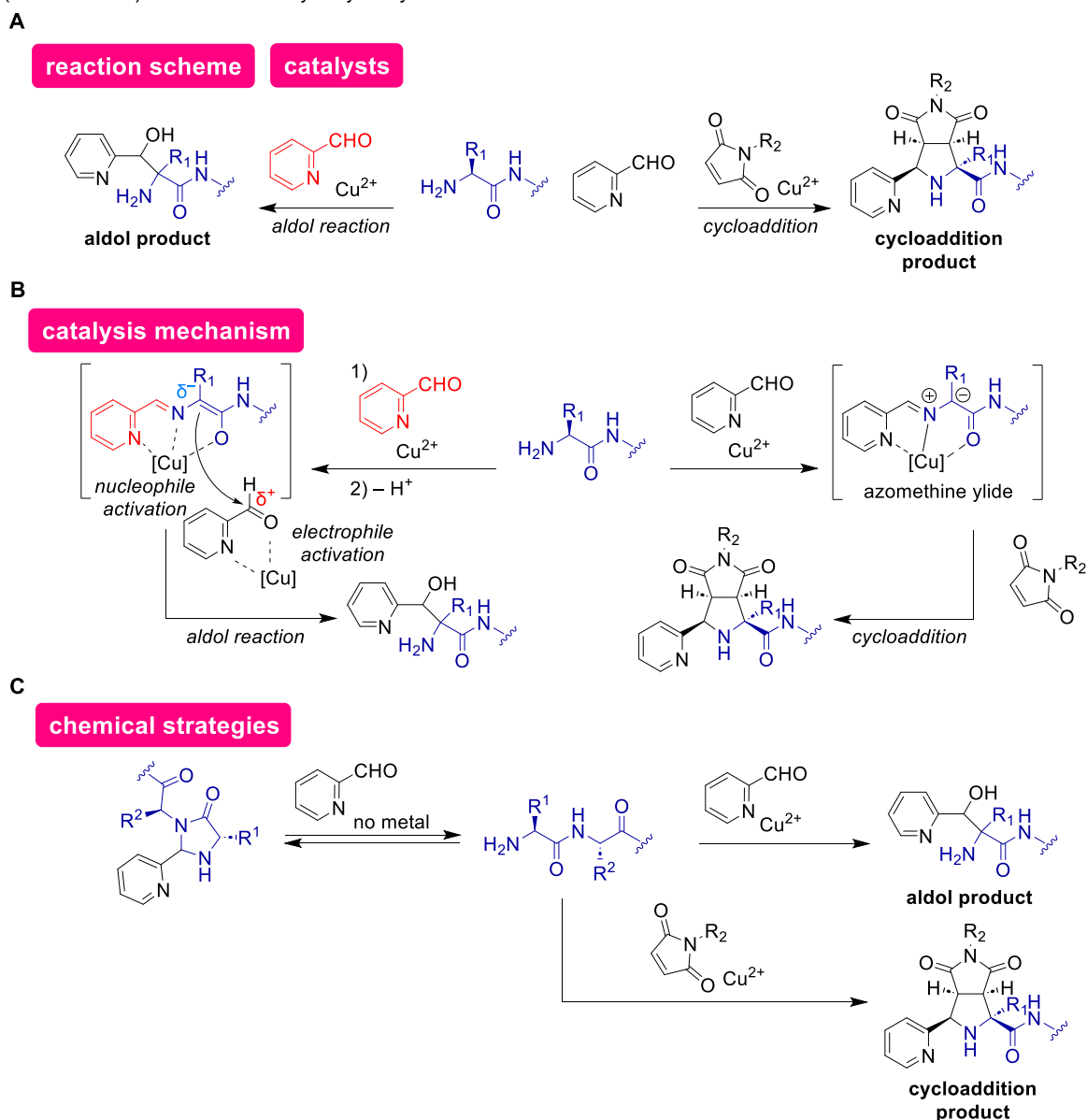
Scheme 14. Dual catalytic aldol reaction (left) and copper-catalyzed cycloaddition (right). (A) Schematic illustrations of copper-/aldehyde-catalyzed aldol-reaction (left) and copper-catalyzed cycloaddition (right). The pyridyl-aldehyde acts as both the reagent and catalyst for the aldol reaction. (B) Catalysis mechanism of the

copper-catalyzed aldol reaction (left) and copper-catalyzed cycloaddition (right). The pyridyl-aldehyde acting as a catalyst is depicted in red and the one as a regent in black. (C) Comparison of reaction products for N-terminal modification by choice of a labeling reagent and catalyst. Top: Alteration of the reaction product by the absence (reversible condensation reaction,[211,212] left) and presence (Irreversible aldol reaction, right) of the copper catalyst. Bottom: Irreversible copper-catalyzed cycloaddition.

6. Organocatalysis

Organocatalytic protein bioconjugation has been achieved by modifying target proteins through organic ligand-assisted reactivity. Organocatalysis has been rapidly growing in the synthetic chemistry fields since the late 1990s.[215] One of the major motivations for development of such catalytic strategies is to overcome the limitations of existing metal catalysts such as toxicity and high cost.[216,217] For example, proline is a readily available, non-toxic natural amino acid that serves as a catalyst for aldol reaction and Mannich reaction.[218] Even though a wide variety of organocatalytic transformation has been reported for small molecule substrates decades after its inception,[219–222] it is interesting that protein bioconjugation driven by organocatalysis has been exclusively by acyl transfer reactions through proximity-accelerated chemistry to date. As also described in the supramolecular catalysis section, such proximity-driven chemistry functions through association and dissociation of an affinity ligand that interacts with a protein of interests. The reported organocatalytic bioconjugation processes generally proceed in two steps (Scheme 15A): (1) association of a ligand to a target protein accelerates nucleophilic attack of ligand-tethered nucleophilic catalyst to an acyl donor reagent. (2) Another set of nucleophilic attacks by proteins to the activated acyl donor occurs, followed by dissociation of the ligand-catalyst. The following paragraphs focus on evolution of acyl transfer-based organocatalytic bioconjugation methods and brief demonstration of their utilities and applications.
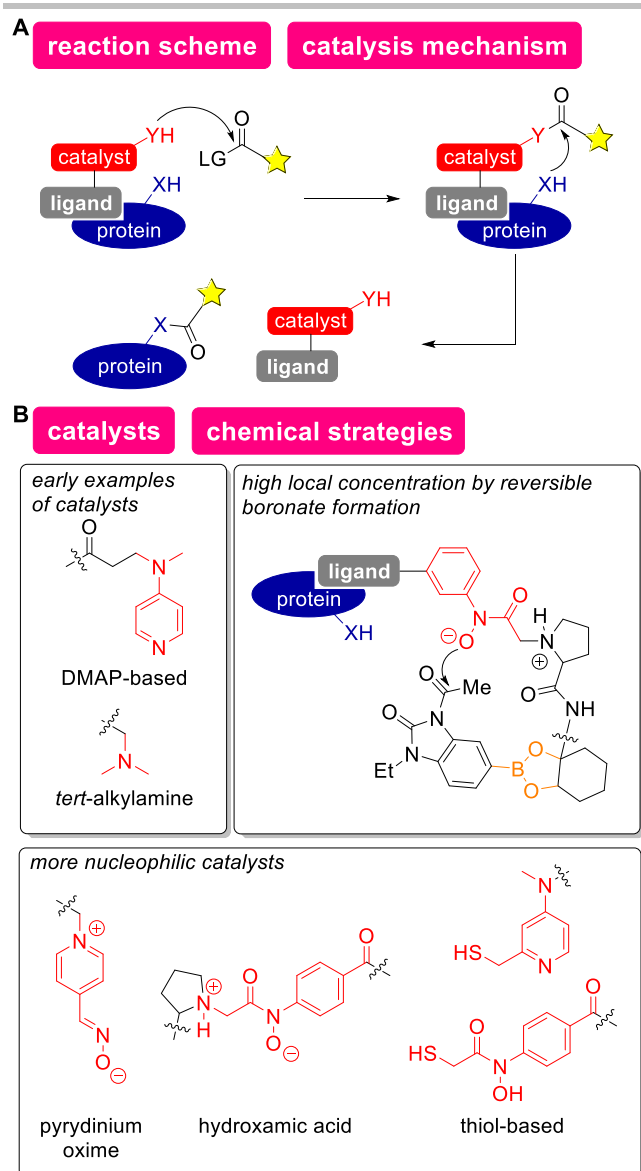
6.1. Organocatalytic acylation

Even in the first-generation labeling systems, organocatalytic acylation reactions through protein-ligand interaction demonstrated their usefulness for site-specific modification of target proteins for live cells and tissue samples (Scheme 15A). The seminal work of the organocatalytic bioconjugation was by Hamachi and co-workers, which demonstrated acylation of a glycoprotein-binding protein, lectin with a dialkylaminopyridine-based catalyst (i.e., dimethylaminopyridine or DMAP-type catalyst, Scheme 15B).[223] The DMAP catalyst was tethered with saccharide-ligand, and thiophenyl esters were employed as acyl donors for the site-specific labeling of lectins. By the virtue of proximity-driven effects, target proteins can be selectively modified even in the presence of other proteins (e.g., in cell and tissue lysates). In addition to saccharide ligands, the DMAP-based catalyst can be conjugated to proteins that bind to protein targets; for instance, DMAP-tethered lectin was utilized for the labeling of glycoproteins on live cell surfaces.[224] As a similar approach, a DMAP-tethered antibody fragment was developed to selectively modify receptors on cell membranes, which enabled epitope mapping of antibodies.[225] Another early example of ligand-directed catalysts is an organocatalyst based on dimethylalkylamine tethered with biotin, which was used for

modification of carboxyl groups of aspartate and glutamate residues of avidin.[226]

More efficient catalysis at physiological pH than the first-generation DMAP-based catalysis was demonstrated with anionic catalysts and thiol-based catalysts with milder acyl donors (Scheme 15B).[227–229] One of the challenges in the DMAP catalysis is that pK_a of the conjugate acid of the DMAP-based moiety is 8.6,[228] and substantial portions of the catalyst could be protonated at physiological pH ranges. To this end, pyrydinium oximes and hydroxamic acids were proposed as alternatives because of their lower pK_as (6.6[230] and 6.5[228], respectively) that could allow faster protein labeling than DMAP at physiological pH. Thiol-based catalysts possess additional benefits, compared to DMAP-based catalysts, that are kinetically favorable thiol-thioester exchange between the catalyst and the acyl donor.[231–235] As an independent approach from the thiol-based catalysis, Kanai and co-workers developed a unique proximity-driven approach through reversible boronate formation to facilitate the organocatalytic modification for site-specific modification of target within live cells (Scheme 15B).[229] Such a range of the second-generation catalysts allowed the use of moderate electrophilic acyl donors (e.g., acetyl-CoA) compared to the one used with DMAP-based catalysts (thioesters derived from thiophenol), attenuating off-target labeling.[227,233] For example, in the DMAP-based catalysis, the reaction usually requires a low temperature (e.g., 4 °C) to minimize the nonspecific labeling arising from the high electrophilicity of the thioester acyl donor.[227] Therefore, the use of milder acyl donors such as *N*-acyl-*N*-alkyl sulfonamides,[227] alkyl thioesters,[231–235] and acyl imidazoles[236] can be beneficial through suppression of the labeling agents' off-target reactivities. A notable application of the second-generation approach is that a hydroxamic acid-thiol-based catalyst conjugated with histone-ligand has been used for acylation of lysine-120 (K120) of histone H2B with endogenous acyl-CoA as an acyl donor.[235] Since acetylation of lysine residues of histone proteins is a naturally occurring post-translational modification that regulates gene expression (epigenetic regulation),[237] this approach could be useful for the chemical manipulation of the epigenetic regulation of gene expression.

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Scheme 15. Organocatalytic acylation reactions. (A) Schematic illustration of organocatalytic acylation facilitated by the ligand-directed chemistry (i.e., binding of a pendant ligand to a protein binding pocket). (B) Representative catalysts and chemical strategies to enhance the organocatalysis. Top left: Chemical structures of early examples of the organocatalyst containing dialkylpyridine (DMAP: dimethylaminopyridine) or tertiary amine units. Top right: Catalysis enhanced by reversible boronate formation. Bottom: Modern strategies to increase catalytic activity/nucleophilicity of the organocatalysts including *N*-oxide with an inherent negative charge and thiol groups.

7. Supramolecular catalysis

Supramolecular chemistry-based strategies such as metal-anion interactions, macrocyclic self-assembly, and ligand-directed affinity labeling can promote proximity-induced reactivities for protein bioconjugation (Scheme 16A). Supramolecular chemistry pertains to molecular assembly through a range of non-covalent interactions.[238] The process can be viewed as host-guest chemistry that causes a covalent bond-forming reaction between a substrate and reagent through supramolecular forces including hydrophobic interaction, hydrogen bond, van der Waals force, π-π stacking, and ion–dipole effect.[239] Enzymatic systems make use of a number of types of supramolecular chemistry to achieve site-

selective modification. For example, a transpeptidase sortase utilizes its domain called β6/β7 loop that recognizes an LPXTG (X = D, E, A, N, Q, or K) motif of a target peptide/proteins for site-selective modification of their C-terminal positions.[240,241] Supramolecular chemistry has been used for non-catalytic bioconjugation reactions including metal-anion ionic interactions by His$_6$-tag/Ni system and DNA/RNA hybridization through base pairings, as such non-catalytic supramolecular bioconjugation reactions have been reviewed in a recent article.[242] As described below, artificial host molecules such as gallium cluster and cucurbituril as well as ligand-directed affinity labeling strategy (see the organocatalyst section) were used as catalytic protein labeling strategies.

7.1. Reductive amination through supramolecular catalysis

Lysine-selective reductive amination can be achieved catalytically by a supramolecular gallium cluster (Scheme 16B).[243] For small molecule substrates, a gallium cluster Ga$_4$L$_6$ (L = *N,N*'-bis(2,3-dihydroxybenzoyl)-1,5-diaminonaphthalene) host were known to cause catalytic Nazarov cyclization and aza-Darzens reactions through assembly of reaction components by hydrophobic effect and stabilization of cationic intermediates (i.e., electrostatic stabilization of cations by the polycationic host Ga$_4$L$_6$).[244,245] Even for peptide and protein bioconjugation purposes, the same anionic gallium cluster can serve as a supramolecular host to catalyze reductive amination reactions on alkylamine groups of lysine residues. The specificity to lysine residues for the supramolecular catalysis contrasts with traditional reductive amination with a borohydride reagent (e.g., NaCNBH$_3$), which often cannot differentiate lysine and N-terminal amines unless there is precise pH control.[246] The selectivity mechanism was not studied in this report, but the supramolecular catalyst's preference toward sterically more accessible ε-amine may be the reason for the observed phenomena.

7.2. Michael addition through supramolecular catalysis

A macropolycyclic catalyst, cucurbit[8]uril can induce proximity-driven Michael addition through the assembly of a tryptophan residue and bipyridinium derivative (Scheme 16B). Cucurbituril is a macropolycyclic compound assembled from glycoluril and formaldehyde.[247] Cucurbituril acts as a host molecule for various guests such as hydrocarbons, saccharides, dyes, amino acids, and proteins through hydrophobic interactions, ion–dipole interactions, and dipole–dipole interactions.[248] Through such supramolecular capability, cucurbituril-mediated chemistry has been employed for protein modification such as azide-alkyne cycloaddition reactions enhanced through the hydrogen bonding network.[249] For modification of proteins with natural amino acid side chains, cucurbit[8]uril—a cucurbituril that contains eight glycoluril units—was used for supramolecular catalysis-based Michael addition reaction modifying a cysteine residue through inclusion of both a bipyridinium group on the labeling reagent and a tryptophan residue simultaneously.[250] Through this approach, cucurbit[8]uril-facilitated modification of a KRas protein was achieved using dehydroalanine-bipyridinium reagent; cucurbit[8]uril served as a host to a tryptophan residue of KRas and bipyridinium for labeling, followed by a proximity-induced thia-Michael reaction between cysteine residue on the substrate and dehydroalanine on the labeling reagent. It should be also noted

that supramolecular catalysis-based chemical backbone hydrolysis has been also achieved using a polymacrocyclic catalyst, although their substrate scope is limited to peptides.[197]

7.3. Ligand-directed labeling

Protein-ligand interactions have been utilized for a variety of catalytic transformation by enhancing inherently slow reactions through proximity-driven effects (Scheme 16B). Early examples of ligand-directed labeling strategies (non-catalytic) were shown to modify active sites of enzymes and antibody binding sites.[251,252] Such early examples were not catalytic, as the bound ligand does not dissociate after the labeling process. More recently, catalytic ligand-directed strategies have been reported where types of ligands range from small molecules to peptide ligands, and

various catalysis, as described in the previous sections (i.e., photocatalysis, transition-metal catalysis, and organocatalysis). The proximity effect is often utilized for sluggish reactions that do not proceed without the rate enhancement mechanism, including examples of ruthenium photocatalysis and DMAP-based organocatalysis.[50,223] In other words, generally unreactive amino acid residues could be modified through the proximity effects, as modification of phenylalanine was achieved by dirhodium catalyst conjugated with STAT3 ligand.[253] The proximity-driven rhodium catalysis was indeed shown to be capable of modification of half of canonical amino acids, including asparagine, phenylalanine, glutamine, and threonine,[254] showcasing the power of the supramolecular chemistry.
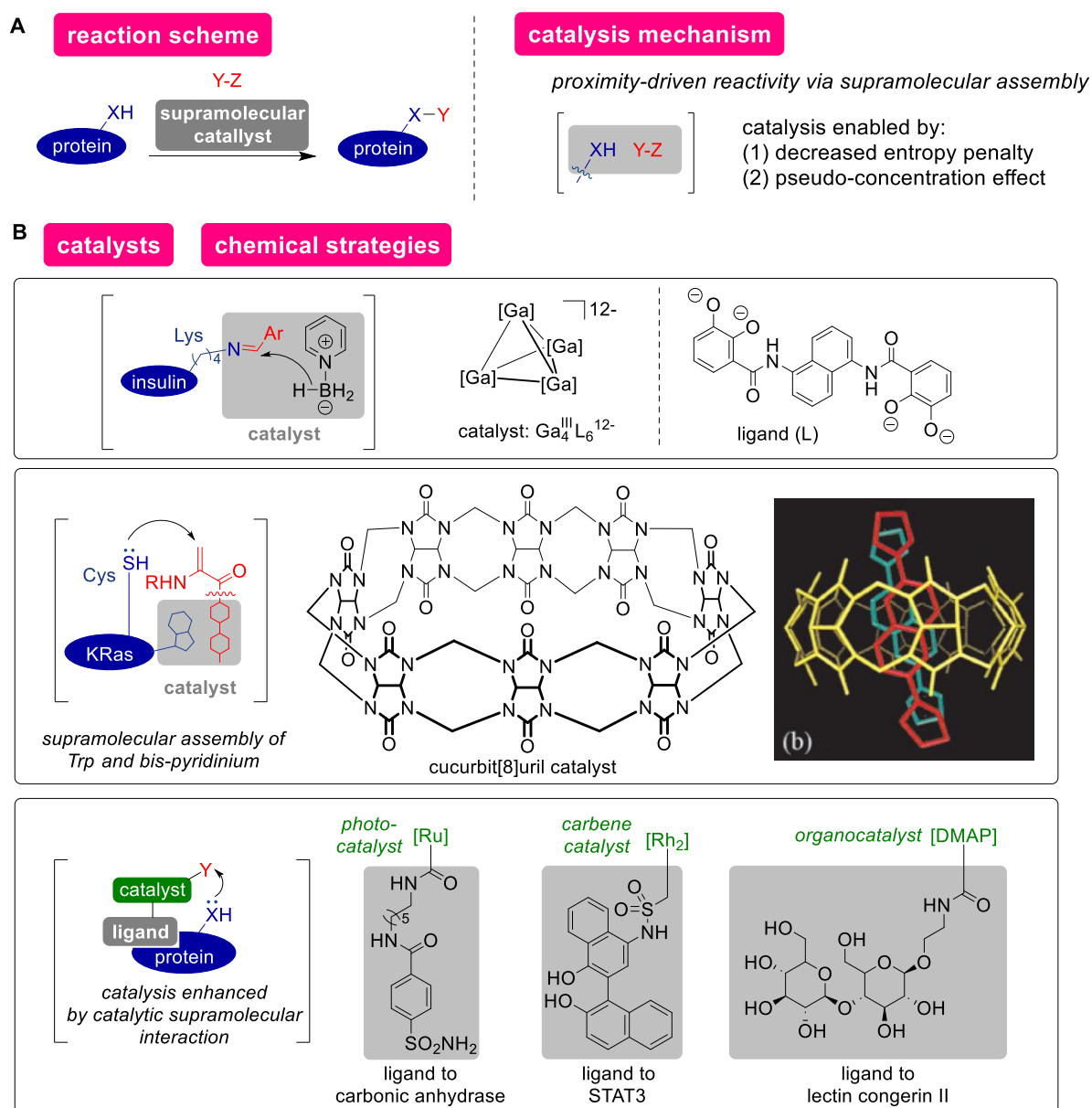
Scheme 16. Supramolecular catalysis. (A) Schematic illustration and mechanism of the protein modification with supramolecular catalysts. (B) Structure of supramolecular catalysts and catalysis mechanisms. Top: Reductive amination catalyzed through the supramolecular interaction between the gallium cluster, pyridine-borane, and imine intermediate. Middle: Thia-Michael addition to a cysteine residue facilitated by the interaction between a tryptophan residue of a protein and bipyridinium unit of the labeling reagent in the cucurbit[8]uril (CB[8]) as a supramolecular host. The image of X-ray crystal structure on the right was reproduced with permission.[247] Copyright 2000, American Chemical Society. Bottom: Proximity-driven catalysis through binding of a ligand to target proteins.

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8. Heterogeneous catalysis

Heterogeneous catalysts could be beneficial for proteolysis applications due to their tunable properties and facile separation from products (Scheme 17A). One of the earliest documented heterogeneous catalysis is Faraday's oxidation reactions by platinum catalysts in the 1800s.[255] The advantages of heterogeneous catalysts are reusability and easier separation of catalysts.[256] While there are many heterogeneous catalytic systems for small molecule substrates,[257–259] catalysts that can be applicable for protein substrates are quite scarce.[260] As described in the following paragraphs, metal-organic framework (MOF)-based platforms are one of the few examples that act as heterogeneous catalysis for protein substrates. MOFs are crystalline materials composed of metal ions and organic ligands. Properties of MOFs such as pore size, type of metal ions, and surface area are tunable.[261,262] As described in the acid catalyst section, artificial proteases composed of non-biomolecule building blocks could be useful for the digestion of proteins because of unique cleavage sites of such approaches. While MOF catalysts are also useful for the same purposes (i.e., hydrolysis of the protein backbones), the protein-hosting ability of MOFs can be an additional advantage for the following two reasons:[260] (1) The space confinement effect of MOF mesopores could mimic enzymes active site, as demonstrated in a few reports.[263,264] (2) The heterogeneous nature of MOFs can be advantageous for proteomics digestion purposes, as the catalysts can easily be separated from products after the reaction. As free metal ions could also be a catalyst for given reaction systems,[200] it is often important that a MOF catalyst possesses chemical and structural stability, so that fragments/components of MOF through decomposition would not induce unwanted processes.[265,266] For instance, one of the early reports for MOF-catalyzed proteolysis described leaching of Cu(II) ions from the MOF,[260] although the Cu(II) ion was not ascribed to the catalytic activity of the system.

Zirconium-based MOF and metal-oxo clusters were demonstrated to mediate backbone cleavage of proteins through their Lewis acidic actions (Scheme 17B, 17C). Parac-Vogt and co-workers introduced a zirconium(IV)-based MOF as a heterogeneous catalyst for proteolysis of model protein substrates.[267] The hydrolysis was proposed to proceed through the activation of the amide backbones by Lewis acidic Zr(IV) centers incorporated in the MOF catalyst. Hexazirconium metal-oxo cluster was a building block of the specific MOF catalyst, which was assembled by capping and interconnection by six benzene-1,3,5-tricarboxylate linker in a trigonal antiprism fashion. The water-soluble metal-oxo cluster, $[Zr_6O_4(OH)_4(CH_3CO_2)_8(H_2O)_2Cl_3]^+$ showed superior hydrolytic activity compared to the zirconium MOF catalyst.[268] Some metal-oxo cluster-based catalysts (e.g., cerium-based polyoxometalate[269] and molybdenum-based polyoxometalate[270]) showed unique properties such as regioselectivity potentially arising from the enzyme-like non-covalent interaction with specific regions of proteins.[271,272] Another explanation for such enzymatic behaviors is that the catalysts might make cleavage sites more accessible through partial unfolding of protein structures of proteins.[269,273,274] As the hexazirconium metal-oxo cluster is a component of the MOFs,[268] the metal-oxo cluster component has been used for mechanistic studies showing the accessibility of the catalyst active site by protein substrates and its similarity of the catalytic action to the hafnium cluster as described below.

Multinuclear hafnium metal-oxo cluster, $[Hf_{18}O_{10}(OH)_{26}(SO_4)_{13} \cdot (H_2O)_{33}]$, would be an effective heterogeneous catalyst for proteolysis specifically at aspartic acid residues (Scheme 17B, 17C).[275] there have been several reports of Hf(IV)-based MOFs as heterogeneous catalysts for organic reactions that involve activation of carbonyl groups.[276,277] The Hf_{18} polynuclear cluster $[Hf_{18}O_{10}(OH)_{26}(SO_4)_{13} \cdot (H_2O)_{33}]$ is insoluble in water and possesses both Lewis and Brønsted acidic moieties (i.e., protic protons of the coordinated water on the Hf centers) that was shown to facilitate hydrolysis of the amide backbones of a protein.[275] The catalysis displayed selective cleavage at aspartate residues (both Asp-Xxx and Xxx-Asp bonds where Xxx is an arbitrary amino acid residue), and the proposed reaction mechanism is by nucleophilic attack of the aspartate COOH to the amide backbone that forms an anhydride intermediate for Xxx-Asp bond cleavage and an imide intermediate for Asp-Xxx bond cleavage, followed by another set of nucleophilic attack by a water molecule to completes the process. It should be noted that presumably due to the Brønsted acidity of the catalyst, negatively charged regions of proteins were effectively cleaved, which was not achieved with the Zr(IV)-based metal-oxo cluster catalyst.[271,275,278] Therefore, hafnium-based catalysis may offer an alternative selectivity for proteolysis applications.

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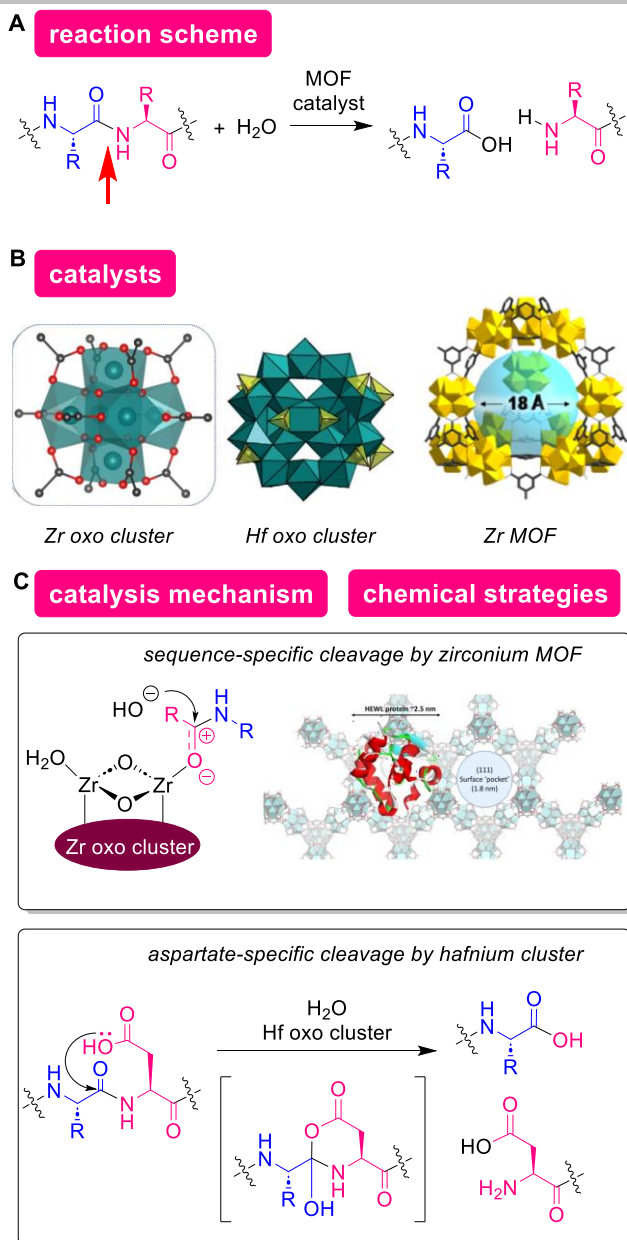
Scheme 17. Heterogeneous catalytic hydrolysis. (A) Schematic illustrations of hydrolysis of the peptide backbone catalyzed by metal clusters and metal-organic framework (MOF) catalysts. (B) Structures of representative heterogeneous and related catalysts. The image of Zr oxo cluster was reprinted with permission.[268] Copyright 2024, American Chemical Society. The image of Hf oxo cluster was reprinted with permission.[275] Copyright 2020, Wiley-VCH Verlag GmbH & Co. KGaA, Weinheim. The image of Zr MOF was reprinted with permission.[267] Copyright 2018, American Chemical Society. (C) Chemical strategies that enhance peptide hydrolysis by MOF and oxo cluster catalysts. Top: size comparison of lysozyme and pore-size of the zirconium MOF, and hydrolysis catalyzed by zirconium oxo-cluster. Bottom: sequence-specific hydrolysis via cyclic hemiaminal intermediate by hafnium cluster. The image of Zr MOF and protein was reprinted with permission.[267] Copyright 2018, American Chemical Society.

3. Summary and Outlook

Catalytic transformations have shown the great utility in chemical modification of proteins from various viewpoints, and the collection and analysis of a set of literature in this review also underscored possible future directions of the realm of the catalytic bioconjugation research. Appearance of redox-mediated chemistry in a multitude of sections is notable, probably implying its growing interests across various catalysis fields. However, because oxidation and reduction reactions are common processes in several canonical amino acid side chains,[279] strategies to suppress unwanted side reactions may be the unavoidable tasks as literature precedents also tackled the issue already (e.g., reactions under inert atmosphere[280] and use of redox-sensitive additives[281]). It became also obvious that some catalytic strategies are limited to only a certain reaction type or virtually non-existent for protein bioconjugation purposes. For instance, organocatalysis and heterogeneous catalysis have been realized only through acyl-transfer reactions and backbone hydrolysis, respectively. We were unable to find any examples of asymmetric catalysis and mechanochemical catalysis that have been used for bioconjugation at a protein level, though potential usefulness of some reactions for small molecule substrates in this areas are indicated in recent literature.[282,283] In particular, the dearth of the asymmetric catalysis is striking given the enantiomeric/geometric importance of post-translational modification in nature (e.g., methionine oxidation[284] and lysine acetylation[285]) as well as increasing studies on d-amino acid/d-proteins.[286] Dehydroalanine functionalization would be an example highlighting this trend, as there have not been examples of protein bioconjugation utilizing dehydroalanine in an asymmetric fashion[143,287] even if a plethora of reports demonstrated asymmetric conjugate addition reactions at a small molecule level.[288] Plausibly, this challenge may have been exacerbated by limited availability of analytical techniques that can be usable to differentiate the isomer forms of a particular amino acid residue in protein substrates. The examination of literature also showed the power of dual catalytic systems, especially those combined with supramolecular catalysis for site-specific or target-specific labeling strategies. Indeed, hybrid catalytic systems have been frequently utilized in chemistry of small molecules and peptides,[289] and protein bioconjugation may benefit from such hybrid systems as well. Finally, it is noteworthy that some catalytic mechanisms would be only possible in polypeptide substrates but not simple small molecule substrates (e.g., copper-catalyzed backbone modification of amide N-H driven by a neighboring histidine residue[132]), and proteins may serve as a platform to expand the boundary of the catalysis domain beyond the small molecule chemistry. Diverse fields spanning bioorganic chemistry, chemical biology, biomedical science, and material science necessitate development of protein bioconjugation to address various scientific and pragmatic challenges, and the chemical strategies and principles mentioned in this review paper may be a catalyst to transcend the limit.

Supporting Information

The list of cited protein modification papers is in the Supporting Information as an Excel spreadsheet.

REVIEW

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Keywords: Proteins • Protein modifications • Bioorganic chemistry

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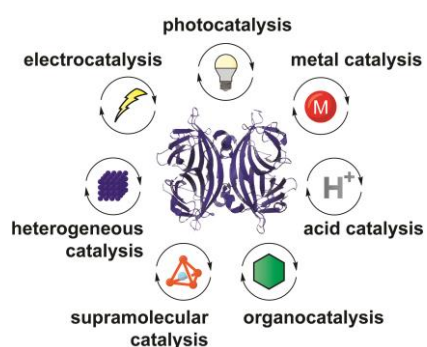
Catalytic principles of synthetic organic chemistry have been increasingly applied to chemical modification of proteins during the recent years. This review article describes how seven types of catalytic systems —electrocatalysis, photocatalysis, metal catalysis, acid catalysis, organocatalysis, supramolecular catalysis, and heterogeneous catalysis—have been leveraged as bioconjugation tools for protein substrates.

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