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Bioorthogonal Ligations and Cleavages in Chemical Biology

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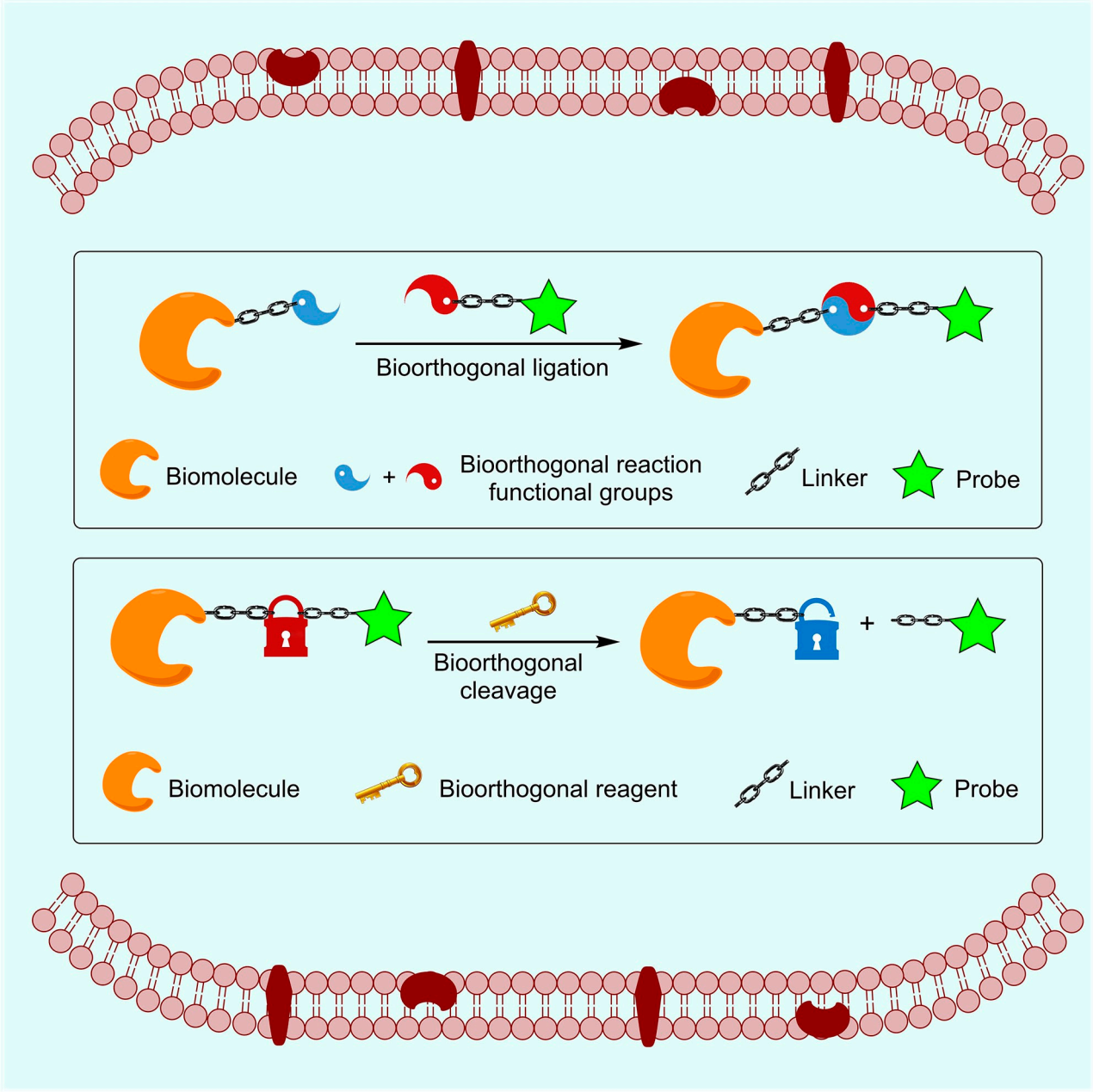

Bioorthogonal reactions including the bioorthogonal ligations and cleavages have become an active field of research in chemical biology, and they play important roles in chemical modification and functional regulation of biomolecules. This review summarizes the developments and applications of the representative bioorthogonal reactions including the Staudinger reactions, the metal-mediated bioorthogonal reactions, the strain-promoted cycloadditions, the inverse electron demand Diels-Alder reactions, the light-triggered bioorthogonal reactions, and the reactions of chloroquinoxalines and *ortho*-dithiophenols.

1. Introduction

To study the life processes of biomolecules in the natural environment by chemical methods has attracted more and more attention. However, it is difficult to find the chemical reactions with good biocompatibility and high selectivity under physiological conditions or in cells or in living organisms due to the vast complexity of biological systems. In 2003, Berztozzi first proposed the term of 'bioorthogonal chemistry', and classified the reactions that meet the above conditions as the bioorthogonal reactions.[1] In the past more than ten years, the continuous growth of bioorthogonal reactions has provided unprecedented opportunities for the study and manipulation of biological processes in biological systems. As an important tool in chemical biology, the bioorthogonal reactions have been widely used in various fields.[2] Among the developed bioorthogonal reactions, the most representative bioorthogonal ligations include the biocompatible Staudinger ligation reactions of azides with modified triphenylphosphines,[3] the Cu(I)-catalyzed azide-alkyne cycloadditions,[4] the strain-promoted azide-alkyne cycloadditions,[5] the inverse electron-demand Diels-Alder (IEDDA) reactions.[6] The initial applications of the reactions are mainly reflected in the formation of chemical bonds, and they are widely used in biomolecule labeling, imaging in vivo and drug design recently. A strategy contrary to this is to develop the biocompatible bond cleavage reactions. Although some bond cleavage reactions based on acids,[7] nucleophiles,[8] oxidants,[9] reducing agents,[10] transition metals promoted[11] or light-induced[12] have been developed successfully, the conditions often are relatively harsh, which limits their applications in biological systems. In recent years, many researchers have paid attention to molecular release or linker shearing reactions, some bioorthogonal reactions (or bioorthogonal ligations) that were originally used in the bond formation have been modified and applied in the biorthogonal cleavages, which improves the application range of the previous bioorthogonal reactions.[2a,c,13] Although some reviews on the bioorthogonal ligations were reported,[2b,14] those on the bioorthogonal cleavages are limited.[2a,c] To the best of our knowledge, the reviews involving with both the bioorthogonal ligations and cleavages in a single paper are very rare thus far.[13,15] Herein, we summarize some representative bioorthogonal reactions and their applications in bioorthogonal ligations and cleavages.

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2. Staudinger Reactions

In 1919, Staudinger and Meyer reported on the Staudinger reactions of azides with triphenylphosphines leading to iminophosphanes, which was initially mainly used in organic synthesis.[16] Until the 21st century, the reactions are gradually applied in chemical biology field.

2.1. Staudinger Reactions Leading to Bioorthogonal Ligations

In 2000, Bertozzi group originally used the Staudinger reaction as the bioorthogonal ligation (Figure 1a).[3] The reaction mechanism is as follows (Figure 1b): Staudinger reaction of methyl 2-(diphenylphosphino)benzoate derivative (**1**) with azide gives phosphorus ylide **2**, and intramolecular nucleophilic attack of the nitrogen anion to carbonyl of the ester group in **2** forms five-membered cyclic intermediate **3** leaving methanol. Treatment of **3** with water and subsequent cleavage of the P–N bond provides the amide product **4**.[3,17]

A few months later, Bertozzi[18] and Raines[19] research groups almost simultaneously reported the traceless Staudinger ligations in the peptide synthesis. As shown in Figure 2, Staudinger reaction of phosphine **5** containing thiol ester group with azide **6** first affords phosphorus ylide **7**, and then intramolecular nucleophilic attack of the nitrogen anion to carbonyl of the thiol ester group in **7** gives intermediate **8**. Finally, hydrolysis of **8** provides amide **10** freeing *o*-sulphydryl triphenylphosphine oxide **9**. Since then, the applications of the Staudinger reactions have been widely reported in chemical biological field.[20]

In 2011, Hackenberger's group reported a chemically selective install of phosphoamidates onto the protein containing azide group through the Staudinger-phosphonite reaction (SPhR), and the reaction exhibits high tolerance of functional groups.[21] They attempted two protocols as follows (Figure 3: (i) Copper-catalyzed coupling of azide (**11**) with ethynylphosphinate borane complex (**12**) forms trizole (**13**), and SPhR of (**13**) with another azide (**14**) linking with the protein provides conjugate (**15**);[22] (ii) SPhR of azide (**11**) with ethynylphosphinate (**16**) containing electron-rich trivalent phosphine gives

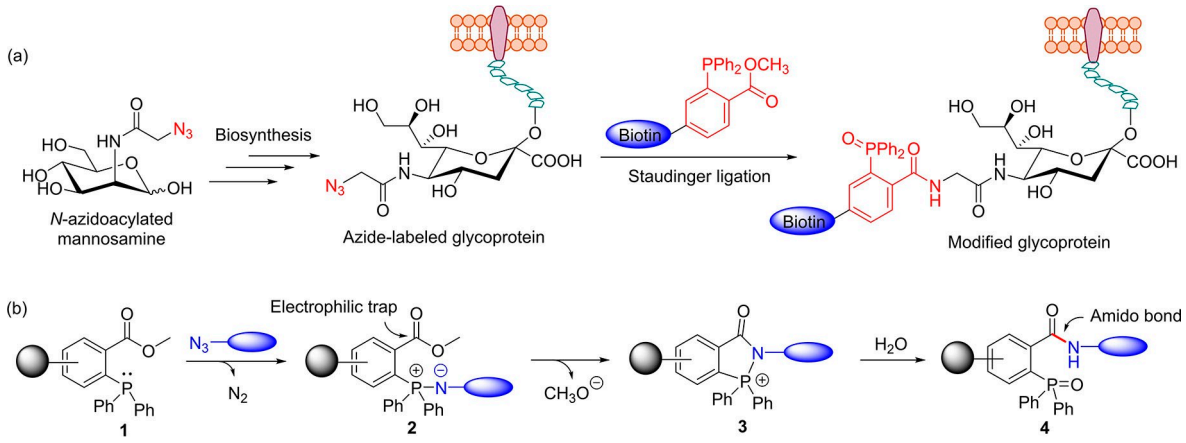

Figure 1. (a) Staudinger ligation of phosphines with azides on the cell surfaces. (b) Mechanism of the Staudinger ligation.

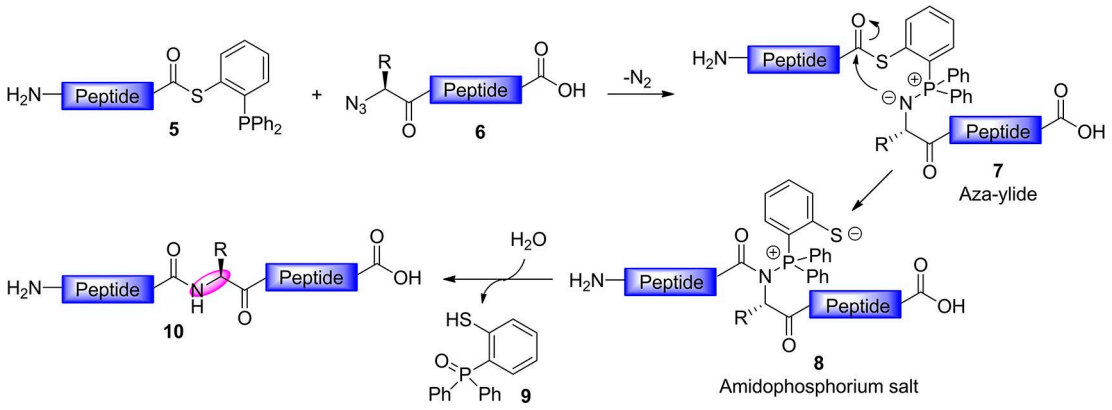

Figure 2. Traceless Staudinger ligation in the peptide synthesis.

ethynylphosphonate (**17**), and Michael addition of sulphydryl on protein **18** to **17** affords conjugate **19**.[23]

In 2017, Ramström and Yan's group developed the rapid Staudinger reaction (PFAA-SR) of perfluoro-substituted aryl azides (PFAAs) and arylphosphines. The reaction rate constant of methyl 4-azido-2,3,5,6-tetrafluorobenzoate (**21**) with 2-(diphenylphosphino) benzoate (**20**) in mixed solution of D_2O/CD_3CN (v/v, 1:1) at 25 °C is 18 $M^{-1}s^{-1}$, and the obtained iminophosphane (**22**) is stable to hydrolysis (Figure 4). The results showed that the PFAA-Staudinger reaction was an excellent bioorthogonal ligation. PFAA-derived mannose and galactose were successfully converted to cell surface polysaccharides, and effectively labeled with phosphine-derived fluorescent bovine serum albumin to provide the clear images with low background noise.[24] In 2019, the PFAA-Staudinger reaction was used to perform multiple orthogonal labeling in combination with a variety of bioorthogonal reactions (Figure 4).[25]

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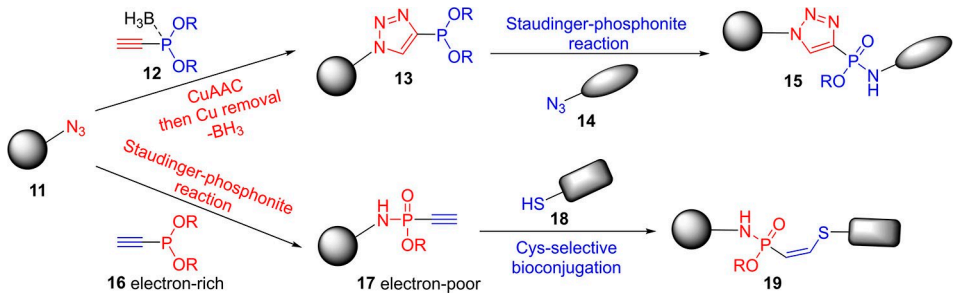

Figure 3. Staudinger-phosphonite ligation.

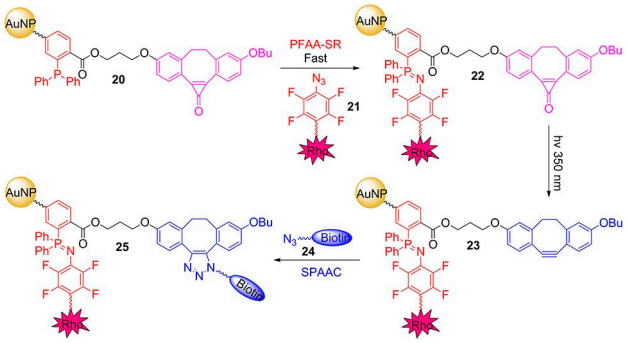

Figure 4. Rapid Staudinger reaction between perfluoro-substituted aryl azide and aryl phosphine.

2.2. Staudinger Reactions Leading to Bioorthogonal Cleavages

The Staudinger reactions are also used in the biorthogonal cleavages,[2c,13] especially in the prodrug release and control of fluorophore activity.[2a,26] Parent drugs are modified with chemically removable functional groups to form the corresponding prodrugs, and activity of the parent drugs is restored after triggering release. In 2006, Florent and co-workers demonstrated that the Staudinger reaction could control the release of doxorubicin prodrugs. The chemical process is as follows: Staudinger reaction of **26** with azide gives **27**, and 1,6-elimination of **27** releases amine **28** (Figure 5a).[27] In 2008, Robillard's group developed the direct transformation of azidobenzyl to the corresponding aniline via the Staudinger reduction, and subsequent 1,6-elimination of anine freed doxorubicin.[28] Deiters's group designed a small molecule switch **29** to control the protein activity, in which the reactions undergo a cascade process including the Staudinger reduction to aniline derivative, 1,4-elimination of aniline derivative to release amino of lysine residue in the protein of living cell (Figure 5b).[29] Other research groups also reported the release of lysine to control the recognition of antigen peptides by T cells[30] or the release of glycine to selectively trigger specific ubiquitination events in living cells via the Staudinger reaction.[31]

The Staudinger reductions are also used in adjustment of fluorophores such as fluorophore activation of nucleic acid detection probes in different forms (Figure 6)[32] and real-time fluorescence imaging of polysaccharides on the living cells.[33] The Staudinger reaction can trigger the cleavage of a linker with an α-azido ether group to convert the α-azido ether into the corresponding acetal ether, which spontaneously dissociates into aldehyde and alcohol (Figure 6b). Kool's group designed linking chain containing α-azido ether to realize the detection of nucleic acid.[32a] Guo's group completed in situ analysis of proteins in single cells by the similar method.[34]

The Staudinger ligations and cleavages show some advantages because the azide is the small size orthogonal group and

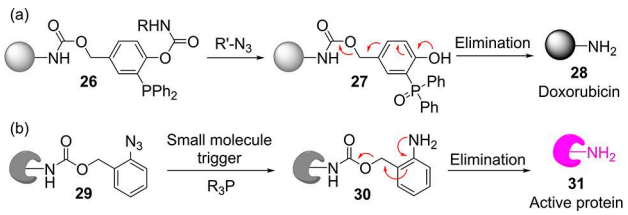

Figure 5. (a) Release of doxorubicin via the Staudinger reaction. (b) Release of fluorescent protein via the Staudinger reaction.

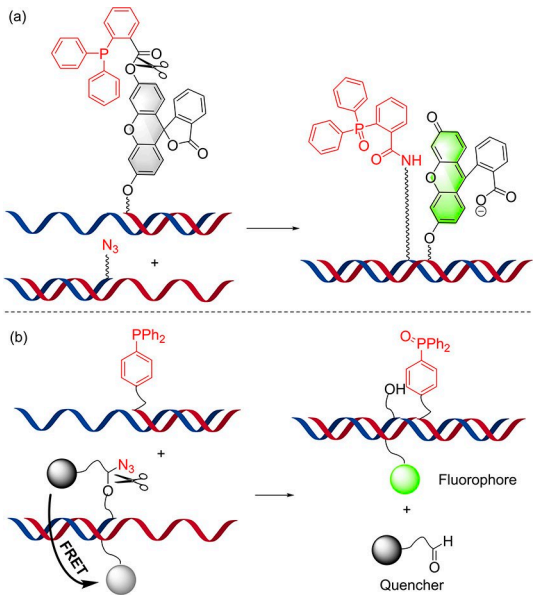

Figure 6. (a) Nucleic acid detection through control of fluorophore via the Staudinger reduction. (b) Cleavage of α-azide via the Staudinger reaction.

exhibits high stability and biocompatibility. However, some drawbacks of the Staudinger reactions limited its wide application such as easy oxidation of phosphines in air, especially in the presence of thiol/disulfide,[35] low reaction kinetics[36] and low water-solubility of phosphines.

3. Metal-Mediated Bioorthogonal Reactions

Transition metal-mediated bioorthogonal reactions have become a powerful toolbox for the selective formation and cleavage of covalent bonds. The reactions at room temperature with good adaptability in aqueous media provide numerous opportunities for bioorthogonal chemistry. The copper-catalyzed azide-alkyne 1,3-dipolar cycloaddition (CuAAC) is a representative method for the transition metal-mediated bioorthogonal reactions. However, other transition metal-catalyzed such as palladium or ruthenium-catalyzed bioorthogonal reactions were used in living cells in order to avoid the cytotoxicity of copper ions.

3.1. Metal-Mediated Bioorthogonal Ligations

3.1.1. Cu(I) Catalyzed Azido-Alkynyl Cycloadditions

In 1963, Huisgen comprehensively reported 1,3-dipolar azide-alkyne cycloadditions under the classic thermal conditions.[37] However, the reaction leads to the formation of two products **34** and **35** (Figure 7). Cu(I)-catalyzed 1,3-dipolar azide-alkyne cycloaddition (CuAAC) has been widely used in the bioorthogonal ligations since the reaction was independently developed by Meldal[4a] and Sharpless[4b] in 2002, in which use of Cu(I) catalysts greatly accelerates the reaction rate and the formation of a single product **36** (Figure 7). Subsequently, Fokin, Sharpless and Finn employed the CuAAC reaction in bioconjugation for the first time[38] and discussed the mechanism of the reaction in details.[39]

Azido and alkynyl groups as the bioorthogonal groups show significant advantages because they are scarce in biological systems and do not constitute biomolecules such as proteins, membrane lipids and nucleic acids. In addition, the two groups are tolerant to biomolecules under physiological conditions. Therefore, it is of great significance to develop efficient and practical methods for introduction of azido and alkynyl groups in biomolecules for the labelling and function control of biomolecules. 40 years ago, Shiner and co-author reported conversion of amino to azido by using trifluoromethanesulfonyl azide (TfN_3) (**38**).[40] However, the easily explosive nature and the relatively poor shelf life of TfN_3 hinder its application. In 2007, Goddard-Borger's group developed an efficient, inexpensive, and shelf-stable diazotransfer reagent: imidazole-1-sulfonyl azide hydrochloride (ISA) (**39**) and completed this transformation.[41] Subsequently, other research groups made improvements in stability of the azido reagents and their applications in the bioconjugations.[42] Very recently, Sharpless and Dong discovered that fluorosulfonyl azide compounds (FSO_2N_3) (**40**) was a powerful azidating reagent for conversion of the primary amines (**37**) to the corresponding azides (**41**) (Figure 8).[43] On the other hand, introduction of terminal alkynes in biomolecules also is important. In 2019, Chang and co-workers developed a unique way to produce a terminal alkyne in bacteria, in which side chain of *L*-lysine transferred into terminal alkynyl group through a multi-step cascade process.[44]

In CuAAC method, Cu(I) can lead to the formation of reactive oxygen species (ROS) that damages biomolecules and exhibits cytotoxicity.[45] To reduce the impact of copper ions on biomolecules, some researchers attempted some new strategies such as optimization of the catalytic conditions and addition of auxiliary ligands. The results showed that the highly water-soluble multidentate triazole-based ligands (**44**–**47**) (Figure 9) could greatly improve the rates of cycloadditions and reduce

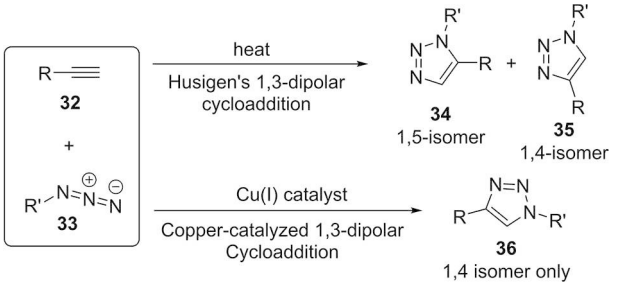

Figure 7. Heating-promoted or Cu(I)-catalyzed azido-alkynyl cycloadditions.

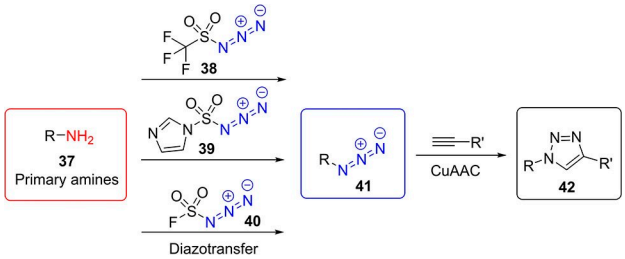

Figure 8. Transformation of primary amines to organic azides.

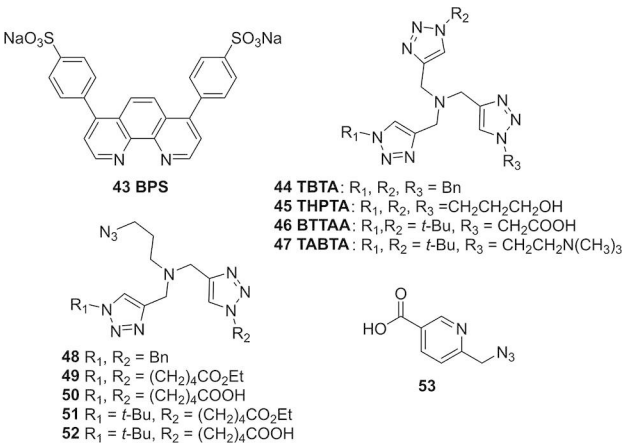

Figure 9. Efficient ligands for CuAAC bioconjugation.

the concentration of Cu(I), which alleviated the degree of oxidative stress in living systems.[46] Finn's group first reported 4,7-diphenyl-1,10-phenanthroline disulfonic acid disodium salt (BPS) (**43**) as the ligand of copper catalysts showing high reaction efficiency in vitro.[47] Meanwhile, Sharpless and Fokin[48] developed tri-(triazolylmethyl) amine (TBTA) (**44**) as the highly efficient ligand of Cu(I), and some research groups used the catalytic systems to realize the bioorthogonal ligations with good biocompatibility.[49]

Another strategy is to increase the directional aggregation of copper ions near the azido and alkynyl groups, introduce chelating groups near the active center and reduce the concentration of copper ions. Zhu and co-workers reported that the method could significantly accelerate the copper-catalyzed cycloaddition by introducing pyridylmethyl azide (**53**) (Figure 9).[50] Ting's group developed a fast, cell-compatible click chemistry with copper-chelating azides for biomolecular labeling.[51] They found that the reaction rate of pyridylmethyl azide was significantly faster than that of benzyl azide, and the ligands could accelerate the cycloaddition reaction and act as a sacrificial reductants to protect cells and biomolecules from ROS.[51] Inspired by the excellent results above, Taran and co-workers made **48**–**52** containing two triazolazolyl and a azido groups that could effectively perform the CuAAC reaction with the biocompatibility at a low concentration in the presence of equivalent of copper salt.[52] Recently, Qu's group adopted a type of catalytic method to construct a heterogeneous copper catalyst on a metal-organic framework. The catalyst could preferentially accumulate in the mitochondria of living cells to perform the CuAAC reaction at the specific sites of mitochondria and achieve the local synthesis of drugs with good biocompatibility.[53]

3.1.2. Palladium-Catalyzed Cross-Couplings and Ruthenium-Catalyzed Cross-Metathesis Reactions

In addition to the CuAAC reactions leading to the biorthogonal ligations, other metal-mediated coupling reactions have been also applied in the biorthogonal ligations of biomolecules[54] such as the palladium-catalyzed Heck reactions,[55] the Sonogashira reactions[56] and the Suzukih–Miyaura reactions,[57] and the ruthenium-catalyzed olefin metathesis reactions (Figure 10).[58]

The palladium-catalyzed cross-coupling reactions paly important roles in modern organic synthetic chemistry.[59] However, the reactions were seldom used in the biorthogonal ligations of biomolecules such as proteins before. Inspired by the successful applications of the CuAAC reactions in biomolecules, some palladium-mediated cross-couplings were attempted in protein modification. In recent years, with the development of various biocompatible catalytic systems and the introduction of suitable bioorthogonal groups on biomolecules, tremendous progress has been made on the palladium-catalyzed bioorthogonal ligations.[60] The palladium-catalyzed bioorthogonal reactions were first used in bioconjugation of peptides via the formation of C–C bonds,[61] which laid the foundation for palladium-mediated modification of biomolecules. In 2006, Yokoyama and co-workers reported the palladium-mediated regioselective protein modification via Mizoroki-Heck cross-coupling (Figure 10b).[62] Unfortunately, the conversion rate of the reaction is low. In 2011, Myers and co-workers developed the stable arylpalladium(ii) reagents (**55**) to air and water, and they kept intact in dimethyl sulfoxide for several months. The Heck coupling of **55** containing indocyanine dye unit with alkenyl group containing lysozyme in aqueous media realized the protein labeling (Figure 11).[55] In 2018, Roelfes's group reported palladium-catalyzed modification of dehydroalanine in the peptides and proteins to get both Heck coupling products and addition products using Pd(EDTA)$(OAc)_2$ as the water-soluble catalyst, and arylboronic acids as the partners of dehydroalanine.[63]

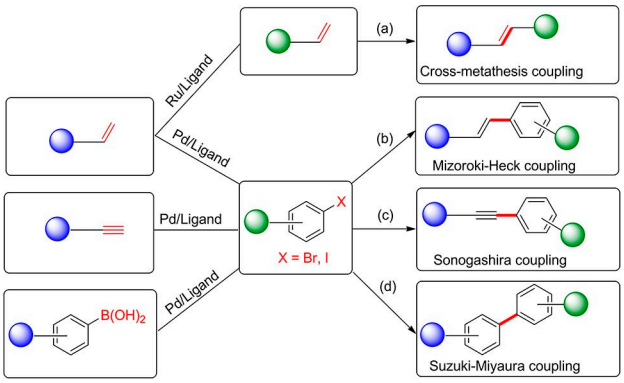

Figure 10. Transition metal-catalyzed cross-coupling reactions.

In 2007, Tachibana and co-workers developed the site-specific functionalization of proteins by the organopalladium Sonogashira reaction (Figure 10c), in which copper catalyst was required.[64] Compared with the previous Mizoroki-Heck reaction,[62] conversion rate of the present Sonogashira reaction (25%) is significantly higher than that of the Mizoroki–Heck reaction (2%). In 2011, Lin's group reported the copper-free palladium-catalyzed Sonogashira arylation of alkyne-encoded proteins in aqueous medium or in bacterial cells using *N*,*N*-dimethyl 2-amino-4,6-dihydroxypyrimidine sodium (**56**) as the effective ligand (Figure 12).[56] The ligand promotes the dissolution of palladium acetate in water and exerts its reactivity in the Sonogashira reaction, and the efficient catalytic system greatly enhances the application of the Sonogashira cross-couplings in bioorthogonal chemistry. It should be pointed out that an

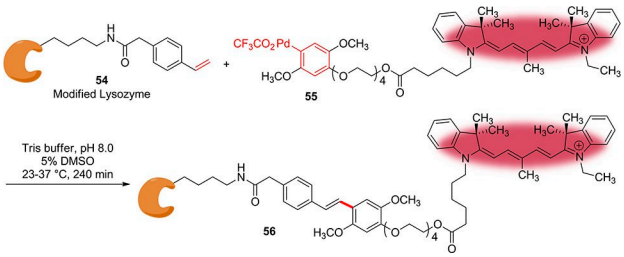

Figure 11. Cross-coupling of stable palladium reagent with olefins in aqueous solution.

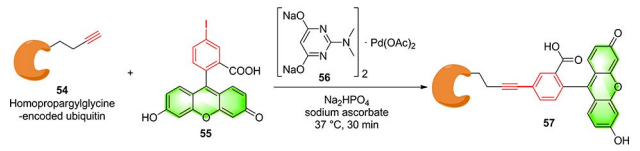

Figure 12. Sonogashira coupling reaction for the protein fluorescent labeling in aqueous solution.

excess of palladium catalyst (approximately 50 eq) is required in the reaction. In 2013, Chen and co-workers developed the ligand-free palladium-mediated site-specific protein labeling inside gram-negative bacterial pathogens with $Pd(NO_3)_2$ as the catalyst, and the reaction showed high efficiency and biocompatiblity.[65]

In 2008, Schultz's group used the genetic codon expansion technology to introduce 4-boronic acid phenylalanine into the proteins through biosynthesis, and realized the bioorthogonal ligation for the first time via the Suzuki-Miyaura reaction using the Pd^0 dibenzylidene acetone (Pd-DBA) catalyst (Figure 10d).[66] However, the high temperature (70 °C) and the low efficiency (30%) of the Pd-DBA catalyst in the reaction limit the wide application in protein modification. In 2009, Davis and co-workers improved the Suzuki-Miyaura bioconjugation with 2-amino-4,6-dihydroxypyrimidine sodium salt (**59**) as the ligand. First, aryl iodide was installed on cysteine residue of the protein, and then Suzuki-Miyaura coupling of the aryl iodide derivative with aryl or vinylboronic acids provided the corresponding protein conjugate in high conversion rate (>95%) within 30 min (Figure 13).[57] In 2014, Chen and co-workers developed the bioorthogonal ligation with *N*-heterocyclic carbene-stabilized palladium complexes as the organometallic catalysts, in which Suzuki-Miyaura coupling of unnatural amino acids with proteins containing *p*-iodophenyl achieved the rapid protein labeling on the surface of mammalian cells.[67] In 2018, Qu's group devloped a general photo-controlled bioorthogonal catalyst by modifying macroporous silica-Pd^0 with supramolecular complex of azobenzene (Azo) and β-cyclodextrin (CD), in which the activity of the catalyst can be adjusted by light-induced structural changes. The palladium catalytic system was successfully applied in the synthesis of fluorescent probe for cell imaging and mitochondria-specific targeting reagent via the Suzuki-Miyaura cross-coupling reaction.[68]

The olefin metathesis reactions, especially ruthenium-catalyzed olefin metathesis reactions are a powerful tool in organic synthesis, and they show some advantages such as mild conditions, wide tolerance of functional groups (Figure 10a).[69]

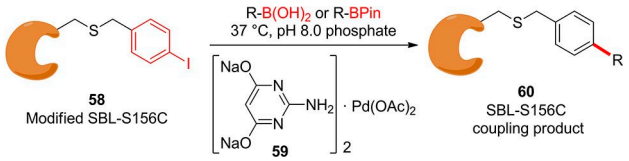

Figure 13. Suzuki-Miyaura coupling reaction for the protein modification in aqueous solution.

In 1995, Grubbs's group made conformationally restricted amino acids and peptides in organic solvents by the olefin metathesis method.[70] Later, some valuable olefin metathesis methods in aqueous media were developed for chemical biology.[71] For example, Davis and co-workers achieved the site-selective protein modification using allyl sulfides (**61**) as the privileged substrates in the aqueous cross-metathesis.[58] Subsequently, the same group developed some excellent bioorthogonal ligations via the olefin metathesis coupling (Figure 14).[72]

3.2. Metal-Mediated Bioorthogonal Cleavages

In addition to the metal-mediated bioorthogonal ligations, some metal-mediated, especially the use of ruthenium or palladium-mediated bioorthogonal cleavages have been rapidly developed in recent years (Figure 15), and some reviews have been summarized.[11,13,54b,73]

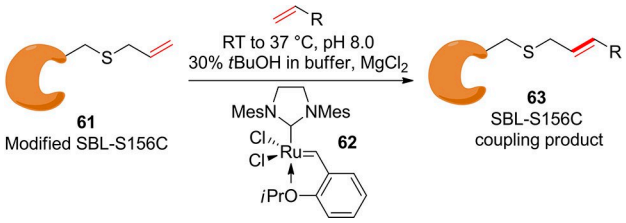

Figure 14. Protein modification via the olefin metathesis coupling in aqueous media.

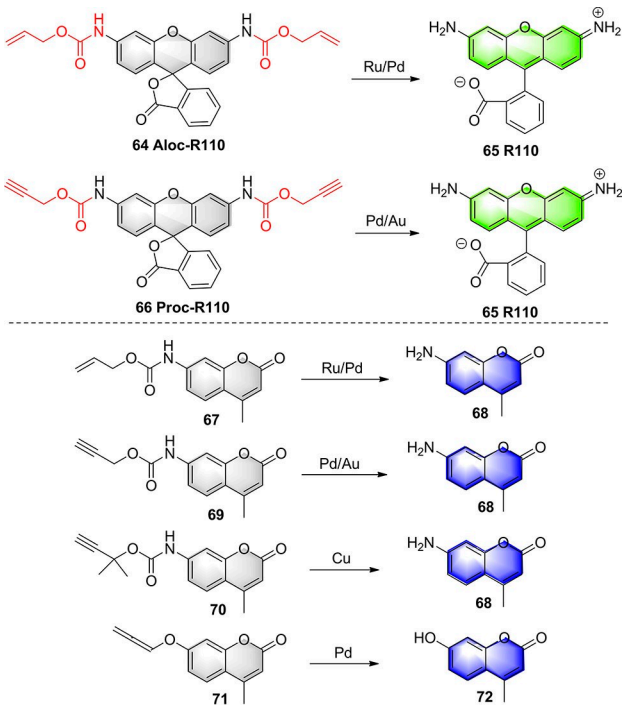

Figure 15. Transition metal-mediated bioorthogonal cleavages for the activation of fluorophores.

3.2.1. Metal-Mediated Deallylation

In 2006, the Meggers's group reported ruthenium-induced allylcarbamate cleavage in the living cells under catalysis of Cp*Ru(cod)Cl for the first time,[74] but an excess of thiophenol was required in the reaction. In 2014, the same group found that catalyst CpRu(QA)(η^3-allyl)]PF_6 showed the better results including good biocompatibility and high drug release efficiency in the ruthenium-induced allylcarbamate cleavage.[75] Rotello and co-workers reported the fabrication of protein-sized bioorthogonal nanozymes using the nanoparticle-embedded transition metal catalysts, and the imaging and therapeutic applications of the gated nanozyme were shown via the Cp*Ru(cod)Cl-catalyzed cleavage of allylcarbamates for pro-fluorophore activation and propargyl groups for prodrug activation inside the living cells.[76] Bradley's group described the palladium-mediated intracellular chemistry.[77] They found that the palladium nanoparticles trapped within polystyrene microspheres could enter cells and catalyzed the allylcarbamate cleavage and the Suzuki-Miyaura cross-coupling, and the method was used in the cellular labelling and synthesis of modulators or inhibitors of cell function. Weissleder's group screened bis[tri(2-furyl)phosphine] palladium(II) dichloride ($PdCl_2(TFP)_2$) from different palladium complexes, and then encapsulated it in the biocompatible poly(lactic-co-glycolic acid)-b-polyethyleneglycol (PLGA-PEG) platform to obtain the palladium nanoparticles. In situ formed Pd(0) catalyst effectively performed the deallylation in vivo.[78]

3.2.2. Metal-Mediated Depropargylation

Compared with the palladium-catalyzed allyloxycarbonyl cleavage, Pd(0) catalysts exhibit higher catalytic activity for the propargyloxycarbonyl cleavage.[77,79] In 2014, Chen and co-workers reported the palladium-triggered deprotection for the protein activation in living cells.[80] They used the biocompatible and efficient palladium catalysts, $Pd(dba)_2$ and $Allyl_2Pd_2Cl_2$, to cleave the *N*-propargyl carbamate of lysine residue in protein, and this deprotection strategy worked well with a range of different cell lines and proteins. Two years later, the same group described the Pd-mediated deallenylation for *O*-allenyl tyrosine residues of the enzymes in vitro and in living cells (Figure 16).[81]

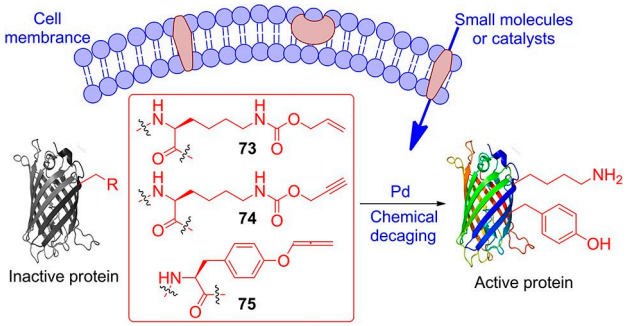

Figure 16. Palladium-mediated cleavages for the activation of protein.

Bernardes's group designed and optimized a bifunctional thioether-propargyl carbamate linker simultaneous allowing the site-specific protein modification and palladium-mediated bioorthogonal decaging. The thioether-directed palladium-catalyzed propargyloxycarbonyl cleavage controlled the drug release from a PEGylated doxorubicin prodrug in cancer cells (Figure 17),[82] which is similar to the previously reported method of palladium-catalyzed cleavage of the allyloxycarbonyl bifunctional chain.[83]

4. Strain-Promoted Cycloaddition Reactions

In recent years, strain-promoted cycloaddition reactions have been developed rapidly. Cycloaddition of highly reactive ring strain alkynes with azides or other 1,3-dipoles provides effective methods for the selective modification of biomolecules.

4.1. Strain-Promoted Cycloadditions Leading to Bioorthogonal Ligations

4.1.1. Strain-Promoted Alkyne-Azide Cycloaddition

Although the copper-catalyzed azido-alkynyl cycloadditions (CuAAC) have been widely investigated, the use of copper affects the function of biomolecules. Another strategy is to reduce the energy barrier of the cycloaddition by using the internal cyclooctynes with twisted tension instead of the terminal alkynes to realize ring strain-promoted azide-alkynyl cycloaddition (SPAAC). Bertozzi and co-workers first established the copper-free azido-alkynyl cycloaddition as the bioorthogonal reaction (Figure 18).[5b] The method shows some advantage such as simple conditions, no addition of copper catalyst, use of stable and biocompatible azides, which make SPAAC to become one of the most popular methods for modification of biomolecules in living cells.[20c,84]

However, the higher concentrations and longer reaction times are usually required during modification of biomolecules because of SPAAC drawback with low reaction rates, which lead to the occurrance of unintended side reactions. Treatment of the ring strain alkynes with sulfhydryl of cysteine residue in proteins led to the addition products.[85] In addition, the high reactive and uneasy available cyclooctynes could rearrange or decompose.[86] Recently, some improvements such as optimization of structures and synthetic routes for ring stain alkynes promoted wide applications of the SPAAC methods.[2b,87]

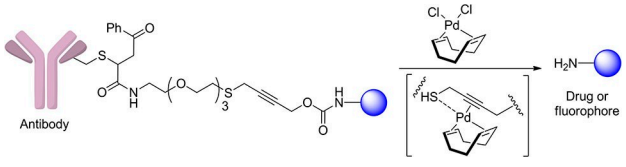

Figure 17. Thioether-directed palladium-mediated propargyloxycarbonyl cleavage.

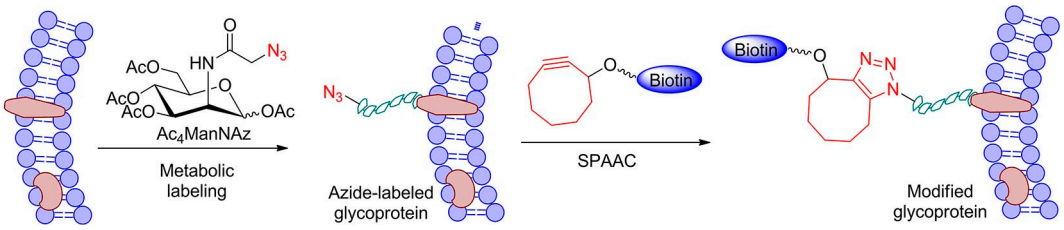

Figure 18. Strain-promoted azide-alkynyl cycloaddition.

As shown in Figure 19, difluorinated cyclooctyne (DIFO) (**83**) with high reactivity was successfully used for fluorescent labeling of polysaccharides on zebrafish cell surface.[5a] Dibenzocyclooctynols (DIBO) such as **86**[88] and **88**[89] with additional ring strain are the ideal partners of azides for labeling of living cells, and their unique structures greatly increase the reactivity of the alkynes in the metal-free [2+3] cycloadditions with azides. In 2010, van Delft and co-workers reported bicyclo[6.1.0]nonyne (BCN) (**85**) as the ring-strained alkyne for the metal-free cycloadditions with azides, and the functionalized derivatives of BCN were used in the labeling of proteins and glycans, as well as in the three-dimensional visualization of living melanoma cells.[90] The diverse strain cyclooctynes also were successfully applied in various fields of chemical biology such as the pretarget imaging in mice[91] or combined with other bioorthogonal reactions for in vivo multiple orthogonal labeling.[92] Very recently, Fierz, Waser and co-workers introduced hypervalent iodine bonds into the peptides and proteins with ethynylbenziodoxolones (EBXs, **92**) via functionalization of cysteine to form **93** with the doubly orthogonal functional groups (hypervalent iodine and azide parts). Palladium-catalyzed Suzuki-Miyaura cross-coupling of hypervalent iodine part with arylboronic acid derivative constructed the C–C bond, and SPAAC reaction of azide part with strain cyclooctyne led to the triazole (Figure 20).[93] In 2018, Luedtke and co-workers used dibenzocycloocta-1,5-diyne containing two morpholino side chains as the partner of azide linking with DNA, and the strain-promoted double click (SPDC) reactions were successfully performed in the living cells.[94]

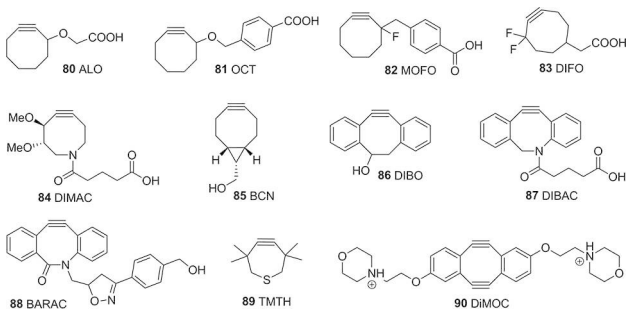

Figure 19. The common ring strain alkynes in SPAAC.

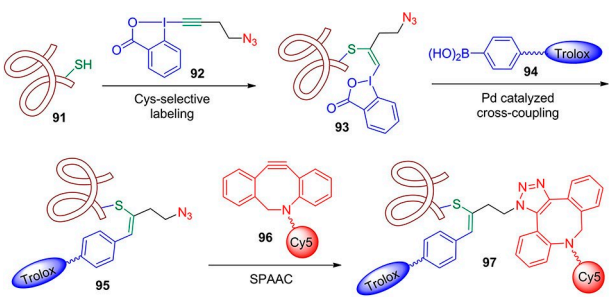

Figure 20. Doubly orthogonal labeling of the peptides and proteins.

4.1.2. Strain-Promoted Cycloadditions of Alkynes with Other 1,3-Dipoles

During the development of the SPAAC reactions, some improvements were attempted such as optimization of strain alkyne structures. Meanwhile, other 1,3-dipoles were applied in the SPAAC reactions instead of azides.[95] Boons and co-workers investigated the 1,3-dipolar cycloadditions of readily available nitrile oxides (Figure 21a) or diazocarbonyl derivatives with cyclooctynes, and they found that the cycloadditions with diazocarbonyl derivatives showed the similar kinetics to azides, whereas the much faster rates were observed for the reaction of cycloadditions with nitrile oxides.[96] It should be noted that the reaction of nitrile oxide with norbornene was used in DNA modification before.[97] In 2015, Raines's group reported the stabilized diazo group, which is approximately half relative to the size of an azido group, and they found that the diazo derivative of *N*-acetylmannosamine endureed the cellular metabolism and labeled the surface of a mammalian cell via 1,3-dipolar cycloaddition of diazo group with strained alkyne (Figure 21c).[98] Pezacki and co-wokers developed the rapid

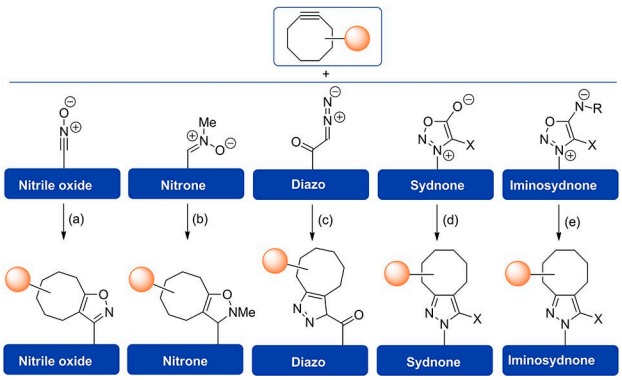

Figure 21. Strain-promoted cycloadditions of other 1,3-dipoles with alkynes.

strain-promoted 1,3-dipolar cycloadditions of nitrones with cyclooctynes and found that the cyclic nitrone compounds showed higher reactivity and better stability.[99] The method were used and improved by many other groups later (Figure 21b).[100] In 2013, Taran's group reported the copper-catalyzed cycloaddition of sydnones with terminal alkynes to generate pyrazoles.[101] Based on the reseach, Chin and co-workers expanded to the phenyl sydnone 1,3-dipole with a bicyclononyne dipolarophile to the strain-promoted cycloaddition in the absence of transition metal catalysis, and the quantitative and specific labelling of a genetically encoded bicyclononyne with a sydnone fluorophore conjugate was shown.[102] In 2016, Taran's group reported the both copper-catalyzed cycloaddition of 4-fluorosydnones with terminal alkynes, and strain-promoted cycloaddition of 4-fluorosydnones with alkynes. The reactions showed very fast reaction rate (rate constant up to 10^4 M^{-1} s^{-1}) and high selectivity giving 5-fluoro-1,4-pyrazoles (Figure 21d).[103] Very recently, Yu and co-workers developed the photoclickable strain-promoted cycloaddition of diarylsydnones with alkynes, which showed the robust selectivity for in vitro and in vivo protein labelling.[104] Furthermore, the method has been successful for the fluorescent molecular imaging[105] and metabolic labeling of sialoconjugates.[106]

4.2. Strain-Promoted Cycloaddition Leading to Bioorthogonal Cleavages

Bayley and co-workers reported the sydnone-strained alkyne cycloaddition for the first time at the single-molecule level at a protein surface, in which the reaction showed the fast rates with carbon dioxide releasing.[107] Taran's group reported the bioorthogonal click-and-release reaction of iminosydnones and strained alkynes, in which the ligation and fragmentation of iminosydnones were found under physiological conditions. The method was successfully used to design the innovative cleavable linkers for protein modification and in the fields of drug release and target-fishing researches.[108] In 2019, the same group described the generation of cleavable micelles able to disassemble by using click-and-release technology via a sequential enzymatic and bioorthogonal activation process, and the results indicated that the method could be applied to deliver the substances encapsulated into micelles in living cells as well as in mice.[109] They also synthesized the group of substituted iminosydnones and explored the structure-activity relationship between the compounds and strain alkynes (Figure 22).[110] Very recently, Kolodych and Wagner achieved the synthesis of monodisperse antibody-conjugated drugs using the reaction of iminosydnones and ring strain alkynes.[111]

As mentioned above, the Staudinger reaction has been used in the bioorthogonal cleavages, but the trivalent phosphines are easily oxided.[2c,13] Gamble and co-worker developed the 1,3-dipolar cycloaddition of *trans*-cyclooctenes (**98**) with azide prodrugs (**98**) to form an unstable triazoline intermediate **100**, and subsequent reactions freed the drugs (Figure 23).[112] They investigated the mechanism by NMR[113] as follows: isomerization of **100** led to **101**, extrusion of nitrogen in **101** formed imine **102**. Hydrolysis of **102** provided aldehyde **103** and amine **104**, and 1,6-elimination of **104** released the active drug **105**. Chen's group achived the protein activation in cells by using the method.[114] Gamble's group investigated the effect of fluorine substituents on aryl azide reactivity and decaging from the 1,2,3-triazoline. The NMR and computational results showed that fluorine substituents on the aromatic ring lowered the transition state energy required for transformation of the triazoline to the imine or aziridine intermediates, which led to the fast reaction rate.[115]

5. Inverse Electron Demand Diels-Alder Reactions

The inverse electron demand Diels-Alder (IEDDA) reactions are an attractive bioorthogonal method due to rapid rates, high yields, good chemoselectivity and biocompatibility in aqueous media. Therefore, the IEDDA reactions are often found in bioorthogonal chemistry.

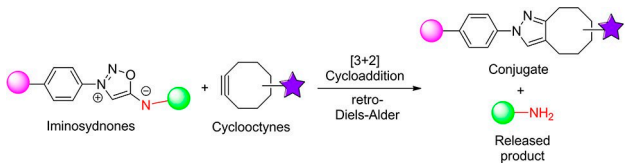

Figure 22. Click-and-release reaction of iminosydnones and strained alkynes.

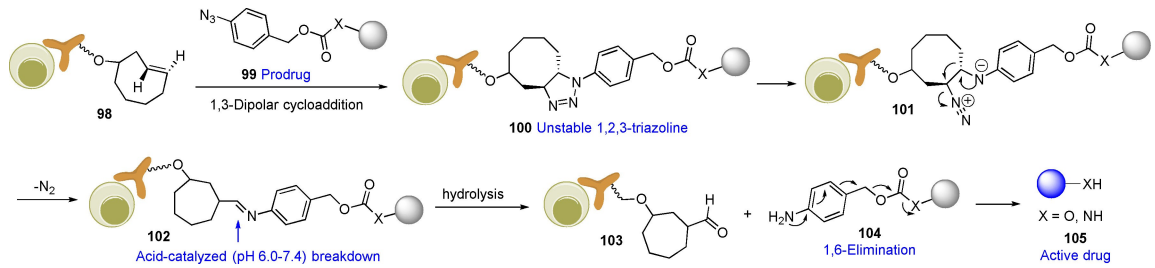

Figure 23. Click-and-release reaction of *trans*-cyclooctenes with azides.

5.1. Cycloadditions of Isonitriles and Tetrazines

5.1.1. Cycloadditions of Isonitriles and Tetrazines Leading to Bioorthogonal Ligations

Cycloaddition reactions involving isocyano group are widely used in organic synthesis. The cycloaddition reactions of isocyanides with conjugated dienes provide many opportunities for the design and synthesis of heterocyclic compounds.[116] Isocyanides are stable in neutral pH aqueous media, and they show the advantage in bioorthogonal reaction for small size of isocyano group.[117] As shown in Figure 24, reactions of isonitriles with tetrazines can undergo the following process: [4 + 1] IEDDA cycloaddition of isonitrile (**106**) with tetrazine forms **107**, release of N_2 in **107** leads to **108**. Isomerization of **108** from primary, secondary isonitriles affords **110**, and hydrolysis of **110** gives aldehyde or ketone **111** and amine **112**.[13,118] Only slow hydrolysis occurs for reaction of isocyanopropionates with tertiary isonitriles in aqueous media.[119] Leeper's group found that the isonitrile-tetrazine reaction rate was high ($57.5 \times 10^{-2}\ M^{-1}s^{-1}$) in a mixed solution of H_2O/THF (1 : 1) at 25 °C, and declared that the isonitrile-tetrazine click reaction was a promising candidate for the ligation reactions in chemical biology and polymer chemistry.[119] Use of tertiary isonitriles can inhibit the tautomerization, which has been applied in the bioorthogonal labeling of polysaccharides containing isocyano group providing the chemical toolbox for metabolic glycan labeling.[120] It is shown that the bulky tetrazine substituents accelerated this cycloaddition. The computational studies by Franzini's group indicated that the dispersion forces between the isocyano group and the tetrazine substituents in the transition state contributed to the atypical structure-activity relationship.[121] Their research showed that the selective reaction of sterically encumbered tetrazines with isonitriles in the presence of strained alkenes/alkynes allowed for the orthogonal labeling of three proteins.

5.1.2. Cycloadditions of Isonitriles and Tetrazines Leading to Bioorthogonal Cleavages

Franzini and co-workers used the removable 3-isocyanopropyl substituents as the masking groups to chemically control the release of bioactive agents and reporter probes.[13,121–122] The cascade reactions of 3-isocyanopropyl derivatives (**114**) with tetrazines (**115**) including [4 + 1] cycloaddition, cycloreversion, tautomerization and hydrolysis led to 3-oxopropyl aldehydes (**117**) and aminodizole (**120**), and their further eliminations liberated phenols and amines near-quantitatively under physiological conditions (Figure 25).[122] The reaction showed biocompatiblity with living organisms and realized the release of a resorufin fluorophore and a mexiletine drug in zebrafish embryos implanted with tetrazine-modified beads. The method shows some advantages including synthetic ease, rapid kinetics, diversity of leaving groups, high release yields, and structural compactness, allowing 3-isocyanopropyl derivatives attractive chemical caging moieties for applications in chemical biology and drug delivery. Very recently, Franzini's group reported the isonitrile-induced removal of tetrazylmethyl derivatives to get amines and phenols. They found that the deprotection was especially effective with (trimethylsilyl)methyl isocyanide, and the elimination serum albumin was catalyzed under physiological conditions. The mechanism invesitigations showed that the imine-tautomerization step was rate limiting, and the Si–C

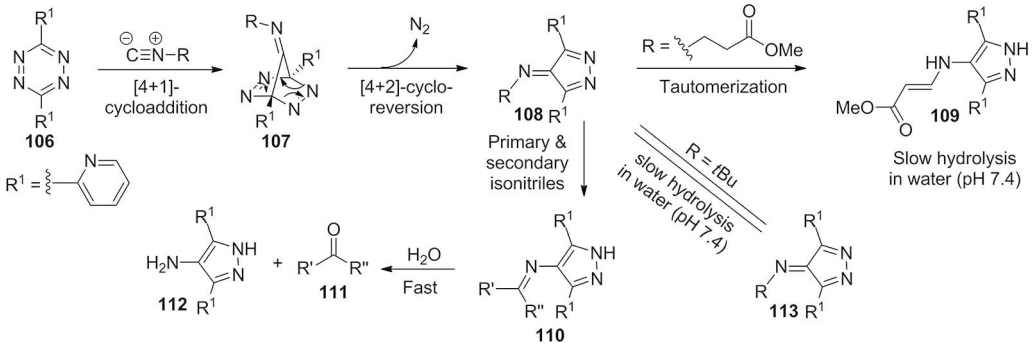

Figure 24. [4 + 1] Cycloaddition between isonitriles and tetrazines.

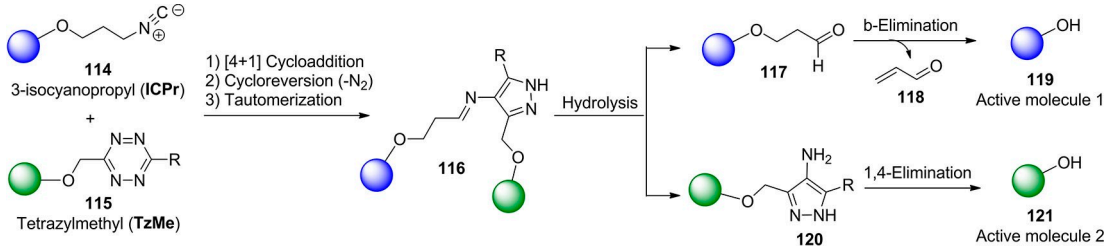

Figure 25. Cycloaddition of isonitriles and tetrazines leading to release of active molecules.

bond cleavage promoted this step in the case with (trimethylsilyl)methyl isocyanide. Tetrazylmethyl-removal showed biocompatiblity in cellular environments, living organisms and zebrafish embryos.[123]

5.2. Inverse Electron Demand [4+2] Cycloadditions

5.2.1. Inverse Electron Demand [4+2] Cycloadditions Leading to Biorthogonal Ligations

The inverse electron demand Diels-Alder reactions (IEDDA) have become a popular biorthogonal method due to their fast kinetics, excellent orthogonality and biocompatibility (Figure 26).[14b,124]

The inverse electron demand Diels-Alder (IEDDA) reaction of 1,2,4,5-tetrazines and olefins was initially used in the synthesis of pyridazines.[125] In 2008, Fox and co-workers installed a protein, thioredoxin (Trx) (**123**) on *trans*-cyclooctene (TCO) **122**

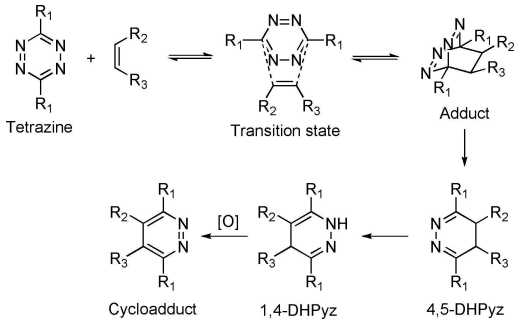

Figure 26. Mechanism on the inverse electron demand Diels-Alder reaction (IEDDA).

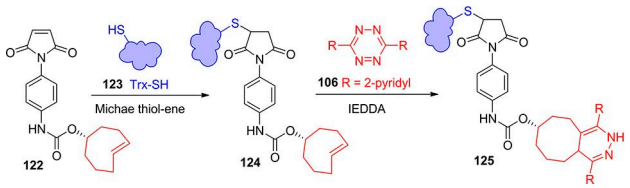

Figure 27. IEDDA of tetrazines and *trans*-cyclooctenes as the bioorthogonal ligation.

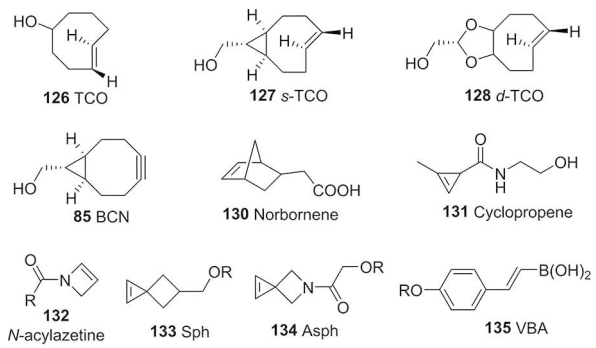

Figure 28. Common alkenes in IEDDA reaction with tetrazines.

via Michael addition of sulfydryl in **123** to maleimide in **122** to get **124**, and rapid IEDDA of **124** with 1,2,4,5-tetrazine (**106**) provided conjugate **125** under physiological conditions (Figure 27).[6] The reaction was used in biorthogonal ligation for the first time without addition of catalyst, and it shows very high reaction rate and good biocompatibility and has been widely used in various fields.[14a,126]

Reactivity of various substrates as the partners of tetrazines has been widely investigated.[14b,124a] Besides *trans*-cyclooctenes, other strain alkenes and alkynes (Figure 28) such as norbornene **130**,[127] (*E*)-bicyclo[6.1.0]non-4-ene (*s*-TCO) (**127**),[128] bicyclononyne (BCN) (**85**),[128] cyclopropane **131**,[129] *N*-acylazetine **132**,[130] and spiro[2.3]hex-1-enes (Sph (**133**) and Asph (**134**))[131] were also uded in the cycloadditions with tetrazines. Meanwhile, Bonger and co-workers reported the iEDDA reaction of 3,6-dipyridyl-s-tetrazines with biocompatible, non-toxic, and highly stable vinylboronic acids (VBAs) as the non-strained, synthetically accessible and water-soluble reaction partners. The reaction showed the high rate (second-order rate constants up to 27 $M^{-1}s^{-1}$) in aqueous environments at room temperature and is suitable for biological labeling applications.[132] Among the olefins, *trans*-cyclooctenes (TCOs) showed very fast reaction rates in IEDDA. Unfortunately, the TCOs can transform into low reactive *cis*-cyclooctenes in the presence of thiols.[111] In order to solve the problem, Fox's group designed dioxolane-fused *trans*-cyclooctene (*d*-TCO) (**128**). **128** exhibited higher reactivity than TCOs, and no isomerization or decomposition was observed after incubation in serum for 4 days at room temperature.[133]

Although various alkenes have been developed in the IEDDA reactions with tetrazines, the investigations on structures of tetrazines are not too much. In 2012, Devaraj and co-workers reported the metal-catalyzed one-pot synthesis of tetrazines directly from aliphatic nitriles and hydrazine.[134] They compared reactivity of different cyclopropene dienophiles with tetrazines and found that the reaction rates significantly increased for the tetrazines containing electron withdrawing groups at the 3 and 6 positions.[134] Unfortunately, the tetrazines with strong electron-withdrawing substituents could be hydrolyzed and showed lower stability.[135] To improve the stability of the tetrazine skeleton, the Prescher's group developed 1,2,4-triazine **137** as the bioorthogonal reagent, and it showed good stability in biological media and fast reaction rate with transcyclooctene (TCO) (Figure 29).[136] Recently, the method was successfully used in the DNA labeling.[137]

In 2019, Boger's group reported the synthesis, characterization, and cycloaddition reactivity of a monocyclic aromatic 1,2,3,5-tetrazine and found that it showed good stability and rapid IEDDA rate with electron-rich or strain-containing dienophiles.[138] In addition, Murphy and Houk reported the cycloaddition reactions of tetrachlorocyclopentadiene ketal (**142**) with bicyclononyne (BCN) (**85**) or *trans*-cyclooctene (TCO) for bioorthogonal labeling (Figure 30).[139]

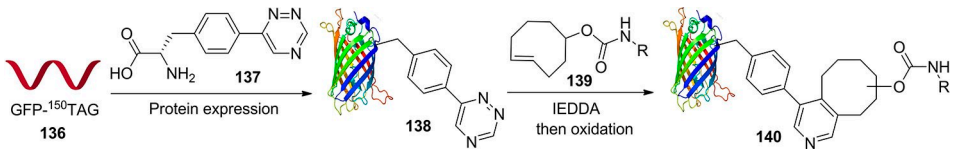

Figure 29. IEDDA reaction of triazine **137** with **139** for the posttranslational modification of recombinant proteins.

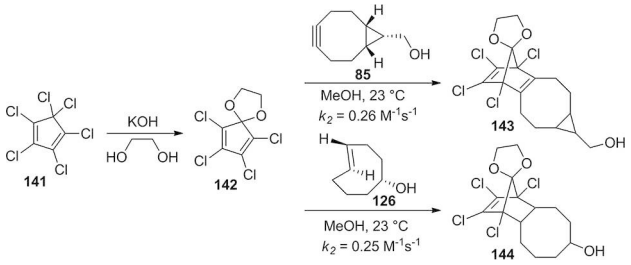

Figure 30. Cycloaddition reactions of tetrachlorocyclopentadiene ketal (**142**) with bicyclononyne (BCN) (**85**) or *trans*-cyclooctene (TCO) (**126**).

5.2.2. Inverse Electron Demand [4+2] Cycloadditions Leading to Biorthogonal Cleavages

In 2013, Robillard and co-workers installed a carbamate group containing doxorubicin at the allyl position of *trans*-cyclooctene to form **145**, and reaction of **145** with tetrazine **146** provided cycloaddition product **147**. Isomerization of **147** led to **148**, and rearrangement of bonds in **148** released active drug, doxorubicin and **149** (Figure 31).[140] The studies indicated that *trans*-cyclooctene derivatives with axial carbamate showed much faster reaction rate than those with the equatorial conformational isomers.[141]

Chen and co-workers reported the small molecule-triggered bioorthogonal protein decaging technique via the inverse electron-demand Diels-Alder reaction for eliminating a chemically caged protein side chain within living cells, and the method provides the efficient activation of the enzyme in its native cellular context in a short time.[142] They systematically investigated 3,6-substituted tetrazine derivatives by using a fluorogenic *trans*-cyclooctene–coumarin reporter and LC-MS analysis. The results showed that the electron-withdrawing groups (EWGs) greatly promoted the initial inverse-electron-demand Diels-Alder cycloaddition step and strongly suppressed the subsequent elimination step. They found that the smaller substituents facilitated the decaging process, and the tetrazines containing EWG group and small non-EWG group at the 3- and 6-position showed the remarkably enhanced decaging rates to lead to the protein activation in living cells.[143]

Weissleder and co-workers investigated the corresponding mechanism and found that acid-functionalized tetrazine (**153**) could markedly accelerate release and ultimately uncover an unexpected dead-end isomer (**156**) for poor release. *N*-Methyl derivative **157** could prevent formation of this dead end and achieve exceptional efficiency (Figure 32).[144] In previous reseaches, the release of amines was widely reported via IEDDA of tetrazines and *trans*-cyclooctenes, but that of alcohols was less investigated. In 2019, Bernardes's group reported the TCO-carbamate benzyl ether self-immolative linker for the release of OH-molecules via IEDDA of tetrazines and *trans*-cyclooctenes, and the stable benzyl ether linker could rapidly liberate alcohols under physiological conditions.[145]

The cycloaddition reaction between vinyl ether and tetrazine can also achieve bioorthogonal decaging. In 2016, Devaraj's group reported a method of activating the fluorescent activity after reacting with tetrazine using a fluorophore **161** coated with vinyl ether (Figure 33a).[146] In 2017, Bernardes's group reported vinyl ether/tetrazine pair for the traceless release of alcohols in cells, in which vinyl ethers containing amino acids, a monosaccharide, a fluorophore, or an analogue of the cytotoxic drug duocarmycin were used as the protecting groups for alcohol-containing molecules and as the reagents for tetrazine-mediated bioorthogonal bond-cleavage reactions, and

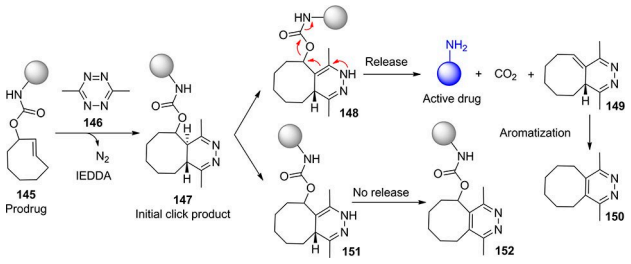

Figure 31. Proposed mechanism for the release of active drug via reaction of doxorubicin-functionalized *trans*-cyclooctene (TCO) with tetrazine.

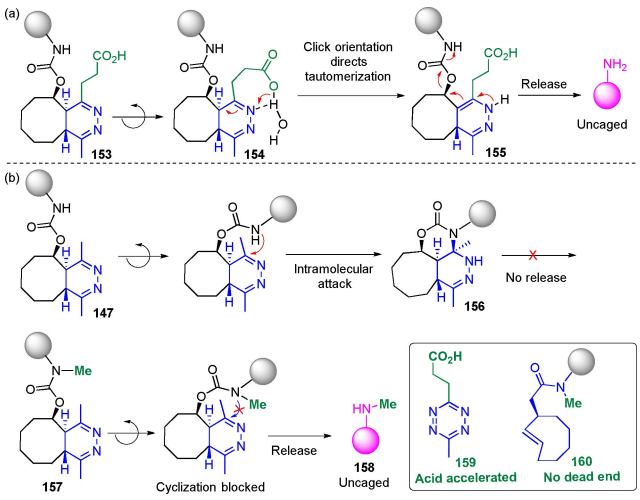

Figure 32. (a) Acid-promoted the release of active amine. (b) Methyl substitution-prevented the formation of dead-end isomer.

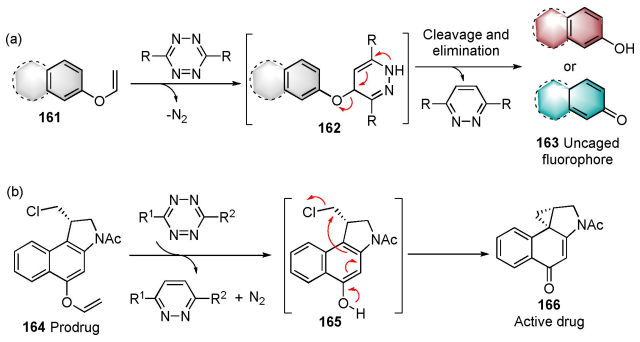

Figure 33. Reaction of vinyl ethers with tetrazines to achieve fluorophore (a) or prodrug (b) release.

the high yields and reasonable kinetics were observed under biocompatible conditions. Importantly, decaging of the non-toxic, vinyl ether duocarmycin double prodrug was successfully achieved in live cells to reinstate cytotoxicity.[147] Bradley and co-workers reported the highly efficient "decaging" of various moieties by water-soluble and stable tetrazine-mediated activation of a "self-immolative" linker such as the controlled delivery of doxorubicin in a cellular context (Figure 33b).[148]

In 2017, Franzini's group reported the bioorthogonal release reactions based on benzonorbornadiene derivative. These carrier molecules with high stablity under physiological conditions reacted rapidly with 1,2,4,5-tetrazines to near-quantitatively release the cargo molecules.[149] In addition, reaction of cyclooctynes with tetrazines was also used for bioorthogonal cleavage. Cyclooctyne **167** containing hydroxyl at its propargyl position reacted with tetrazine **168** to provide **169** and **170**, and intramolecular nucleophilic attack of hydroxyl to amide in **170** led to the release of active molecule **172** (Figure 34).[150]

6. Light-Triggered Bioorthogonal Reactions

Since the light-triggered bioorthogonal reactions show high controllability of time and position, the initiation and process of the reactions can be effectively controlled through variation of light wavelength and intensity. The common methods include the light-induced transformation of small molecule structures to trigger the bioorthogonal reactions and introduction of photo-cleavable protecting groups to control the activity and function of biomolecules.

6.1. Light-Triggered Bioorthogonal Ligations

The light-induced generation of highly active free radicals and carbenes is widely used in chemical biology. However, applications of the light-triggered bioorthogonal methods are limited due to the lack of reaction selectivity of the photoactive groups.[151] The other type of light-triggered reactions with bioorthogonal characteristics show their unique advantages in some fields, and the initiation and process of the reactions can be controlled through the choice of suitable wavelength and intensity of light.[152]

In 1967, Huisgen and co-workers reported the photo-activated the 1,3-dipolar cycloaddition between 2,5-diphenyltetrazole (**173**) and methyl crotonate,[153] and the reaction undergoes photoinduced release of N_2 in the diaryl tetrazole to generate in situ a nitrile imine dipole (**174**) followed by spontaneous cyclization with an alkene dipolarophile to provide a pyrazoline cycloadduct (**175**) (Figure 36a). In 2008, Lin and co-workers developed the photoinducible 1,3-dipolar cycloaddition reaction of the tetrazole group attached to the proteins with alkene to afford the labelled proteins in biological media. The reaction showed fast rate and tolerance of proteinaceous groups, and the fluorescent adducts were formed (Figure 35b).[154] In 2010, the same group developed the genetic incorporation of photoreactive *p*-(2-tetrazole)phenylalanine (*p*-Tpa) into myoglobin site-specifically in *E. coli* to form **177** by evolving an orthogonal tRNA/aminoacyl-tRNA synthetase pair, and then the photoclick reaction of *p*-Tpa in **177** as the bioorthogonal chemical "handle" achieved fluorescent labeling of *p*-Tpa-encoded myoglobin (Figure 35b).[154] The highly unstable nitrile imines in Figure 36a can quickly decompose or participate in other side reactions.[154] In 2018, they reported the bioinspired strategy of molecular shape controls the selectivity of the highly reactive nitrile imine dipole. They found that the sterically shielded nitrile imine favored the 1,3-dipolar cycloaddition over the competing nucleophilic addition, and the extraordinarily long half-life of 102 s was observed for the photo-generated nitrile imine in aqueous medium. The sterically shielded nitrile imine was used in rapid (~1 min)

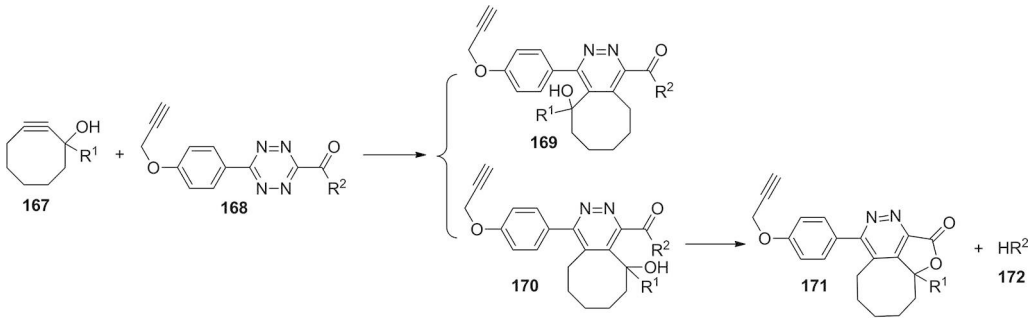

Figure 34. Reaction of cyclooctyne containing hydroxyl with tetrazine leading to release of active molecule.

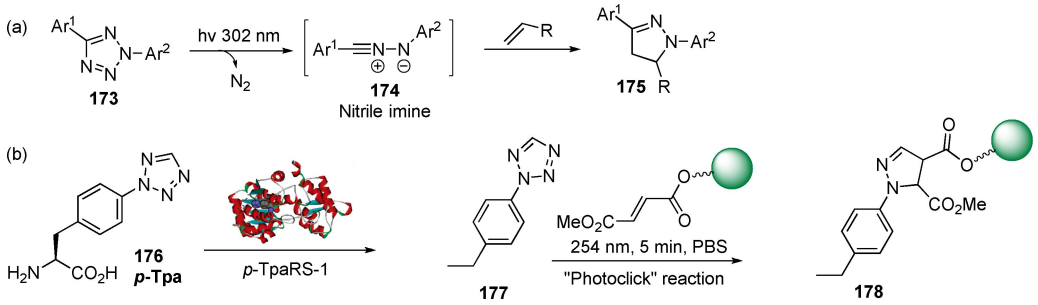

Figure 35. (a) Photoactivated 1,3-dipolar cycloaddition between 2,5-diaryl tetrazole and substituted alkene dipolarophile. (b) Photoinduced site-specific labeling of protein with genetically encode tetrazole.

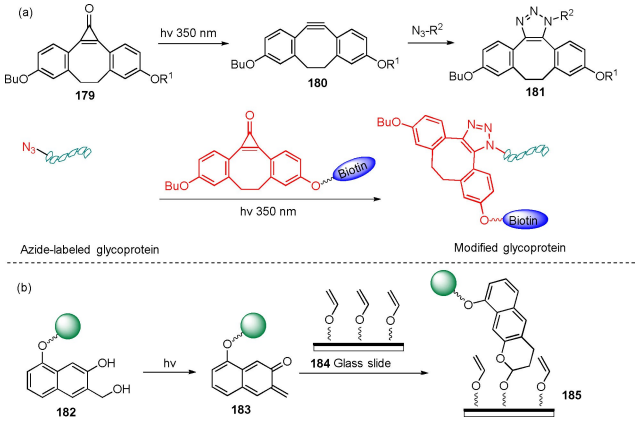

Figure 36. (a) Cyclopropenones (**179**) as the precursors of dibenzocyclooctynes (**180**) in the strain cycloaddition. (b) 3-(Hydroxymethyl)-2-naphthol as the precursor of 2-napthoquinone-3-methide in the photochemically induced hetero-Diels-Alder reaction.

bioorthogonal labeling of glucagon receptor in live mammalian cells.[155]

In 2009, Boons and Popik developed cyclopropenones (**179**) as the precursors of dibenzocyclooctynes (**180**). The cyclopropenones do not react with azides in the dark, and the light irradiation of **179** formed the corresponding dibenzocyclooctynes, which then underwent cycloadditions with azides to provide the corresponding triazoles (**181**) under ambient conditions. The strategy was used in the labelling of living cells expressing glycoproteins containing *N*-azidoacetyl-sialic acid (Figure 36a).[156] Popik's group developed the photochemically induced hetero-Diels-Alder reaction for the light-directed surface derivatization and patterning. Aqueous solution of substrate (**182**) containing 3-(hydroxymethyl)-2-naphthol (NQMP) was adsorbed on glass slides functionalized with vinyl ether moieties. 3-(Hydroxymethyl)-2-naphthol part transferred into reactive 2-napthoquinone-3-methide (*o*NQM) (**183**) via irradiation of shadow mask, and the fast hetero-Diels-Alder addition ($k_{D\text{-}A} \approx 4{\cdot}10^4\ M^{-1}\ s^{-1}$) of *o*NQM with vinyl ether part immobilized on the glass slides provided the photochemically stable conjugate (**184**). The rapid hydrolysis of unreacted oNQM groups formed NQMP (Figure 36b).[157] In 2018, Zhang and coworkers developed a visible light-triggered cycloaddition of 9,10-phenanthrenequinone with electron-rich alkenes to provide fluorogenic [4+2] cycloadducts under irradiation of a hand-held LED lamp. The bioorthogonal photoclick fast reaction showed biocompatiblity without observable side reactions such as nucleophilic additions by water or common nucleophilic species, and the reaction was used in the temporal and spatial labeling of live cells.[158] Besides, other building blocks such as *o*-nitroanisole and *o*-nitrobenzyl alcohol were also applied in photo-induced bioconjugation reactions.[159]

6.2. Light-Triggered Bioorthogonal Cleavages

No additional chemical reagents are required in the light-triggered bioorthogonal cleavages, and the method shows the high selectivity and stability under physiological conditions.[12,160] Among them, the readily available and UV light-cleavable *o*-nitrobenzyl ether derivatives are widely used in the proteomics researches.[161] Recently, Chan developed a light-controlled formaldehyde donor to achieve the quantitative release of formaldehyde in the living cells. Both silaxanthene and *o*-nitrobenzyl parts were connected through the acetals (**186**–**188**), and under UV light irradiation the acetals released hemiacetals, which hydrolysis provided formaldehyde and silaxanthene containing hydroxyl with strong fluorescence signal (Figure 37).[162]

In 2018, Chenoweth's group reported the reversible control of protein localization in the living cells using the photocaged-photocleavable chemical dimerizer (**189**). They developed the chemical inducer of protein dimerization that could be quickly turned on and off with single pulses of light at two orthogonal wavelengths, and the utility of the molecule by controlling peroxisome transport and mitotic checkpoint signaling was performed in the living cells. (Figure 38).[163] In 2019, Schultz's group developed the photorelease of 2-arachidonoylglycerol in the living cells, in which the rapid photorelease of the signaling lipid was performed.[164]

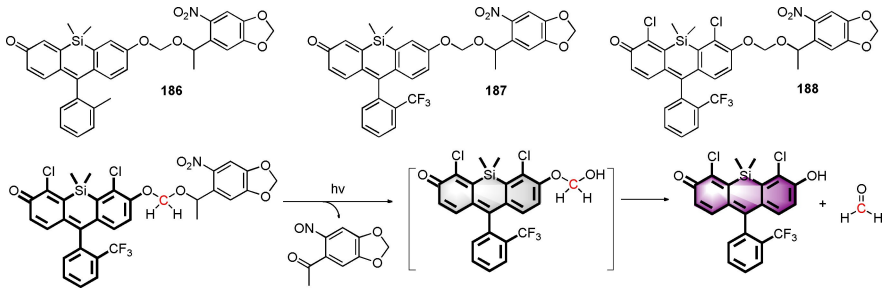

Figure 37. Light-triggered cleavage of acetals containing *o*-nitrobenzyl ether part.

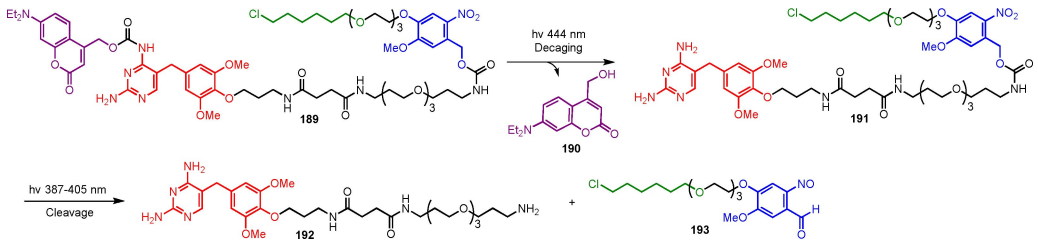

Figure 38. Light-triggered bioorthogonal cleavage of **189** at two orthogonal wavelengths.

7. Reactions of Chloroquinoxalines and *ortho*-Dithiophenols Leading to Bioorthogonal Ligations and Cleavages

Very recently, Fu and co-workers reported the bioorthogonal ligations and cleavages via the selective reactions of readily accessible chloroquinoxalines (CQ) and *ortho*-dithiophenols (DT). Double nucleophilic substitutions of DT to CQ provided the conjugates containing tetracyclic benzo[5,6][1,4]dithiino [2,3-*b*]quinoxalines with strong built-in fluorescence with releasing the other active molecules (Figure 39). The CQ-DT bioorthogonal reactions were used in the bioorthogonal ligations, bioorthogonal cleavages and simultaneous bioorthogonal ligations and cleavages, and the method exhibits some advantages including the readily accessible unnatural orthogonal groups, the appealing reaction kinetics ($k_2 \approx 1.3\ M^{-1}s^{-1}$), excellent biocompatibility of the orthogonal groups and high stability of the conjugates.[165]

8. Conclusions and Outlook

In this review, the advances on the six kinds of representative bioorthogonal reactions have been summarized. The methods show extreme selectivity and biocompatibility in richly functionalized biological systems, in living cells or in living organisms. Although great achievements have been made, there are still some challenges existing in this field, such as the needs for higher selectivity, faster reaction rates, lower concentration of bioorthogonal handles, wavelength-controlled visible light-triggered biorthogonal reactions, more efficient time- and site-related release of active molecules, no excess of the partners, using uncommon elements in life systems, and method

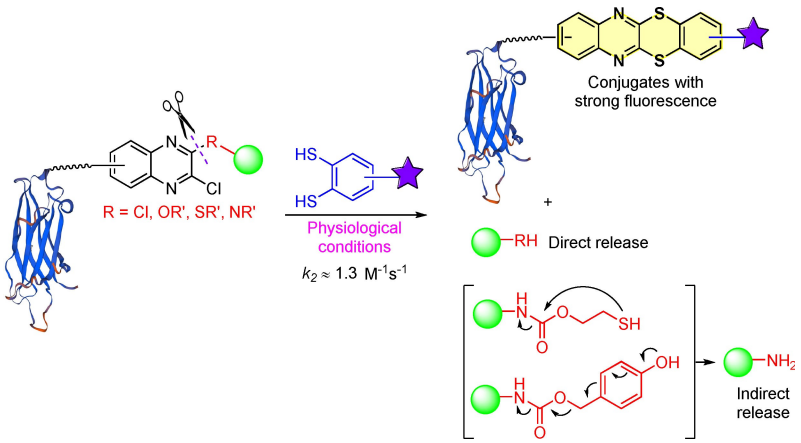

Figure 39. Bioorthogonal ligations and cleavages by reactions of chloroquinoxalines with *ortho*-dithiophenols.

universality. In these regards, we anticipate that the problems will be solved as intimat cooperation and continuous exploration of chemists and biologists. We believe that the developments and applications of bioorthogonal ligations and cleavages still is an active area of research in chemical biology, and more new and efficient methods will be discovered in the future. It should be noted that the examples for other bioorthogonal ligations and cleavages are existing, and any omissions on this wide topic are unintentional for the length limition of this review.

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Conflict of Interest

The authors declare no conflict of interest.

Keywords: bioorthogonal reactions · bioorthogonal ligations · bioorthogonal cleavages · biomolecules · chemical modification

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