HHS Public Access

Author manuscript

Nat Rev Chem. Author manuscript; available in PMC 2021 July 20.

Published in final edited form as:

Nat Rev Chem. 2020 ; 4: 476–489. doi:10.1038/s41570-020-0205-0.

Developing bioorthogonal probes to span a spectrum of reactivities

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Abstract

Bioorthogonal chemistries enable researchers to interrogate biomolecules in living systems. These reactions are highly selective and biocompatible and can be performed in many complex environments. However, like any organic transformation, there is no perfect bioorthogonal reaction. Choosing the “best fit” for a desired application is critical. Correspondingly, there must be a variety of chemistries—spanning a spectrum of rates and other features—to choose from. Over the past few years, significant strides have been made towards not only expanding the number of bioorthogonal chemistries, but also fine-tuning existing reactions for particular applications. In this Review, we highlight recent advances in bioorthogonal reaction development, focusing on how physical organic chemistry principles have guided probe design. The continued expansion of this toolset will provide more precisely tuned reagents for manipulating bonds in distinct environments.

I. Introduction

A comprehensive view of living systems requires tools and methods to probe biomolecules in their native habitats. Fluorescent proteins and other genetic tags have long been used in this capacity[1]. While powerful, such tools are not amenable to direct monitoring of non-proteinaceous targets, including small molecule metabolites. The need for more generalizable platforms spurred the development of bioorthogonal chemistries—reactions that are so selective that they can be used to covalently tag targets in live cells and, in some cases, living organisms. For decades, bioorthogonal reactions have been used to visualize and profile a broad spectrum of biomolecules. These studies have revealed fundamental new insights into various aspects of cell and organismal biology. Such studies also revealed limitations in the bioorthogonal toolkit that inspired the development of even more selective and finely tuned probes.

Central to all applications of bioorthogonal chemistry are reactions that are robust and compatible with living systems. The development of such transformations can be quite difficult. The solvent and temperature are fixed, and the reactions must proceed in the midst

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of a multitude of interfering functionality. The reactions often cannot be accelerated simply by “heating up” the subject or adding more reagent. Thus, the canonical rules for fine-tuning chemistries in round-bottom flasks often fail to translate to physiological environments, where few parameters can be varied[2]. Nonetheless, generations of chemists have been inspired to control bond formation in live cells and organisms. Their efforts have provided transformations that can be executed without detriment in living systems.

This article reviews the development of bioorthogonal reactions, with an emphasis on how mechanistic insights have driven the field. Like other areas of chemistry, no “one-size-fits-all” transformation exists. Rather, each reaction has its pros and cons, with limitations continuing to propel new advances. In the first section, we provide a brief history on bioorthogonal chemistry and introduce common tactics that facilitated early probe development. The bulk of the review then showcases recent achievements in bioorthogonal reaction design. We also highlight efforts to engineer bioorthogonal chemistries to be used concurrently for multi-component labeling. These and other innovations will continue to expand the collection of biocompatible and mutually orthogonal reagents.

II. Setting the stage: Classic bioorthogonal transformations

Chemists rely on robust and versatile reactions to craft complex molecules. Synthetic routes are drafted with reagent accessibilities, yields, and selectivities in mind. Ideally, the transformations are fast, selective, and applicable to a broad range of substrates. In reality, most reactions are not universally efficient and require tweaks to temperature, pH, or stoichiometry in different contexts. Catalysts and solvents are also varied to achieve optimal yields. Limitations in reaction scope often become the inspiration for new transformations. This iterative cycle of reaction discovery and refinement has provided a compendium of methods for controlling bond formation in various contexts. In some cases, hundreds, if not thousands, of specialized reagents have been developed to address shortcomings in reaction scope.

Iterative refinement has also been used to tune reagents for use in living systems, with certain considerations in mind (Figure 1)[2,3]. Bioorthogonal functional groups must toe the line of being kinetically and metabolically stable, yet prone to rapid reaction with complementary probes under physiological conditions. Such reactions must also be tolerant of water and other biological functional groups[4–6]. The constraints imposed by cells and tissues exclude many organic transformations. Several biological applications also demand reagents that impart a minimal steric “footprint”[7,8]. Thus, developing chemistries that feature small reagents is another important goal.

So, where does one begin? Hunting for unusual functional groups in heterologous organisms is a good starting point. Microbes and other species often have access to chemistries and functionality not present in mammalian cells. Thus, these motifs and chemistries can survive in living systems and are immediately “orthogonal”. A classic example is the alkyne, a motif that is present in numerous microbial metabolites[9], but absent in higher eukaryotes. The alkyne has been applied as a bioorthogonal motif in numerous settings[2]. Popular bioorthogonal functional groups also comprise some unlikely candidates from synthetic

chemistry[10,11]. Organic azides and strained alkynes were often viewed as too unstable for use in living systems. However, careful tuning provided reagents now recognized among the gold standards in the field. These examples provided important lessons for subsequent reagent development[12]. In this section, we provide a brief perspective on how some seemingly "misfit" functional groups became stalwarts of the two most common classes of bioorthogonal chemistry: polar reactions and cycloadditions. We highlight early obstacles and key advances in the development of these probes. Initial successes provided a roadmap for continued reagent refinement.

Polar reactions

Aldehydes and ketones were among the first reagents employed as bioorthogonal labels. Such carbonyl groups were attractive for biomolecule tagging, given their small size and compatibility in living systems[13]. The electrophiles were also readily condensed with α-nucleophiles. Aldehyde and ketone condensations have been used to label a wide variety of biomolecules[14–17]. However, the reactions are pH sensitive and quite slow in physiological settings. Aniline catalysts[18–21] can boost the rates, although the transformations remain difficult to execute in cellular environments.

While aldehydes and ketones have been less employed in cells, only a few other bioorthogonal functional groups rival their minimal size. Among the most influential has been the organic azide. This motif is abiotic and comprises just three atoms. Azides are remarkably inert in biological settings, but exhibit unique manifolds of reactivity. In one case, they can react with soft nucleophiles, including triarylphosphines (via Staudinger reduction). This reaction proceeds via an aza-ylide intermediate, which can be intercepted with neighboring electrophiles. Bertozzi and coworkers capitalized on this feature, installing an ester on the phosphine to trap the aza-ylide. The transformation ultimately linked the two reactants via an amide bond. This variant – termed the Staudinger ligation – was amenable to tagging azides in a variety of complex environments, including live cells[22]. Early applications featured glycans and post-translational modifications, although many other biomolecules have since been targeted[23–27]. The Staudinger ligation was also the first of its kind to be used in live rodents, a particularly demanding environment[28].

The versatility of the Staudinger ligation propelled an entire field of reaction development. Many early studies were directed at improving ligation speed[4,5]. While the reaction was robust enough for certain *in vivo* applications, the slow rate proved challenging for imaging studies in rodents and higher organisms. Large boluses of reagent were required to drive covalent bond formation on reasonable time scales. Such quantities were not easily achieved, due to the limited water solubility of many phosphine probes. These shortcomings generally sidelined the Staudinger ligation *in vivo*, but proved a fruitful ground for inspiring other types of transformations with organic azides.

Cycloadditions

Cycloadditions are popular bioorthogonal transformations owing to their exquisite chemoselectivity. One of the earliest exploited was [3+2] cycloaddition with azides. In addition to being mild electrophiles, organic azides are 1,3-dipoles subject to react with

alkynes. As noted earlier, alkynes are rare in higher eukaryotes, rendering them suitably orthogonal. However, azide-alkyne cycloadditions typically require high temperatures or pressures to proceed, conditions that are not biocompatible. A key breakthrough was the recognition that the cycloaddition could be accelerated via Cu(I). The copper-catalyzed azide-alkyne cycloaddition (CuAAC) is ubiquitous in chemical biology and other disciplines[29,30], and is still inspiring new transformations[31]. Cu(I) cytotoxicity[32–34] has precluded CuAAC application *in vivo* in some cases, although more biofriendly catalysts have been developed. Many feature water-soluble ligands that stabilize Cu(I) and prevent the formation of reactive oxygen species[35–38]. Polytriazole ligands, in particular, enabled CuAAC reactions to be conducted in live cells and zebrafish[36].

The constraints posed by copper catalysts also inspired chemists to devise new strategies to ligate 1,3-dipoles. One of the most fruitful approaches relied on strain energy – bending the alkyne from its normal linear geometry[39]. The smallest of the stable cycloalkynes, cyclooctyne, was found to react with azides in the absence of a copper catalyst[40]. This work gave rise to an entire family of strain-promoted azide-alkyne cycloadditions (SPAACs). Such reactions have been widely used in complex biological settings, including plants[41], *C. elegans*[42], and mice[43–46]. While SPAAC reactions minimize toxicity in living systems, some of the alkynes react with biological thiols[47]. Moreover, the fastest rate constants with the most reactive strained alkynes plateau at rates of ~1 M^{-1} s^{-1}, which are not suitable for some *in vivo* applications.

Other cycloadditions have been developed to address the need for fast-acting, biocompatible reagents. One notable class comprises inverse electron-demand Diels–Alder (IEDDA) cycloadditions. The IEDDA reaction between tetrazine and *trans*-cyclooctene (TCO), in particular, has gained prominence[11,48–50]. This reaction pair boasts rates up to 10^6 times faster than the most reactive SPAAC pair. Such rapid reactions have widespread use in cells and living organisms, as only small amounts of material are required to drive covalent bond formation. The kinetic profile of the tetrazine-TCO cycloaddition has enabled the exploration of new imaging platforms *in vivo*, including PET imaging[51,52] and other radiolabels[53–55]. Tetrazine-TCO reactions have also been widely employed to tag biomolecules, release prodrugs[56–59], and activate enzymes[60–62].

III. Fine-tuning bioorthogonal reagents and reactions

Despite remarkable achievements in bioorthogonal reaction design over the past two decades, limitations remain. Many reagents are insufficiently stable for use in the most demanding biological settings. Others remain too slow to transition to *in vivo* applications. And while many of the reactions work well on single targets, most cannot be used in combination due to cross-reactivity concerns. Simultaneous tracking of multiple biomolecules remains a longstanding challenge. Collectively, these limitations underscore the need to not only develop new reactions, but also fine-tune existing chemistries. The next sections highlight recent approaches to address voids in the bioorthogonal toolbox and broaden the spectrum of reactivities (Table 1).

Tuning polar reagents and reactions

As noted previously, some of the earliest bioorthogonal transformations involved condensation reactions with carbonyls and α-nucleophiles. The reversible nature of these reactions presented challenges in biomolecule labeling. Efforts to forge more stable adducts have been undertaken, including the use of proximal stabilizing groups[63–69]. One example showcases boronic acids. These functional groups can coordinate ligation adducts, including hydrazones[64] and oximes[65,66], preventing hydrolysis. Boronic acids have also been used to stabilize ligation adducts from other α-nucleophiles[67–69], and have recently been applied in tagging N-terminal cysteine residues on peptides and small proteins[70,71].

Polar reactions with azides have also been tuned for specific applications. The Staudinger ligation was an early target for optimization, with efforts focused on improving rates and reagent solubility. Introducing electron-withdrawing groups on the azide or electron-donating groups on the phosphine boosted the ligation speed. For example, electron-deficient aryl azides with fluorine[72] and chlorine[73] substituents exhibited faster rates compared to their unsubstituted counterparts. Interestingly, the key aza-ylide formed in these cases was a stable adduct, obviating the need for an electrophilic trap on the phosphine. The halogenated aryl azides have since been used to label glycans and proteins in live cells[72,73]. Generating suitable electron-rich phosphines for Staudinger ligation has been less straightforward. More nucleophilic scaffolds can reduce disulfide bonds in proteins and are prone to rapid oxidation. Despite this liability, numerous phosphine probes have been tuned for cellular and other applications[74–76].

Phosphine reagents have also been tuned for improved biocompatibility. For example, Ren and co-workers synthesized phosphines comprising a fluorosulfate group[77]. This handle functions similarly to the ester trap in the original Staudinger ligation, by intercepting the aza-ylide. After ejection of fluoride and hydrolysis, the ligation provides an aryl sulfamate ester. The modified phosphine displayed markedly improved water solubility compared to initial Staudinger ligation probes. An additional benefit was that the sulfamate ester products mimic phosphate backbones, present in many biomolecules and metabolites.

Entirely different phosphorus nucleophiles have also been examined for azide ligation, with an eye towards forming mimics of biological functional groups. For example, phosphites[78–80] and phosphonites[81–83] react similarly with aryl azides to provide phosphoramidate and phosphonamidite adducts, respectively. These reactions can be used to selectively modify proteins and other biomolecules with a variety of probes.

Tuned phosphines have been exploited in other chemical contexts. Phosphines can react with Michael acceptors, forming stable phosphonium adducts. This transformation has been employed to label biomolecules. In one study, tris(2-carboxyethyl)phosphine (TCEP), a water-soluble reductant widely used in biology, was reacted with electron-deficient alkenes. This reaction was used to study protein glycosylation in live cells[84] and crotonylation patterns on histone proteins[85].

A more recent addition to the phosphine ligation kit comprises cyclopropenone derivatives (CpXs)[86–89]. In the case of cyclopropenones (X = O), the ligation proceeds via an initial

Michael-type addition, followed by ring-opening to form a reactive ketene-ylide. The ketene can be trapped by pendant nucleophiles on the phosphine to furnish α,β-unsaturated carbonyls. Mono-substituted CpOs were subject to react with biologically relevant thiols[86], but further tuning provided more bioinert scaffolds comprising dialkyl motifs[87]. These latter reagents can be used in live cells.

The reactivity of CpOs can be further modulated via heteroatom replacement, a common strategy for probe tuning in bioorthogonal chemistry. The sulfur variant of CpO – the cyclopropenethione (CpS) – also reacts rapidly with phosphines via thioketene-ylide intermediates[88]. Thiocarbonyl products are formed, which can be useful biophysical probes to study protein stability and function[90,91]. Nitrogen CpO heteroanalogs, cyclopropeniminium (CpN^+) ions, were also explored as bioorthogonal reagents. Interestingly, CpN^+ ions react with phosphines via a different mechanism than CpO or CpS derivatives. The initial Michael addition provides an enamine, which undergoes proton transfer instead of ring opening to give phosphonium bicycles[89] (Figure 2). Thus, small changes to a core scaffold can have profound effects on reactivity.

Tuning dipoles for bioorthogonal cycloaddition

The azide-alkyne cycloaddition remains one of the most popular 1,3-dipolar cycloadditions for examining biological systems. The success of this ligation has served as inspiration for exploring new 1,3-dipoles (Figure 3A). One example includes nitrones[92], reagents that can react more rapidly than azides with certain alkynes[93–95]. Nitrone-alkyne cycloadditions, similar to their azide-alkyne counterparts, can be accelerated by copper catalysis. Such reactions have been used to image sugar metabolism and receptor-ligand interactions in mammalian cells, as well as peptidoglycan structures in bacteria[96–98].

Another newcomer to the bioorthogonal dipole set is sydnone[99]. This 1,3-dipole reacts with alkynes to afford pyrazole adducts. Although initial applications required copper catalysis[100], subsequent work identified scaffolds that were also capable of copper-free reactions with strained alkynes[101]. Sydnones have undergone further modification to modulate their reactivity for biological application. Chlorine substituents were found to increase reaction rates (30-fold) with a popular strained alkyne, bicyclo[6.1.0]nonyne (BCN)[102]. Fluorine substitution further boosted reaction rates of sydnones with a variety of strained alkynes[103].

Close relatives of organic azides, diazo compounds have been similarly tuned as dipoles for bioorthogonal application[104]. The small size of the diazo group makes it attractive for biomolecule labeling, but such motifs were long thought to be too reactive for cellular use. However, diazo motifs are stable when in conjugation with aryl systems or electron-withdrawing groups (e.g., esters, amides)[105,106]. They ligate strained alkynes with second-order rate constants similar to azides. Diazo-cyclooctyne reactions have been used to tag cellular glycans among other targets[107,108]. Importantly, the resulting pyrazole adducts are stable to a number of biological nucleophiles[109]. Further diazo tuning has provided scaffolds that react with acyclic electron-deficient alkenes, a transformation that can be performed in the presence of azides[110].

More reactive 1,3-dipoles have also been harnessed for bioorthogonal application. Most are masked until an external trigger (often light) is applied, enabling the reactant to be released "on demand"[106,111–113]. One such class of dipoles comprises nitrile imines. These motifs react robustly with strained alkenes such as norbornene[114] and cyclopropene[115,116], but they are prone to rapid hydrolysis in the absence of a ligation partner. Nitrile imines can be caged as tetrazoles or sydnones, functional groups that are more stable in biological environs. UV irradiation can liberate the reactive species. The starting tetrazole chromophores can be tuned for photolysis – and thus nitrile imine release – using different wavelengths of light[117,118]. This added layer of control has inspired the exploration of other "photo-click" reactions to expand the compendium of bioorthogonal chemistries[119–121]. Nitrile imines have also been extensively tuned via electronic[122] and steric modification[123].

Tuning dipolarophiles for bioorthogonal cycloaddition

Tuning bioorthogonal cycloadditions is perhaps best exemplified in the context of the strained cycloalkynes as dipolarophiles[10,12]. Many efforts have been directed at modifying ring strain, with an eye towards increasing reaction rates or *in cellulo* stability. Examples include modulation of ring size or conformation[124–131], electronic perturbation[132–135], installation of endocyclic heteroatoms[136–138], and combinations thereof (Figure 3B)[139,140]. There have also been significant efforts to improve the water solubility of these relatively greasy probes. Towards this end, cycloalkynes featuring sulfamate backbones, as well as larger heterocyclic derivatives (up to 12-membered rings) have also been explored[141–146]. The heteroatom variants were generally more water soluble and stable. However, the benefits of ring expansion came at the cost of rate, a trade-off that is prevalent in bioorthogonal reagent tuning[2]. With the reactivity-stability axis in mind, one of the more impactful cycloalkynes has been BCN[131]. This scaffold has increased strain energy compared to the original cyclooctyne (due to the fused cyclopropane ring)[147], but is remarkably stable in cellular environments. Coupled with its synthetic accessibility, BCN has become a staple strained alkyne for cycloaddition chemistries in a number of fields. In a recent example, BCN-fluorophore conjugates were used for super-resolution imaging in live cells (Figure 3C)[148]. FtsZ, a protein involved in bacterial cell division, was enzymatically outfitted with an azide. Subsequent treatment with cell-permeable BCN derivatives (comprising photoswitchable rhodamines) enabled protein localization patterns to be observed.

Efforts to modulate cycloalkyne reactivity have been bolstered by computation. Calculations can readily predict combinations of steric and electronic features to tune scaffolds for desired outcomes. One well-established approach for modulating cycloaddition partners relies on the Distortion/Interaction model[149,150]. This analysis computes the activation barrier for a given reaction by calculating the difference between a distortion energy (i.e., the energy required for reactants to adopt their ideal transition state geometries) and an interaction energy (i.e., favorable orbital overlap between the two reaction partners). The calculated activation energy is then correlated to a predicted rate constant, which can ultimately guide reagent tuning. One of the earliest demonstrations of the Distortion/Interaction model in bioorthogonal reagent design involved a series of biarylazacyclooctynone (BARAC) analogues. This study revealed structural features that

impeded BARAC reactivity with azides and set the stage for the development of improved cyclooctynes[151]. Similar computational studies have been used to fine-tune sydnone-cycloalkyne reactions[152] and other bioorthogonal cycloaddtions[149,153,154].

Tuning dienophiles for bioorthogonal cycloaddition

Parallel developments in the realm of IEDDA have expanded the number of bioorthogonal chemistries in recent years. Reactions featuring TCO, in particular, have found widespread use in cells and *in vivo*[11]. Such transformations have also been the targets of extensive reagent tuning[155–159]. Many efforts have focused on pushing the kinetics of the tetrazine-TCO ligation (Figure 4). Increasing TCO strain was hypothesized to boost reaction rate, similar to the strained alkynes. Toward this end, Fox and coworkers designed scaffolds wherein TCO was forced to adopt a half-chair conformation (e.g., d-TCO[155] and s-TCO[155]). Such motifs were predicted to be 5.6–5.9 kcal mol^{-1} higher in energy relative to the more stable crown conformation of TCO[160]. The more reactive d-TCO and s-TCO variants display bimolecular rate constants as high as 3×10^6 M^{-1} s^{-1}.

Similar to the strained alkynes, ring contraction strategies have been employed in the context of strained alkenes. Recently, *trans*-cycloheptene (TCH) analogs have been reported as voracious dienophiles in IEDDA cycloadditions with tetrazines[161,162]. TCH readily isomerizes under ambient conditions but can be isolated as a stable complex with Ag(I). In addition, incorporation of an endocyclic silicon atom provided a more stable seven-membered cycloalkene, sila-*trans*-cycloheptene (SiTCH). The longer Si-C bond lengths in SiTCH relieved some ring strain, enabling facile isolation. Computational studies further revealed that the activation barrier for SiTCH reactivity with diphenyltetrazine was significantly lower compared to s-TCO, enabling rapid ligation. The second-order rate constant for SiTCH and a model tetrazine was 1.14×10^7 M^{-1} s^{-1}, the fastest bioorthogonal ligation on record. Despite their impressive rates, though, TCH and SiTCH degrade rapidly in the presence of cellular thiols[162].

1,3-Disubstituted cyclopropenes, alternative dienophiles for IEDDA reactions, have also been tuned for a variety of applications. We and others reported that these scaffolds react with tetrazines in biological environments[163,164]. Compared to TCO, the reaction between tetrazines and cyclopropenes is much slower. However, the small size and cellular stability of cyclopropenes have rendered them attractive for interrogating biomolecules in complex environments[165]. One notable example from the Chin lab showcased a cyclopropene-lysine analog to monitor nascent protein biosynthesis in *Drosophila*[166].

The reactivity profile of the cyclopropene can be dramatically influenced by steric tuning. The Distortion/Interaction model predicted that increasing steric bulk at C3 on cyclopropene would drastically reduce its reactivity with tetrazines. Diminished reactivity was attributed to geometric constraints in the transition state. The poor orbital overlap between the cycloaddition partners was predicted to slow the ligation. Indeed, no reaction was observed even when 3,3-disbustituted cyclopropenes were subjected to a variety of functionalized tetrazines. Such cyclopropenes still reacted with nitrile imines, though, and this differential reactivity was exploited to label two proteins in tandem[116].

Further cyclopropene tuning was achieved upon introduction of spirocycles at C3. Based on crystallographic data, cyclopropene **2** exhibited a reduced bond angle between the two C^3-groups compared to parent cyclopropene **1** (Figure 5). The decreased bond angle drove the substituents away from the incoming nitrile imine, increasing ligation rates by 15-fold[167]. Interestingly, the spirocyclic cyclopropenes also exhibited reactivity with some tetrazines, and have been used to label cell-surface receptors in live cells[168]. Heteroatoms within the spirocycle further boosted cyclopropene reactivity by lowering LUMO energies[169]. Spirocyclic cyclopropenes have recently been outfitted with light-sensitive cages. Upon uncaging, the scaffolds become available for IEDDA ligation. These masked reagents can be used for biomolecule labeling with spatiotemporal control, but their syntheses remain challenging[170,171].

Tetrazines have also been explored as reactants for other biocompatible cycloadditions. Recent work has featured transformations with isonitriles. These dienophiles react with tetrazines via [4+1] cycloaddition to provide imine products. With primary and secondary isonitriles, the imines undergo facile tautomerization and hydrolysis to liberate amines. Such isonitriles have proven useful as cages for amino fluorophores and small-molecule drugs[172,173]. Hydrolysis can be mitigated with appropriately tuned isonitriles[174,175]. For example, tertiary scaffolds react with tetrazines to form stable ligation adducts, as they cannot react further[175]. Because of their small size and versatility, isonitriles have been used to label biomolecules, including proteins[176] and glycans[177].

Simple vinyl groups have been shown to undergo IEDDA reactions with tetrazine probes. The small size of the vinyl motif is attractive for biomolecule tagging strategies. However, even with electron-rich vinyl reagents, most of the alkenes examined to date suffer from low aqueous solubility and slow rates[178,179]. Vinylboronic acids (VBAs) react significantly faster with tetrazines. The ligation liberates boronic acid, a major driving force for the reaction[180]. Even more rapid cycloadditions can be achieved with electron-rich VBAs and tetrazines outfitted with hydroxy groups. These latter groups coordinate the boronic acid motifs[181,182]. The VBA-tetrazine ligation has been used to profile the efficacy of proteasome inhibitors in live cells[183]. Many other cyclic[184,185] and acyclic[186] alkenes have been similarly explored as tetrazine ligation partners[187].

Tuning dienes for bioorthogonal cycloaddition

The second half of the IEDDA reaction, the diene (most often, tetrazine), has also been thoroughly modified to achieve altered stability and reactivity profiles[62,182,188–191]. In one example, the stability of the tetrazine probe was modulated using electronic perturbations. An amino acid comprising a tetrazine motif was initially found to hydrolyze in the cellular milieu. The hydrolysis was facilitated by a labile secondary amine linkage. The electron-donating amine group also slowed IEDDA rates. Simple removal of the amine handle addressed both of these limitations.[190] In another example, researchers synthesized a panel of tetrazines for amine uncaging with TCO. The tetrazines were screened with model enzymes bearing TCO-caged amino acids. The best tetrazines facilitated near quantitative uncaging in under 4 minutes with as little as 50 μM reagent[62].

The rates that some tetrazine ligations achieve are unparalleled. Such rates, though, often come at the cost of probe stability. Many of the most reactive tetrazines are known to react with thiols, which can lead to off-target effects and high background labeling in cells[192]. To address this liability, some groups have drawn inspiration from caged bioorthogonal reagents. These efforts are focused on liberating the reactive tetrazine and take advantage of the redox properties of the motif. The reduced form, dihydrotetrazine, is unreactive towards dienophiles and stable in biological contexts. Dihydrotetrazines can then be oxidized to tetrazines *in situ* via enzymatic[193], or photocatalytic[193] approaches. Such strategies provide an avenue to employ even the most reactive tetrazines in biological environments. Caged tetrazines have also been employed to decorate electrode surfaces with biomolecules[194].

The instability of some tetrazine motifs inspired pursuits of less electron-deficient dienes, including 1,2,4-triazines. Like its tetrazine counterpart, the triazine reacts via IEDDA with strained π-systems such as TCO and BCN[195,196]. While the rates of these reactions are markedly slower, the triazine is stable in the presence of biological thiols for over 24 hours at elevated pH. These scaffolds have since been installed in proteins[195,197], as well as enzymatically appended onto DNA *in vitro*[198,199]. 1,2,4-Triazines have also been electronically tuned to achieve faster kinetic profiles[200,201]. For example, electron-withdrawing pyridinium groups have been used to improve cycloaddition rates. These scaffolds have been recently used to label mitochondria in live cells[200]. Subtle steric modifications to the triazine core also provided altered modes of reactivity with strained alkynes. In a recent study, the Distortion/Interaction model predicted that triazine substitutions at C3 and C6 would diminish reactivity with sterically encumbered strained alkynes[202]. Such reactions were predicted to remain facile with C^5-substituted isomers, as steric clashes were minimized at the bond-forming centers (i.e., C3 and C6). The predictions were confirmed experimentally via simultaneous, dual labeling of two protein targets.

The number of dienes that can participate in IEDDA cycloadditions is also growing. A nitrogenous heterocycle, 4*H*-pyrazole, was recently shown to ligate the strained alkyne BCN[203]. At the outset, the pyrazole required further tuning to elicit robust reactivity. Addition of fluorine substituents was hypothesized to impart negative hyperconjugative effects on the pyrazole ring. This tuning would increase the antiaromatic character of the scaffold, resulting in destabilization of the pyrazole ring and more rapid ligation. Computational analyses verified that a *gem*-difluoro group decreased the LUMO values of the 4*H*-pyrazole, resulting in fast reactivity with BCN. Such antiaromaticity considerations along a reaction coordinate could be more generally exploited in bioorthogonal reaction design.

Another class of dienes comprises *ortho*-quinones. These motifs react with dienophiles via strain-promoted oxidation-controlled cyclooctyne–1,2-quinone (SPOCQ) cycloadditions[204]. Early iterations involved converting 1,2-catechols to the corresponding quinones using an exogenous oxidant[205]. SPOCQ reactions were orders of magnitude faster than azide-alkyne cycloadditions (second-order rate constants of ~500 M^{-1} s^{-1}), on par with some tetrazine-TCO ligations. Later studies demonstrated that genetically encodable tyrosine tags could be selectively oxidized to quinones *in situ* using tyrosinase. These motifs could then be used to append small molecules for antibody-drug conjugate formation. The strategy enabled control

over the location and number of warheads that were appended to the antibody[206]. *ortho*-Quinones were also found to react efficiently with strained alkenes, such as cyclopropenes[207]. However, the need to generate *ortho*-quinones *in situ* can impose constraints. *ortho*-Quinoline quinone methides were explored as more versatile alternatives, as these scaffolds can be generated *in situ* without any external triggers in biological environments. The motifs were found to react robustly with vinyl thioethers via hetero Diels–Alder cycloaddition. The unusual thioacetal adduct formed was found to be stable in aqueous solution at various pH values[208,209].

IV. Combining mutually orthogonal reactions

As evident from above, the past decade has seen a surge in the number of transformations available for biological application. Despite the expanded toolkit, it remains challenging to apply more than one reaction at a time[210]. New reaction development has largely focused on labeling single targets. Identifying collections of compatible bioorthogonal chemistries would enable multicomponent labeling studies, and allow a broader set of biological processes to be examined. Finding such combinations of reactions has historically been challenging, as many popular reagents cross-react with one another. The search for orthogonal reactions has accelerated in recent years, aided by computational tools and reaction tuning.

Bioorthogonal reactions that feature unique mechanisms are well suited for multicomponent labeling studies. Reagents with distinct modes of reactivity can often mitigate cross-reactivity issues. For example, azide-alkyne cycloadditions can be used in tandem with hydrazine/ketone condensations[211], various IEDDA reagents[163,178,212–214], some 1,3-dipoles[98,110], and other motifs[180,215–217]. Efforts to employ three mutually compatible bioorthogonal groups have also been pursued[202,213,218–220]. One recent example featured azide-, cyclopropene-, and alkyne-containing sugars to study the heterogeneity of glycan metabolism in plant cells[219]. The ligations could not be performed simultaneously, though, owing to cross-reactivities among the reaction partners. Cumbersome washes were also required between ligations, eroding temporal resolution. Only three studies to date have been able to achieve simultaneous triple labeling[221–223]. One notable example features two tetrazines, one that is sterically encumbered and reacts selectively with a small isonitrile. The second tetrazine ligates TCO in a typical IEDDA cycloaddition. The tetrazine reactions were combined with an azide/strained alkyne pair in a triple labeling experiment. Three model proteins, labeled with either a bulky tetrazine, a less encumbered tetrazine, or an azide were mixed and reacted with isonitrile-, TCO-, and strained-alkyne fluorophores. The matched adducts were detected by in-gel fluorescence, with no evidence of cross-reactivity (Figure 6)[223]. While this study showcased triple component labeling in a model context, the reagents should be applicable in other biological settings.

V. Exploring new genres of reactivity

Identifying additional genres of bioorthogonal chemistry will continue to bolster multicomponent labeling studies. Recent efforts to explore new areas of chemical space – coupled with additional tuning of existing ligations – are proving fruitful. Boron reagents are

gaining traction. In recent work, a diboron probe was found to react selectively with *N*-oxides. Importantly, the ligation proceeded efficiently inside cells, one of the harshest biological environments[224]. The unique mechanism of this reaction is further compatible with several existing bioorthogonal chemistries.

Perhaps the most noteworthy developments in polar reagent design involve sulfur(VI) fluorides. These motifs are remarkably stable in cellular environments, and react robustly with oxygen and nitrogen nucleophiles through sulfur(VI) fluoride exchange (SuFEx) chemistry[225,226]. SuFEx reagents have been used for drug design[227,228], activity-based profiling[229] and other protein targeting studies[230]. They have also been employed for examining protein-protein interactions via proximity-driven crosslinking[231,232]. SuFEx electrophiles also provide a mild and convenient method to introduce bioorthogonal azides onto a variety of amine targets, further exemplifying the utility of these motifs[233].

Explorations into new cycloaddition platforms are also expanding the bioorthogonal toolkit. One example involves the quadricyclane ligation[221]. This reaction is a formal [2σ+2σ+2π] cycloaddition with Ni-bis(dithiolene) complexes, a rather unique mode of reactivity. Quadricyclanes are highly strained molecules (~80 kcal mol^{-1} of strain energy[234]), but can survive in aqueous conditions and in the presence of thiols for extended periods. Quadricyclanes have even been used for intracellular labeling applications via genetic code expansion[235]. The novelty of the quadricyclane reaction is likely to inspire continued exploration of other cycloaddition manifolds for bioorthogonal application.

Bioorthogonal platforms of reactivity have also expanded to include transition metals. These metals are virtually absent in the cellular milieu and can forge stable C-C bonds, making them attractive for selective labeling applications. Focused reviews on this topic have been covered recently, but a few examples are described below[236,237]. Water soluble Au(I) complexes have been developed to drive covalent bond formation in aqueous media[238]. Gold nanoparticles have also been employed for imaging in zebrafish[239]. More reactive Au(III) species can be harnessed for selective amidation reactions, although the metal complexes must be associated with scaffolding proteins[240]. Inspired by copper catalysis, several groups have optimized ligands to form water soluble, minimally toxic palladium complexes. Some have been used for Suzuki-Miyaura[241,242] couplings in cells, along with Sonogashira reactions[243,244], and alkynyl-carbamate deprotections[245,246]. Palladium nanoparticles[247] and assemblies[248] have also been developed for conducting various transformations *in cellulo*. Similar advances in ruthenium chemistry[249–251] and iridium photocatalysis[252] are enabling biomolecule targeting.

New developments in protein bioconjugation are also being leveraged for more general bioorthogonal application. One example features aryl diazonium ions[253,254]. These electrophiles react robustly with electron-rich aromatic side chains such as tyrosine. Site-specific modification with aryl diazoniums was historically challenging owing to the abundance of tyrosine residues in proteins. Further exploration revealed a selective reaction between 5-hydroxytryptophan (5HTP) and aryl diazonium reagents. The additional electron density in 5HTP enabled the use of less reactive diazonium reagents, preventing background labeling of other aromatic side chains. This reaction has since been used to label

recombinant proteins and antibody derivatives[255], and additional targets are anticipated. Further tuning of other protein-labeling strategies is likely to provide more bioorthogonal reagents[256,257].

Conclusions

At its core, bioorthogonal chemistry is focused on controlling reactive functional groups in harsh environments. How does one design a pair of reagents to form a single adduct, in the confines of a cell or whole organism? The strict requirements imposed on these reactions have often forced researchers to explore unconventional handles. Historic work provided an initial set of reagents, many of which are still employed for examining biomolecules in vivo.

The toolbox has greatly expanded in recent years via iterative tuning of established probes. Some common themes from these studies have emerged. For example, the rates and stabilities of first-generation tools can be modulated for particular applications, drawing on common physical organic chemistry principles. The impacts of certain modifications can often be predicted computationally. Computational analyses can also be invaluable to the hunt for collections of mutually compatible transformations.

After decades of work on bioorthogonal chemistries, there is still no “one-size-fits-all” reaction. Rather, a spectrum of reactivities exists and the challenge lies in knowing how best to apply the probes. Recent successes in tuning highly reactive chemical handles suggest that other “fringe” functional groups can be harnessed for understanding biology. There is a further need for not just single-component reactions, but also collections of bioorthogonal chemistries that can be used in tandem[210]. The continued exploration of unique modes of reactivity[256–260] will be useful in this regard. The strategies and examples highlighted in this Review provide a roadmap for continued expansion of the bioorthogonal toolkit, taking chemistries beyond flasks and into living systems.

Acknowledgements

S.S.N. is an Allergan Graduate Research Fellow. J.A.P. is a Cottrell Scholar, Alfred P. Sloan Fellow, and Dreyfus Scholar. This work was funded by the National Institutes of Health (R01 GM126226). We thank members of the Prescher laboratory for helpful discussions during the manuscript preparation.

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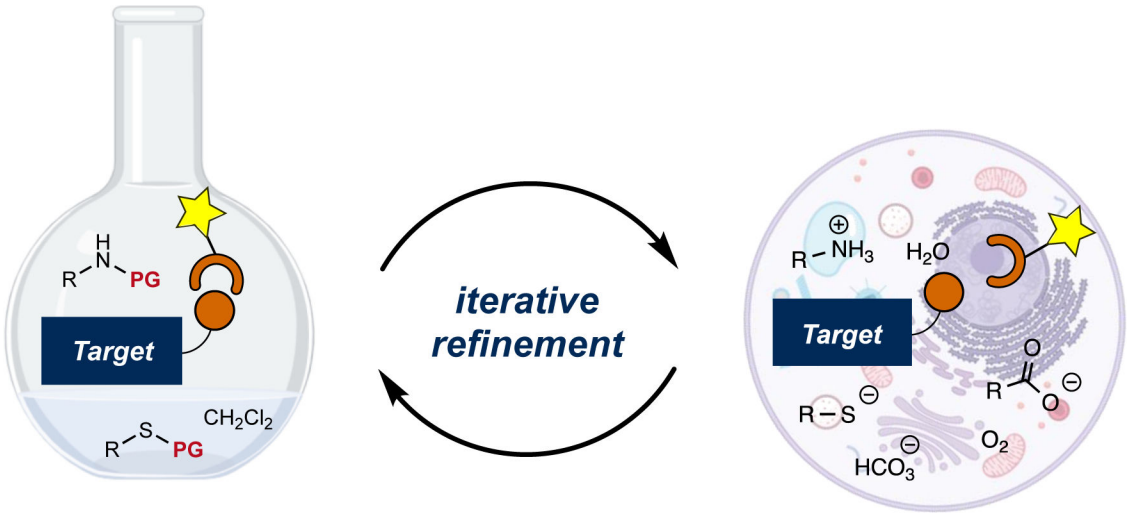


Figure 1.

Translating reactions from round-bottom flasks to living systems. Reactants (orange circle and arc) used in cells and tissues must be compatible with cellular functionality. Challenging applications continue to drive the refinement of bioorthogonal reactions. Optimization in either flasks or cells invokes similar principles, although variables that are easily controlled in flasks are often invariant in living systems.

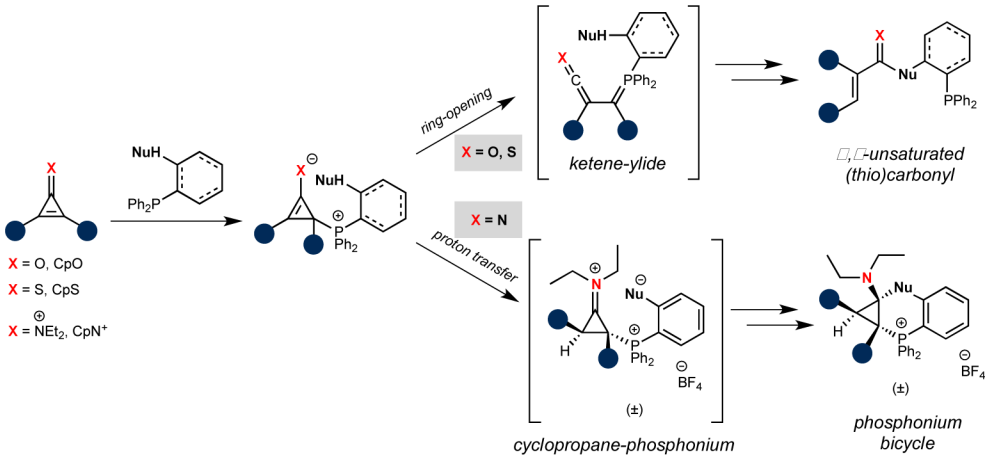


Figure 2.

Cyclopropenone (CpX) derivatives exhibit unique reactivities. Analogs comprising oxygen or sulfur atoms react robustly with phosphines via ketene-ylide intermediates. Nitrogenous scaffolds react to form phosphonium bicycles.

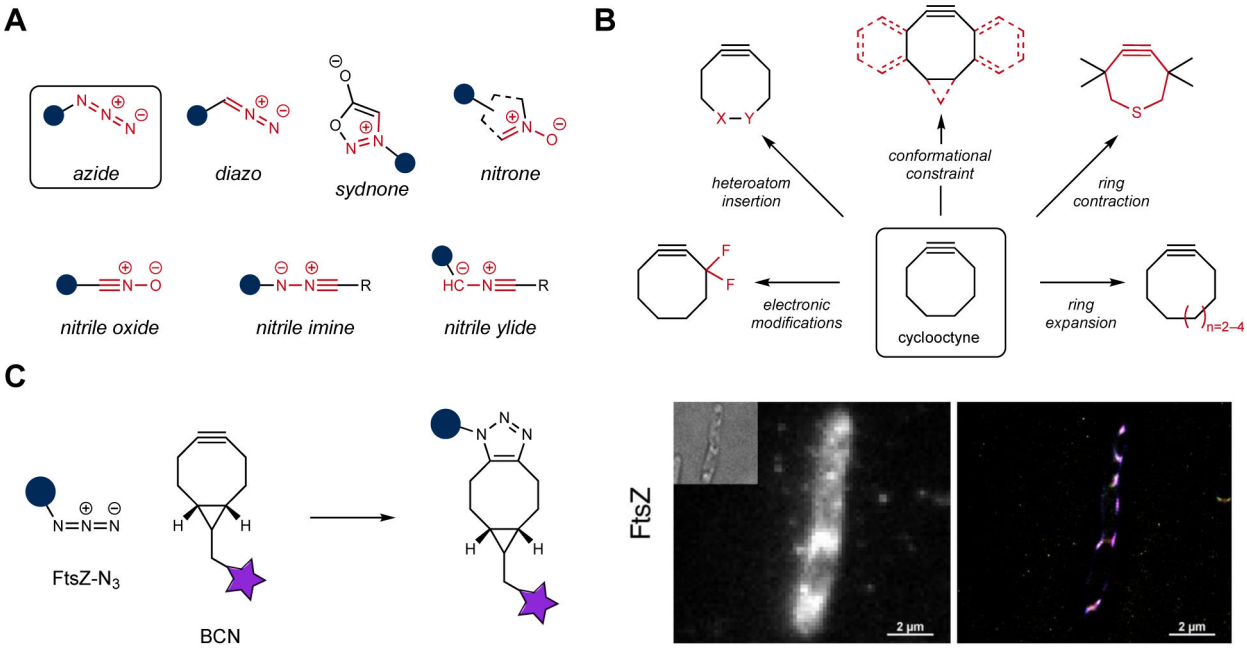


Figure 3.

An expansive set of reagents for dipolar cycloaddition. (A) Several 1,3 dipoles are available for bioorthogonal labeling. Organic azides were among the first to be exploited for cycloadditions in cells. More recently, diazo, sydnone, and nitrone motifs have been developed for biomolecule labeling. Highly reactive dipoles (including nitrile oxides, imines, and ylides) are also viable ligation partners. They are typically produced "on demand" via photolysis or chemical uncaging. (B) Several classes of strained alkynes are available for bioorthogonal cycloaddition. These reagents derive from cyclooctyne, the first-reported variant. Extensive modifications to cyclooctyne provided a suite of reagents with altered reactivities. (C) Sample application of bioorthogonal cycloadditions. FtsZ, a bacterial protein involved in cellular division, was outfitted with an azide handle (left). Subsequent ligation with BCN-photoswitchable fluorophore conjugates enabled super-resolution microscopy in live *E. coli* (right). Images were adapted with permission from ref.[148] The American Chemical Society

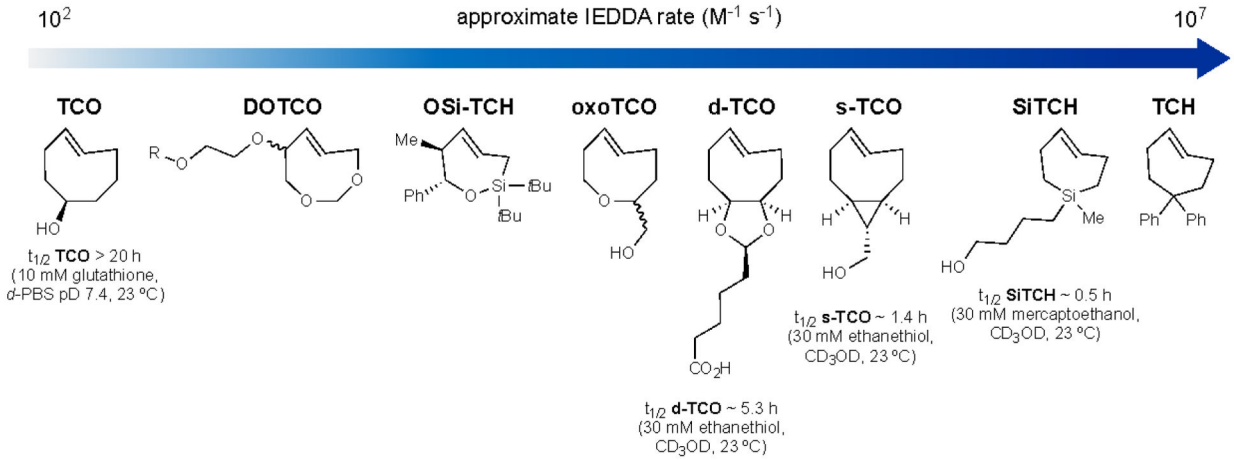


Figure 4.

A collection of strained alkenes for IEDDA reactions. These reactions exhibit a range of reaction rates with tetrazines. *trans*-Cyclooctene (TCO) was the original member of this group. TCO has since been modified to access faster and more biocompatible reagents. Some of the scaffolds feature endocyclic heteroatoms (DOTCO[156], OSi-TCH[161], oxoTCO[158]), conformational constraints (d-TCO[155], s-TCO[155]), and contracted rings (SiTCH[154], TCH[154]). In general, the fastest reacting scaffolds exhibit the shortest half-lives in the presence of biological thiols.

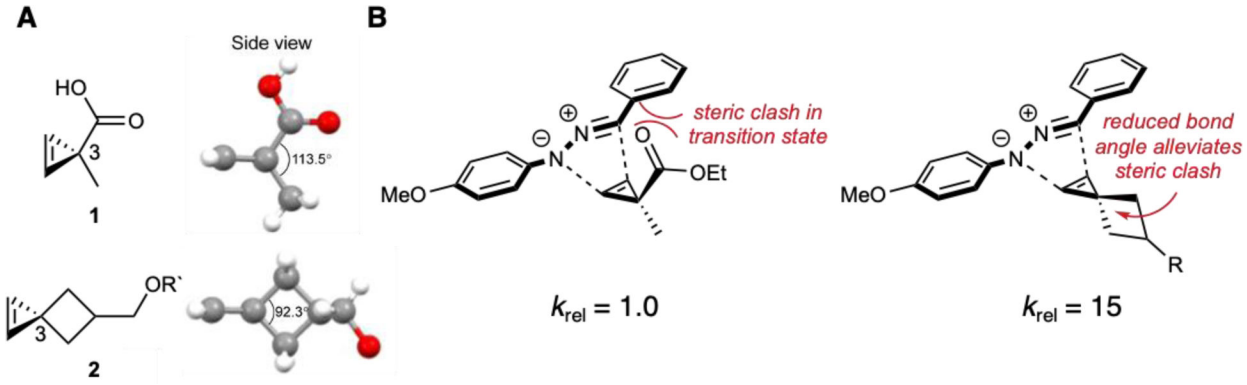


Figure 5.

Steric modifications tune cyclopropene reactivity. (A) The angle between C^3-substituents is reduced in spirocyclic cyclopropene **2** compared to parent compound **1**. (B) The decreased bond angle minimizes steric clashes in the transition state, resulting in faster reactions with nitrile imines. Part **A** was adapted with permission from ref.[167], The American Chemical Society.

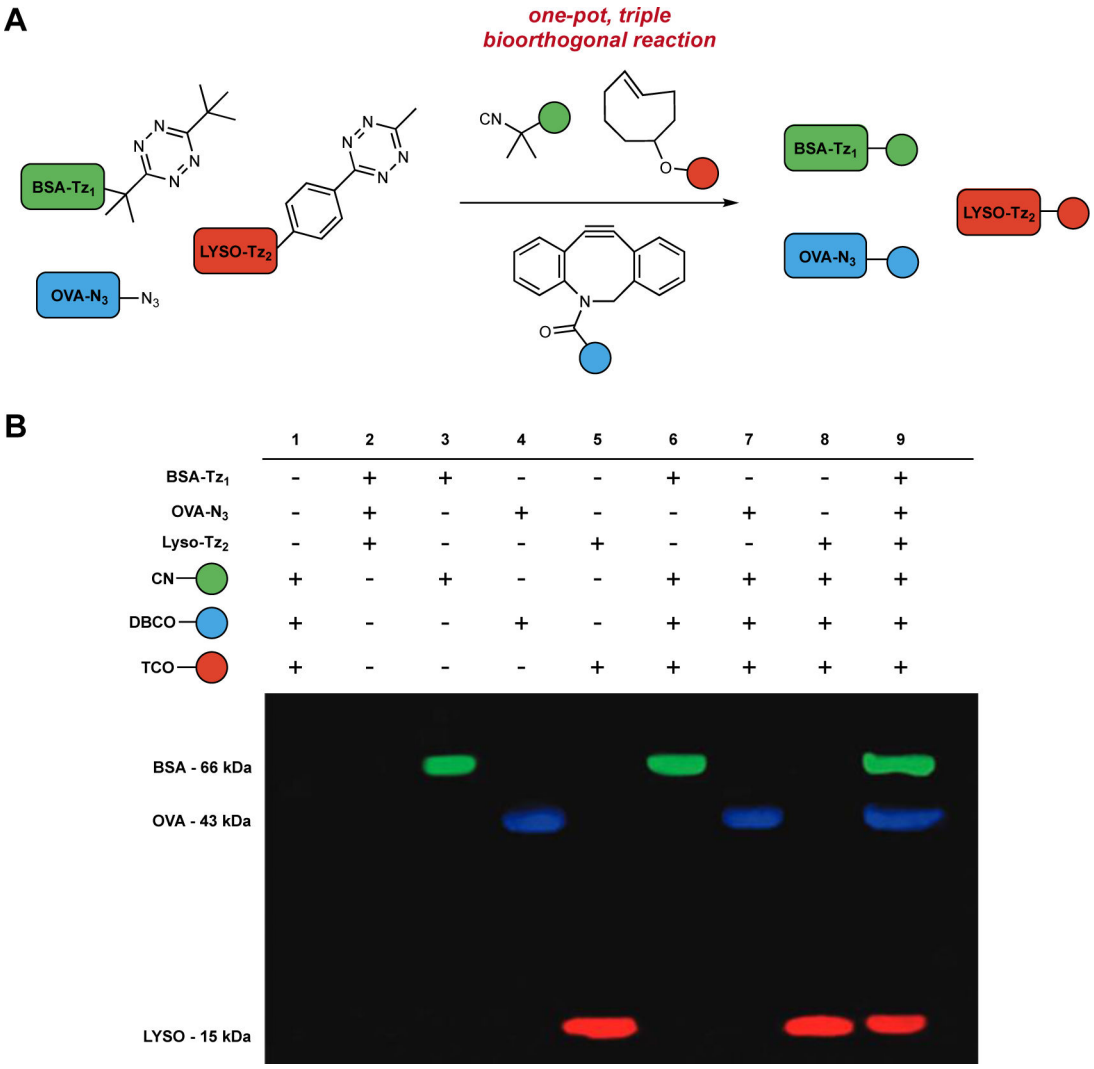
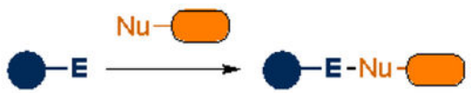
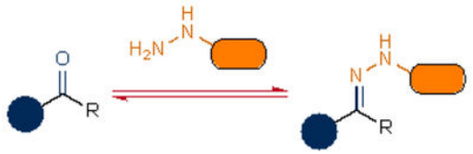
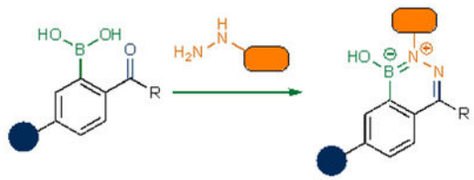
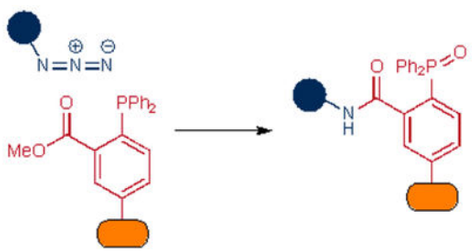
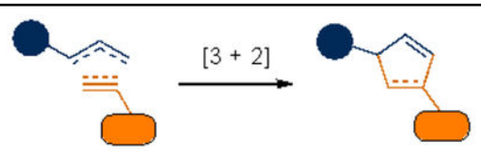
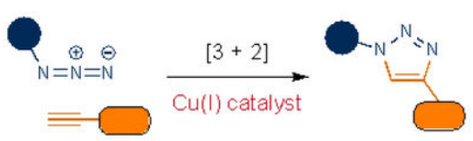
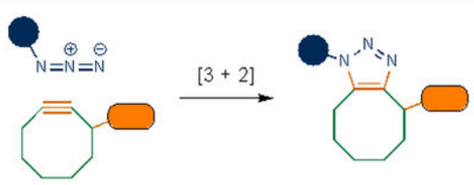


Figure 6.

Collections of bioorthogonal chemistries for multicomponent labeling. (A) Three mechanistically distinct reactions – [4+1] tetrazine (Tz_1)-isonitrile cycloaddition, [4+2] tetrazine (Tz_2)-TCO cycloaddition, and [3+2] azide-alkyne cycloaddition – were used to simultaneously label three model proteins (BSA, OVA, and lysozyme). (B) Covalent protein adducts were visualized via gel electrophoresis followed by fluorescence scanning. No cross-reactivities were detected. Part **B** was adapted with permission from ref.[223], Wiley-VCH.

Table 1.

Tuning transformations for biological application. Bioorthogonal chemistries largely fall into two categories: polar reactions and cycloadditions. Examples of how limitations spurred the development of new reactions are shown.

Reaction type	Overall transformation	Early example	Tuned variant
Polar		 Reversible, pH dependent	 Product stabilization
		 Limited phosphine solubility	 Improved phosphine solubility
Cycloaddition	 1,3-Dipolar	 Cytotoxic catalyst	 Strain-promoted cycloaddition

Reaction type	Overall transformation	Early example	Tuned variant
Inverse electron-demand Diels–Alder	[4 + 2]	:NuH [4 + 2] Off-target reactivity	[4 + 2] Stereoelectronic tuning