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From Mechanism to Mouse: A Tale of Two Bioorthogonal Reactions

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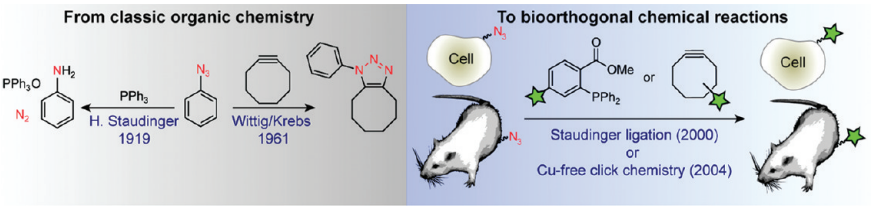

Bioorthogonal reactions are chemical reactions that neither interact with nor interfere with a biological system. The participating functional groups must be inert to biological moieties, must selectively reactive with each other under biocompatible conditions, and, for *in vivo* applications, must be nontoxic to cells and organisms. Additionally, it is helpful if one reactive group is small and therefore minimally perturbing of a biomolecule into which it has been introduced either chemically or biosynthetically. Examples from the past decade suggest that a promising strategy for bioorthogonal reaction development begins with an analysis of functional group and reactivity space outside those defined by Nature. Issues such as stability of reactants and products (particularly in water), kinetics, and unwanted side reactivity with biofunctionalities must be addressed, ideally guided by detailed mechanistic studies. Finally, the reaction must be tested in a variety of environments, escalating from aqueous media to biomolecule solutions to cultured cells and, for the most optimized transformations, to live organisms.

Work in our laboratory led to the development of two bioorthogonal transformations that exploit the azide as a small, abiotic, and bioinert reaction partner: the Staudinger ligation and strain-promoted azide–alkyne cycloaddition. The Staudinger ligation is based on the classic Staudinger reduction of azides with triarylphosphines first reported in 1919. In the ligation reaction, the intermediate aza-ylide undergoes intramolecular reaction with an ester, forming an amide bond faster than aza-ylide hydrolysis would otherwise occur in water. The Staudinger ligation is highly selective and reliably forms its product in environs as demanding as live mice. However, the Staudinger ligation has some liabilities, such as the propensity of phosphine reagents to undergo air oxidation and the relatively slow kinetics of the reaction.

The Staudinger ligation takes advantage of the electrophilicity of the azide; however, the azide can also participate in cycloaddition reactions. In 1961, Wittig and Krebs noted that the strained, cyclic alkyne cyclooctyne reacts violently when combined neat with phenyl azide, forming a triazole product by 1,3-dipolar cycloaddition. This observation stood in stark contrast to the slow kinetics associated with 1,3-dipolar cycloaddition of azides with unstrained, linear alkynes, the conventional Huisgen process. Notably, the reaction of azides with terminal alkynes can be accelerated dramatically by copper catalysis (this highly popular Cu-catalyzed azide–alkyne cycloaddition (CuAAC) is a quintessential "click" reaction). However, the copper catalysts are too cytotoxic for long-term exposure with live cells or organisms. Thus, for applications of bioorthogonal chemistry in living systems, we built upon Wittig and Krebs' observation with the design of cyclooctyne reagents that react rapidly and selectively with biomolecule-associated azides. This strain-promoted azide–alkyne cycloaddition is often referred to as "Cu-free click chemistry". Mechanistic and theoretical studies inspired the design of a series of cyclooctyne compounds bearing fluorine substituents, fused rings, and judiciously situated heteroatoms, with the goals of optimizing azide cycloaddition kinetics, stability, solubility, and pharmacokinetic properties. Cyclooctyne reagents have now been used for labeling azide-modified biomolecules on cultured cells and in live *Caenorhabditis elegans*, zebrafish, and mice.

As this special issue testifies, the field of bioorthogonal chemistry is firmly established as a challenging frontier of reaction methodology and an important new instrument for biological discovery. The above reactions, as well as several newcomers with bioorthogonal attributes, have enabled the high-precision chemical modification of biomolecules *in vitro*, as well as real-time visualization of molecules and processes in cells and live organisms. The consequence is an impressive body of new knowledge and technology, amassed using a relatively small bioorthogonal reaction compendium. Expansion of this toolkit, an effort that is already well underway, is an important objective for chemists and biologists alike.

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Introduction

As scientists, we have much to learn regarding the molecular interactions and chemical transformations that enable life. Genomic data have illuminated many aspects of protein and nucleic acid expression and regulation. However, other biomolecules such as glycans, lipids, and metabolites, on their own or as posttranslational modifications, are impossible to interrogate using genomic data alone. Additionally, biology-driven experimental approaches do a poor job in these sectors of biochemistry, particularly when the goal is to monitor spatiotemporal dynamics of the target biomolecules in cells or model organisms. This technology deficit prompted us to look toward chemical means to study biological processes, and ultimately, motivated our interest in developing bioorthogonal chemical reactions.

A reaction classifies as bioorthogonal if it neither interacts nor interferes with a biological system (Figure 1A).[1] Our first published use of the term "bioorthogonal" occurred in 2003,[2] although we often used this concise descriptor in public presentations during our earlier work in the late 1990s. Historically, the concept of bioorthogonality has strong roots in the much older field of bioconjugation, wherein a classic challenge was to identify selective behaviors of amino acid side chains that could be exploited for chemical modification *in vitro*. However, targeted modification of a protein or any other biomolecule *in vivo* would require a chemical reaction among functionalities that are not so prevalent among (and ideally are absent from) natural biomolecules. A few isolated reports from the 1990s suggested that such chemical reactions might exist or least could be invented with some clever mechanistic thinking. As far back as 1990, Rideout and co-workers demonstrated that the selective condensation of hydrazine and aldehyde groups could be harnessed to assemble toxins from inactive prodrugs within live cells.[3] Then, in 1998, Tsien and co-workers rocked the chemistry world with the first example of live cell protein labeling using bisarsenical dyes.[4] These early examples foreshadowed the power of bioorthogonal chemistry as an instrument for biological discovery and biotechnology. But perhaps more importantly, they empowered chemists to consider developing chemical reactions explicitly tailored for use in biological systems. Growing interest in this challenge is underscored by widespread adoption of the term "bioorthogonal" by the chemistry community since that time, as evidenced by an expanding number of publications containing this term (Figure 2).

Nowadays, the use of bioorthogonal chemistry to probe biomolecules in living systems typically follows a two-step process. First, a metabolic substrate, small molecule ligand, or enzyme inhibitor is adorned with one of the bioorthogonal functional groups and introduced to the biological system. The structural perturbation imposed by that functional group, also referred to as a "chemical reporter", must be minimal so as not to undermine the molecule's natural bioactivity. Once the labeled molecule has been delivered to its target (e.g., a metabolically labeled glycan, lipid, or protein or an inhibitor-bound receptor or enzyme), the second step involves a bioorthogonal chemical reaction with an appropriately functionalized probe (Figure 1B). A number of creative approaches have now been developed to deliver bioorthogonal functional groups to biomolecules in cells and model organisms.[1] In retrospect, this aspect of the experimental platform has been relatively straightforward. By contrast, developing and optimizing bioorthogonal reactions, the synthetic methodology component of the platform, continues to be a significant challenge.

From a chemist's perspective, bioorthogonal reaction development has unusually restrictive boundary conditions. The reaction must form a stable covalent linkage between two functional groups that are bioinert and ideally nontoxic. The reaction must have fast kinetics so that product is formed at a reasonable rate even when reactant concentrations are very low, as is required in many biological labeling experiments. Also, such fast kinetics must be achieved in the physiological ranges of pH and temperature. For optimal

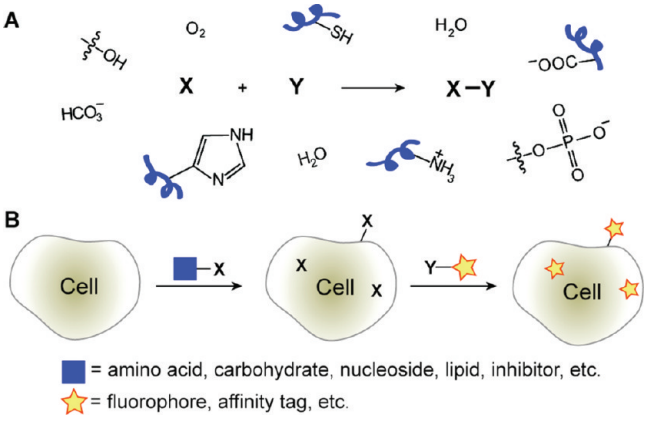

FIGURE 1. (A) A generic bioorthogonal chemical reaction between X and Y that proceeds in biological systems. (B) A common experimental platform for biomolecule probing using bioorthogonal chemistry. First, a non-native functional group, often called a "chemical reporter", is installed in a biomolecule of interest. The modified biomolecule is subsequently labeled using a bioorthogonal chemical reaction.

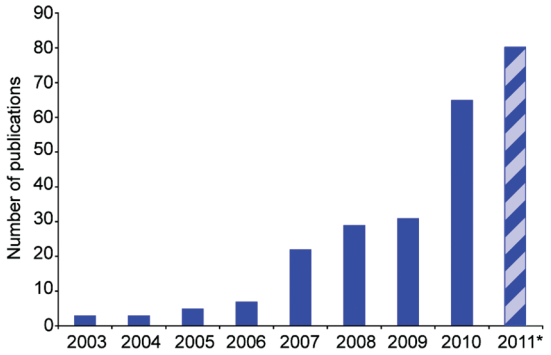

FIGURE 2. The number of publications containing the word "bioorthogonal" categorized by year of publication. *The 2011 value is projected based on publications from the first half of the year. Source: SciFinder Scholar.

utility as chemical reporters, at least one of these functional groups should be small as well.

Considering all of the above requirements, one might argue that the perfect bioorthogonal reaction has yet to be reported, though a number of transformations are approaching the ideal. In this Account, we propose a strategy to develop bioorthogonal reactions that has found validation in stories from our lab, specifically the Staudinger ligation and strain-promoted azide–alkyne cycloaddition, also termed "Cu-free click chemistry". We also discuss gaps in existing reaction methodology where there is need for future optimization. Applications of these reactions and the development of new bioorthogonal chemistries are highlighted in other contributions to this special issue of *Accounts of Chemical Research*.

A Guide for Bioorthogonal Reaction Development

The process of bioorthogonal reaction development and optimization is a journey that requires a critical understanding of mechanistic chemistry, biochemistry, and, for *in vivo* applications, pharmacology and metabolism. The effort begins with an analysis of those functionalities and reaction types that are not represented among Nature's repertoire. From this abiotic chemical space, a prototype reaction among functional groups with inherent stability toward biological moieties, nucleophiles, reductants, and of course, water, is identified (Figure 3, step 1). In our experience, the chemical literature from the early to mid-20th century is fertile ground for unearthing prototype reactions. During this period, physical organic chemists were intrigued by the properties of exotic structures outside of mainstream of organic synthesis, and the practical utility of some of these mechanistic oddities was generally

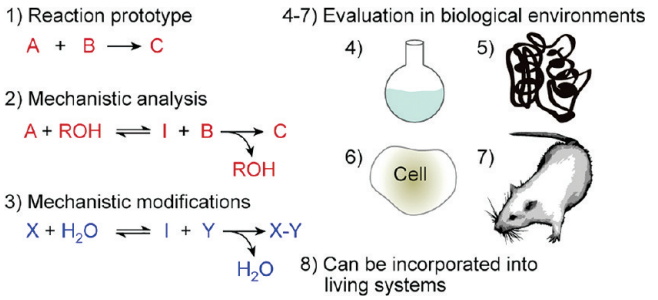

FIGURE 3. A step-by-step guide to developing a bioorthogonal reaction.

not of primary importance. Understanding the fundamental behaviors of organic molecules, how structure relates to reactivity, was sufficient justification for such mechanistic explorations, a testament to a time when society was more forgiving of curiosity-driven science.

Once a prototype reaction is selected, an in-depth mechanistic analysis is essential to guide the requisite adaptations for use in biological systems and to anticipate potential pitfalls (Figure 3, step 2). Each elementary step of the reaction must be compatible with water and the large excess of nucleophilic functionalities found in Nature (e.g., amines, thiols, hydroxy groups). These elementary steps must proceed at reasonable rates under physiological conditions. In practice, reactions with a second-order rate constant smaller than 10^{-4} M^{-1} s^{-1} will be too slow for practical use when reagents are held at the low concentrations necessary to label biomolecules with minimal background. For this reason, rate enhancement is a common initial goal in transforming a prototype reaction to a *bona fide* bioorthogonal transformation.

The next step (Figure 3, step 3) is to modify the reagents, and in some cases the overall mechanism, to solve whatever problems are revealed in step 2. Adjustments might include the addition of steric bulk for protection from biological nucleophiles, exchange of heteroatoms to promote optimal orbital interactions, or activation of the reagents by strain enhancement or electronic perturbation. The mechanistic modifications are the most difficult part of the reaction development process, and chemists often find themselves pursing numerous iterations of a reaction along the way.

Once the optimized candidate reaction proceeds efficiently in a flask, it must be tested against the standards of bioorthogonality in environments of increasing complexity (Figure 3, steps 4–7). The first test is whether the reaction proceeds reliably in aqueous media alongside biological metabolites such as amino acids and sugars (step 4). Next, the reaction must be evaluated on biomolecules (step 5), in

live cells (step 6), and ultimately, in model organisms such as zebrafish or mice (step 7). Not all bioorthogonal reactions developed to date have succeeded in live animals, or even in live cells, but these decisive measures of bioorthogonality should always be considered a central goal.

The final criterion of a superior bioorthogonal reaction is that at least one of its participating functional groups can be incorporated into biomolecules in living systems (Figure 3, step 8). In reality, step 8 is often pursued in parallel to steps 6 and 7. Numerous methods for installing unnatural functional groups within proteins, glycans, lipids, nucleic acids, and other metabolites have been developed.[1] The functional groups with access to the most extensive list of biomolecules, typically the smallest functional groups, are those whose bioorthogonal reactions will ultimately be the most useful.

The Staudinger Ligation Initiates a New Era in Bioorthogonal Chemistry

The Staudinger ligation essentially launched the field of bioorthogonal chemistry, not because it was the first bioorthogonal reaction *per se*, but because it was the first among entirely abiotic functional groups and therefore had the potential for translation to live organisms.[5] Its prototype reaction was the iconic Staudinger reduction of azides with triphenylphosphine and water (Figure 4A), a famously mild transformation that was reported by Hermann Staudinger in 1919.[6] Features that caught our attention were the small size of the azide, its kinetic stability, and its absence from biological systems. Also, the azide's behavior as a "soft electrophile" that prefers "soft nucleophiles" (such as phosphines) situates the functional group in a reaction space that is distinct from most of biology, wherein nucleophiles are typically "hard". That organic azides would be well tolerated by cells and organisms was hinted at by the established use of aryl azides as photo-cross-linkers and by the favorable toxicity profiles of commercially approved drugs such as azidothymidine. Additionally, phosphines, the other reactive group, are naturally absent from living systems.

Mechanistically, the classic Staudinger reduction (Figure 4A) proceeds through nucleophilic attack of the phosphine (**2**) on the azide (**1**) followed by loss of nitrogen to yield an aza-ylide species (**3**). In aqueous environments, the aza-ylide is rapidly hydrolyzed to produce a phosphine oxide (**4**) and an amine (**5**). The Staudinger reduction appeared well-suited as a prototype for bioorthogonal reaction development because the two participants were abiotic, mutually and selectively reactive, mostly unreactive with biological functionalities, and tolerant

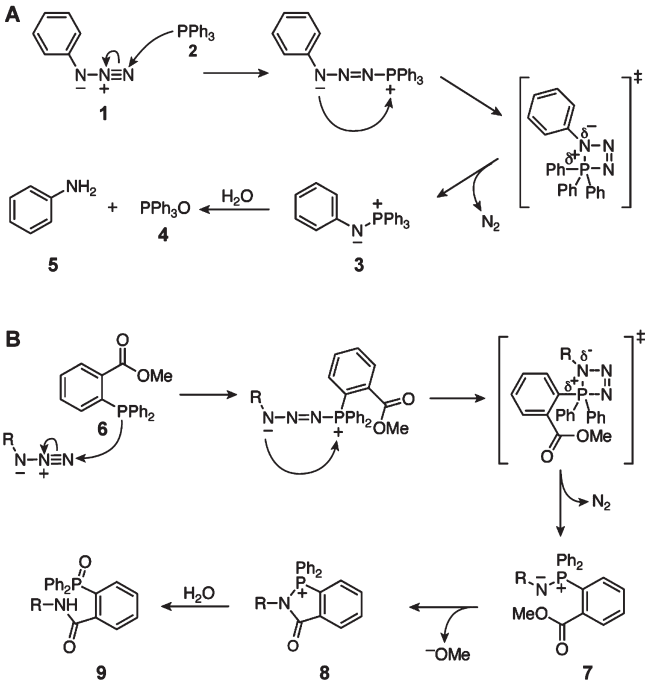

FIGURE 4. The mechanism of the Staudinger reduction (A) and Staudinger ligation (B).

of water. The main problem was that the initial covalent linkage formed (intermediate **3**) was later lost to hydrolysis. Thus, a mechanistic modification was needed to redirect the aza-ylide intermediate to a stably ligated product. This was achieved by introducing an ester group *ortho* to the phosphorus atom on one of the aryl rings (**6**, Figure 4B). Formation of the aza-ylide intermediate (**7**) proceeded analogously to the Staudinger reduction; however, the ester group offered a new path of reactivity in which the nucleophilic nitrogen atom reacted with this electrophilic trap to form intermediate **8**, which, upon hydrolysis, yielded a stable amide-linked product (**9**).[5]

This adjustment to the prototype reaction was sufficient to generate a bioorthogonal chemical reaction. The engineered phosphines were exquisitely selective for azides even when surrounded by biofunctionality as demonstrated by the selective tagging of azide-labeled glycoproteins with phosphine probes in cell lysates (Figure 5A,B).[7] Additionally, the two reactants proved to be surprisingly nontoxic, and therefore the Staudinger ligation can be performed on live cells. Flow cytometry data from a typical experiment in which cell-surface glycans were labeled with azidosugars and then reacted with a phosphine probe are shown in Figure 5C.[4] Finally, the Staudinger ligation was performed in live mice, enabling the selective *in vivo* covalent modification of cell-surface glycans with chemical probes (Figure 5D).[8] This unprecedented feat was a

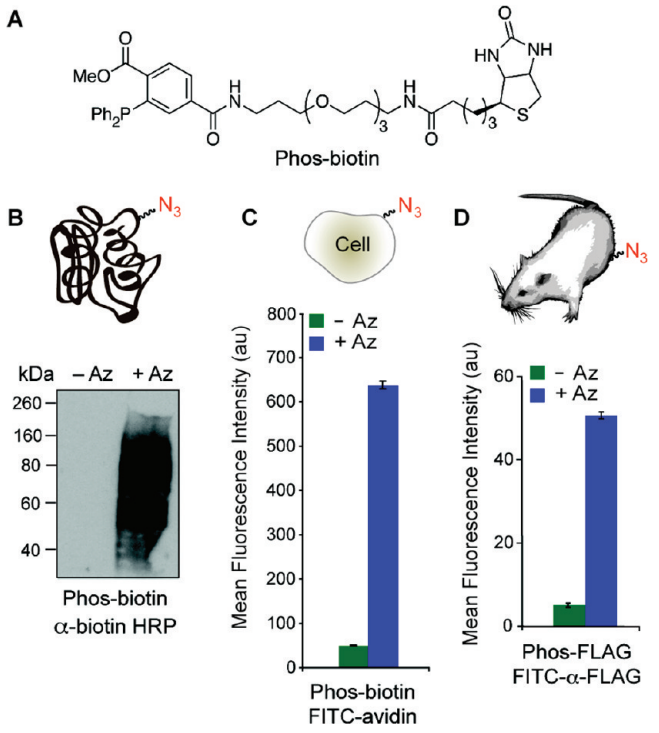

FIGURE 5. The Staudinger ligation enables selective biomolecule labeling in a variety of environments. (A) A phosphine–biotin (Phos-biotin) probe for detection of azides through the Staudinger ligation. (B,C) Selective labeling of azide-modified glycoproteins in lysates and on live cells. Jurkat cells were treated with (blue bars) or without (green bars) peracetylated-*N*-azidoacetyl mannosamine (Ac_4ManNAz), which is metabolized to *N*-azidoacetyl neuraminic acid and incorporated into glycoproteins. (B) Lysates were treated with Phos-biotin (250 μM) overnight and analyzed by Western blot probing with an anti-biotin–horse radish peroxidase (HRP) antibody. (C) Live cells were treated with Phos-biotin (250 μM) for 1 h, followed by incubation with a fluorescent avidin protein (FITC-avidin) and analyzed by flow cytometry. (D) Mice were injected with (blue bars) or without (green bars) Ac_4ManNAz once daily for 7 d. On the eighth day, phosphine conjugated to the FLAG peptide (Phos-FLAG) was injected into the mice. After 3 h, the mice were sacrificed, and their splenocytes were isolated, incubated with a fluorescent anti-FLAG antibody (FITC-anti-FLAG), and analyzed by flow cytometry. Au = arbitrary units.

testament to the mutual selectivity of the Staudinger ligation reagents; no previously reported reaction could have reliably formed products in such a complex reaction vessel. But the real gem of this early work was the azide. The benefits of its small size were immediately apparent as we found that several glycan biosynthetic pathways were quite accommodating of azidosugar substrates. Since that initial work, many other groups have used the azide as a chemical reporter of protein biosynthesis, lipid posttranslational modifications, nucleic acid biosynthesis, enzyme activity, and the list keeps growing (many examples are highlighted in this issue).[1]

The Staudinger ligation possessed unmatched capabilities, but it fell short of perfection. The phosphine reagents

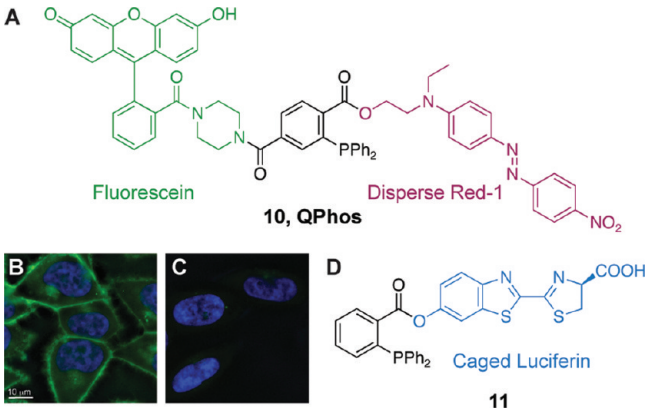

FIGURE 6. (A) A FRET-based fluorogenic phosphine for the Staudinger ligation. (B,C) HeLa cells were grown in the presence (B) or absence (C) of Ac_4ManNAz. The cells were washed, incubated with 50 μM **10** for 8 h at 37 °C, and imaged. Green = fluorescein. Blue = Hoechst 33342 nuclear stain. Images were originally published in ref 11. Copyright 2008, WILEY-VHC. (D) A phosphine–luciferin probe for bioluminescence imaging of azides.

slowly underwent air oxidation within biological systems and were probably metabolized by cytochrome P450 enzymes in mice. Additionally, the kinetics of the reaction were somewhat slow (typical second-order rate constant of 0.0020 M^{-1} s^{-1}), which necessitated the use of high concentrations of phosphine reagent. This, in turn, was found to be problematic for fluorescence imaging applications since excess probe reagent was difficult to wash away, resulting in high background signal.[9]

A detailed mechanistic study revealed that the rate-determining step of the Staudinger ligation is the initial nucleophilic attack of the phosphine on the azide.[10] Thus, increasing the electron density on the phosphorus atom could, in principle, increase the rate of the Staudinger ligation. While the addition of electron-donating groups to the aryl substituents did indeed increase the rate of the desired reaction, these more reactive substrates were also rapidly oxidized in air.

Frustrated by our inability to improve the intrinsic kinetics of the Staudinger ligation, we turned to alternate means of reducing background fluorescence in cell imaging experiments. A key step in the reaction mechanism is intramolecular amide bond formation with concomitant ester cleavage. Our mechanistic studies indicated that the alcohol leaving group could be varied widely in structure without detriment to rate or yield.[10] We exploited this feature to design a fluorogenic phosphine reagent (**10**, QPhos, Figure 6A).[11] Fluorescein was conjugated to one of the phosphine's aryl substituents through an amide linkage; a FRET quencher, disperse

red-1, was appended through the ester linkage. Upon reaction with an azide, the quencher was released to yield a fluorescent product. QPhos allowed for direct imaging of azides on live cultured cells (Figure 6B,C).

The slow reaction kinetics of the Staudinger ligation, coupled with the imperfect spectral properties of fluorescein for *in vivo* imaging, have to date undermined the use of fluorogenic phosphine **10** in live animals. Red-shifted variants of the fluorogenic phosphine have been synthesized, but these compounds undergo rapid nonspecific phosphine oxidation.[12] Recently, we redirected our *in vivo* imaging efforts to the more sensitive imaging modality of bioluminescence, and toward this end, we reported bioluminogenic phosphine reagent **11** (Figure 6D).[13] Like fluorogenic reagent **10**, compound **11** releases luciferin during its Staudinger ligation with phosphines. Once liberated, luciferin readily enters cells wherein heterologously expressed luciferase catalyzes its oxidation and the concomitant emission of light. Compound **11** enabled very sensitive detection of azides within cell-surface glycoproteins and is a promising reagent for *in vivo* imaging in luciferase transgenic mice.

Since its original inception, the Staudinger ligation has found utility far beyond glycan imaging. The reaction has been employed for glycoproteomics studies[14] and for immobilization of azide-labeled proteins on surfaces.[15] Additionally, a modified version of the Staudinger ligation, the "traceless" Staudinger ligation, has been used for protein synthesis in a manner reminiscent of native chemical ligation.[16,17] For this application, another mechanistic modification was made so that the phosphine oxide is expelled during the reaction, leaving an unencumbered amide-linked product.

While still the reaction of choice for a wide range of bioconjugation applications, the slow kinetics of the Staudinger ligation remains an unsolved problem and an obstacle for *in vivo* chemistry. Consequently, during the mid-2000s, we and others turned our attention to the other mode of bioorthogonal reactivity exhibited by the azide: its 1,3-dipolar cycloaddition with alkynes.

Cu-Free Click Chemistry

The cycloaddition reaction of azides and alkynes to form triazoles (Figure 7A) was first reported by Michael in the late 1890s[18] and later studied in depth by Huisgen in the mid-20th century.[19] Huisgen spent a great deal of his career analyzing the mechanism of this and other [3 + 2] cycloaddition reactions,[19] and consequently, there was a large body of physical organic chemistry one could exploit in converting

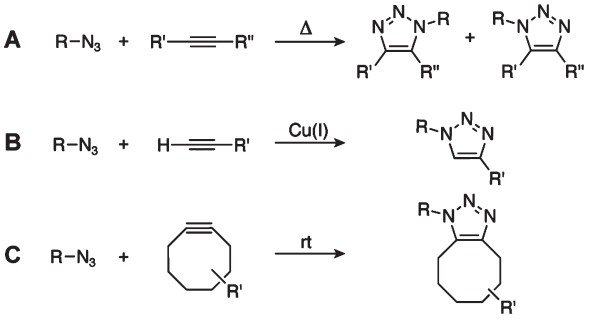

FIGURE 7. (A) The 1,3-dipolar cycloaddition of azides and linear alkynes to form regioisomeric triazole products. (B) The Cu(I)-catalyzed formal azide–alkyne cycloaddition to yield 1,4-triazole products, also known as CuAAC, a paradigm example of "click chemistry". (C) The strain-promoted cycloaddition of azides and cyclooctynes to give triazole products, also known as Cu-free click chemistry.

this prototype to a bioorthogonal reaction. Similar to the Staudinger ligation, the major deficiency of the canonical azide–alkyne cycloaddition was its sluggish kinetics using conventional unactivated alkynes.[20] Indeed, the standard Huisgen reactions were typically performed at elevated temperatures and pressures that are far beyond the limits of biological systems.

In the early 2000s, Sharpless and Meldal also noted the potential utility of the Huisgen cycloaddition as a means to selectively couple highly functionalized molecules. They independently reported that a dramatic rate enhancement of the reaction with terminal alkynes can be achieved using a Cu(I) catalyst (Figure 7B).[21,22] Today this reaction is considered a paragon of "click chemistry" and has been used in many fields of chemistry, including chemical biology.[23] It is nearly bioorthogonal, with the major liability being that the Cu(I) catalyst is cytotoxic. Several laboratories are working toward decreasing the cytotoxicity or increasing the reactivity of the catalyst through ligand optimization. Recent success in this area has allowed for live cell imaging of azide and terminal alkyne chemical reporter groups.[24,25]

We sought to avoid the use of tranisition metal catalysts altogether, hoping that a more biofriendly method of activating alkynes toward reaction with azides could be found by mining the classic mechanistic literature. Sure enough, in 1961, Wittig and Krebs reported that cyclooctyne, the smallest stable cycloalkyne, reacted "like an explosion" with phenyl azide.[26] We inferred from this statement that a good portion of the ~18 kcal/mol of ring strain associated with cyclooctyne was released in the transition state of the cycloaddition reaction.

Motivated by this report of a putative "Cu-free click chemistry", we embarked on the synthesis of strained

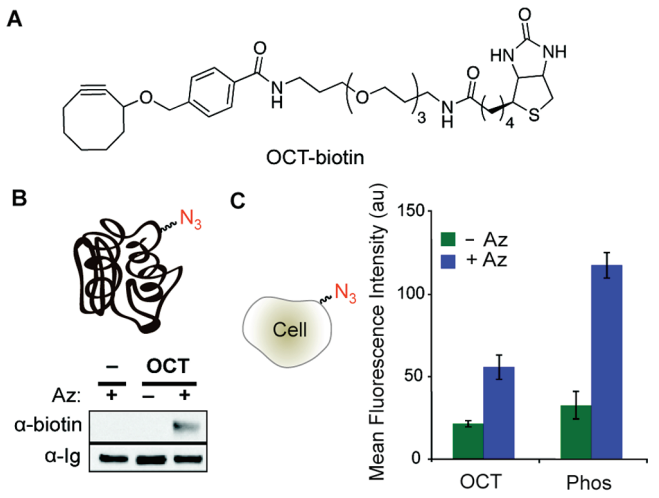

FIGURE 8. Cyclooctyne selectively reacts with azides through a strain-promoted cycloaddition. (A) A cyclooctyne–biotin probe (OCT-biotin). (B) OCT selectively labels an azide-modified form of the recombinant glycoprotein GlyCAM-IgG. Purified GlyCAM-IgG or azido-GlyCAM-IgG was incubated with 0 or 250 μM OCT-biotin overnight at rt. The samples were analyzed by Western blot probing with an anti-biotin antibody conjugated to HRP. An anti-IgG antibody confirmed equal protein loading. Western blot reprinted with permission from ref 27. Copyright 2004 American Chemical Society. (C) OCT labels live cells in an azide-dependent manner. Jurkat cells were grown in the presence (blue bars) or absence (green bars) of Ac_4ManNAz. The cells were incubated with OCT-biotin or Phos-biotin (100 μM) for 1 h at rt, followed by treatment with FITC-avidin, and analyzed by flow cytometry.

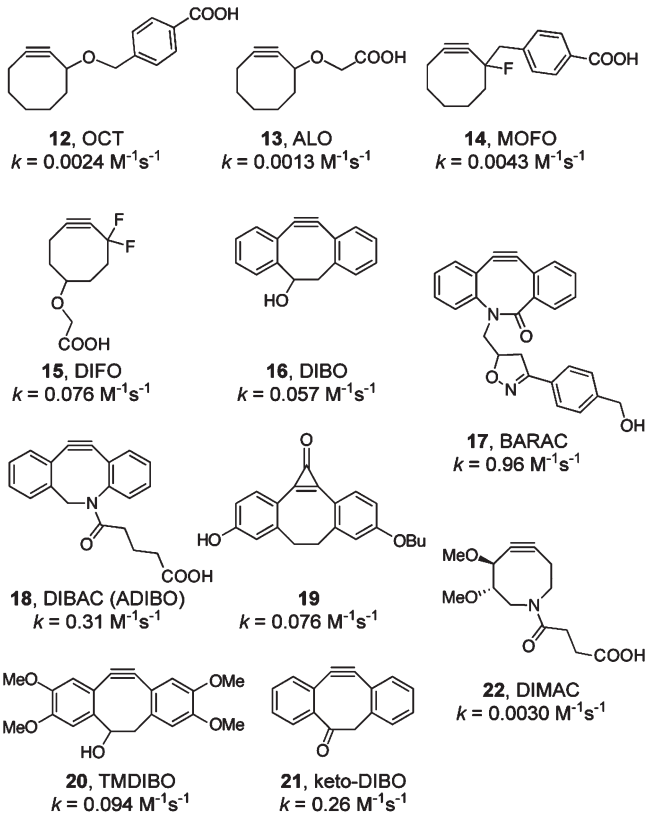

FIGURE 9. Cyclooctynes synthesized for Cu-free click chemistry in living systems. The second-order rate constants are for the reaction with benzyl azide in acetonitrile (**12**,[27] **13**,[28] **14**,[28] **15**,[29] **17**,[31] **22**[42]) or methanol (**16**,[36] **18**,[32] **19**,[34] **20**,[35] **21**[36]).

cyclooctynes that were also functionalized for the attachment of biological probes (Figure 7C). The first in class was the compound we call OCT, which we conjugated to biotin for cell labeling studies (Figure 8A).[27] Linear alkynes are essentially unreactive with azides at physiological temperature, but OCT-biotin readily reacted with azide-labeled glycans on proteins, within cell lysates, and on live cultured cells (Figure 8B,C). Most importantly, the compound exhibited no apparent toxicity, in stark contrast to the reagents for the Cu-catalyzed reaction. However, with a second-order rate constant of 0.0024 M^{-1} s^{-1} in model reactions, OCT (**12**, Figure 9) was no faster than the Staudinger ligation. The compound also had limited water solubility.

We embarked on a series of mechanism-based modifications to accelerate the reaction and improve its physical properties for *in vivo* imaging applications. The "aryl-less octyne" **13** (ALO) had better water solubility, but its kinetic properties were similar to those of OCT.[28] The first significant rate enhancement was achieved by addition of an electron-withdrawing fluorine atom at the propargylic position to yield a monofluorinated cyclooctyne (MOFO, **14**). MOFO proved more reactive than OCT and ALO ($k = 0.0043$ M^{-1} s^{-1}) and, accordingly, labeled azides in cell lysates and on cell-surfaces more rapidly.[28] Even more dramatic was the addition of a *gem*-difluoro group at the propargylic position, creating difluorinated cyclooctyne **15** (DIFO), which increased the rate of Cu-free click chemistry by more than an order of magnitude ($k = 0.076$ M^{-1} s^{-1}).[29] Boons and co-workers later reported that a similar rate enhancement can be achieved by fusing two aryl rings to the cyclooctyne core, resulting in a highly strained dibenzocyclooctyne **16** (DIBO, $k = 0.057$ M^{-1} s^{-1}).[30] We were able to achieve another order of magnitude rate increase through the addition of an amide bond to the DIBO scaffold, yielding a biarylazacyclooctynone (BARAC, **17**, $k = 0.96$ M^{-1} s^{-1}).[31] A version of BARAC with an exocyclic amide (**18**) was prepared independently by the Van Delft and Popik groups (named DIBAC or ADIBO) and its reaction with azides was associated with a rate-constant of 0.31 M^{-1} s^{-1}.[32,33] Additionally, photocaged[34] and tetramethoxy[35] versions of DIBO (**19**, **20**) have been reported, as well as a keto-DIBO (**21**) that undergoes spectral changes upon

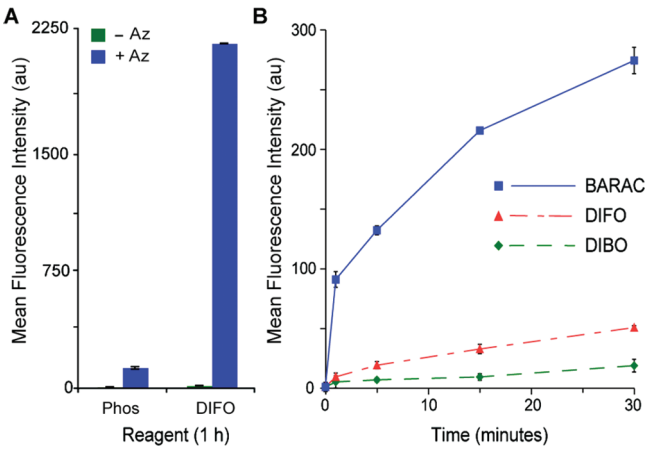

FIGURE 10. The cyclooctynes are superior reagents for labeling azides on cell surfaces. (A,B) Jurkat cells were grown in the presence or absence of Ac_4ManNAz. (A) The cells were incubated with Phos-biotin or DIFO-biotin (100 μM) for 1 h at rt, followed by treatment with FITC-avidin, and analyzed by flow cytometry. (B) The cells were treated with BARAC-biotin, DIFO-biotin, or DIBO-biotin (1 μM) for various amounts of time. Each sample was incubated with FITC-avidin and analyzed by flow cytometry. Each point represents the difference between the azide-treated and untreated cells. Au = arbitrary units.

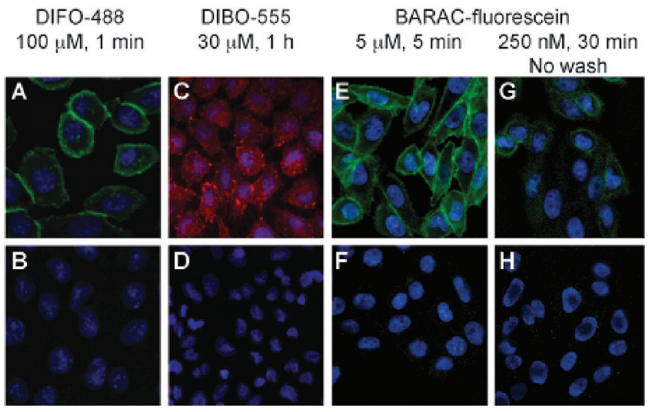

FIGURE 11. Cyclooctyne–fluorophore conjugates label cells in an azide-dependent manner. CHO (A, B, E–H) or U-2 OS (C, D) cells were grown in the presence (A, C, E, G) or absence (B, D, F, H) of Ac_4ManNAz. (A,B) The cells were incubated with DIFO conjugated to Alexa Fluor 488 (DIFO-488, 100 μM) for 1 min at 37 °C, washed, and imaged. (C,D) The cells were incubated with DIBO conjugated to Alexa Fluor 555 (DIBO-555, 30 μM) for 1 h at rt. The cells were then washed, fixed, and imaged. (E,F) The cells were incubated with BARAC conjugated to fluorescein (BARAC-fluorescein, 5 μM) for 5 min, washed, and imaged. (G,H) The cells were incubated with BARAC-fluorescein (250 nM) for 30 min and immediately imaged without washing. Green = DIFO-488 or BARAC-fluorescein; Red = DIBO-555; Blue = Hoechst 33342 nuclear stain. (A,B) Images were reprinted with permission from ref 29. Copyright 2008 National Academy of Sciences, USA. (C,D) Images reprinted with permission from ref 37. Copyright 2011 Life Technologies Corporation. (E–H) Reprinted with permission from ref 31. Copyright 2010 American Chemical Society.

triazole formation with azides.[36] These diverse cyclooctynes demonstrate the value of mechanistic modifications in transforming an obscure chemical reaction from the mid-20th century literature into a highly efficient bioorthogonal ligation.

The second-order rate constants for their cycloaddition reactions with azides accurately reflected the cyclooctynes' ability to detect azides in biological labeling experiments. As shown in Figure 10A, DIFO reagents chemically labeled cell surface azides with far greater sensitivity than comparable phosphine reagents. As well, cell surface labeling efficiencies of DIFO, DIBO, and BARAC directly correlated with their relative reactivities (Figure 10B).[29–31] All three cyclooctynes have been conjugated to fluorophores for direct imaging of azidosugars on live cells (Figure 11).[29–31,37] The heightened reactivity of BARAC enabled imaging with such low probe concentrations that removal of excess probe via washing steps was not necessary.

The first application of bioorthogonal chemistry to *in vivo* imaging, a landmark in the field, was achieved using the reaction of DIFO with azides. We employed DIFO–Alexa Fluor conjugates (DIFO-488, DIFO-555, etc.) to probe spatiotemporal changes in cell-surface glycosylation in *Caenorhabditis elegans* and in developing zebrafish. With peracetylated *N*-azidoacetylgalactosamine (Ac_4GalNAz) as a metabolic label, glycoproteins were imaged during three stages of *C. elegans* development, and significant labeling was observed in the pharynx, vulva, and anus (Figure 12A).[38]

In a similar manner, GalNAz-labeled glycoproteins in zebrafish embryos were imaged between 60 and 73 h postfertilization (hpf), and dynamic labeling was monitored in the pectoral fins, olfactory pit, and jaw. Glycan trafficking between 60 and 72 hpf was further analyzed through pulse–chase experiments with spectrally distinct DIFO conjugates (Figure 12B).[39] Glycans expressed during earlier stages of zebrafish embryogenesis could be detected by direct microinjection of GalNAz or the advanced metabolite UDP-GalNAz into the yolk of single-cell embryos. Using this technique, azidoglycans could be imaged as early as 7 hpf (Figure 12C).[40]

The mouse is a more versatile model for studies of human disease, particularly cancer, and therefore we sought to use DIFO probes for *in vivo* imaging in this organism. However, the fast kinetics of the reaction of DIFO with azides *ex vivo* did not translate to an efficient reaction in mice. Indeed, in a head-to-head comparison with phosphine probes, DIFO reagents reacted less efficiently with azide-labeled glycoproteins on mouse splenocytes *in vivo* (Figure 13A).[41] Why did the sluggish Staudinger ligation outperform the speedy Cu-free click chemistry with DIFO? We found that DIFO, a hydrophobic hydrocarbon, binds strongly to the abundant

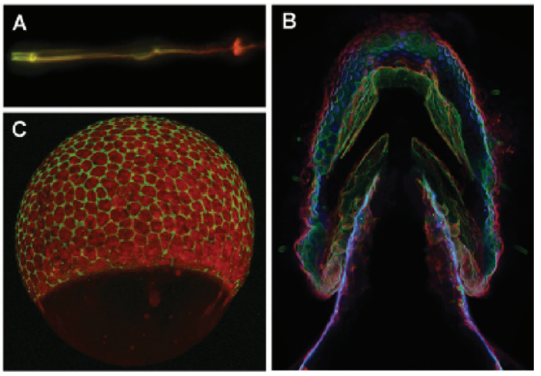

FIGURE 12. DIFO–Alexa Fluor conjugates label azides in higher organisms. (A) *C. elegans* were grown in the presence of Ac_4GalNAz and reacted with DIFO-488 (100 μM) followed by DIFO conjugated to Alexa Fluor 568 (DIFO-568, 100 μM) and imaged at their adult stage. Image reprinted with permission from ref 38. Copyright 2009 American Chemical Society. (B) Zebrafish embryos were metabolically labeled with Ac_4GalNAz from 3 to 60 hpf. The fish were sequentially incubated with 100 μM DIFO conjugated to Alexa Fluor 647 (DIFO-647, 60–61 hpf), DIFO-488 (62–63 hpf), and DIFO-555 (72–73 hpf) and imaged by confocal microscopy. During periods in which the zebrafish were not being labeled with DIFO, the fish were bathed in a solution of Ac_4GalNAz. Blue = DIFO-647, Green = DIFO-488, Red = DIFO-555. (C) Zebrafish embryos were injected with UDP-GalNAz and a rhodamine–dextran tracer dye. At 7 hpf, the embryos were incubated with DIFO-488 (100 μM) for 1 h and imaged by confocal microscopy. Green = DIFO-488, red = rhodamine–dextran. Image originally published in ref 40.

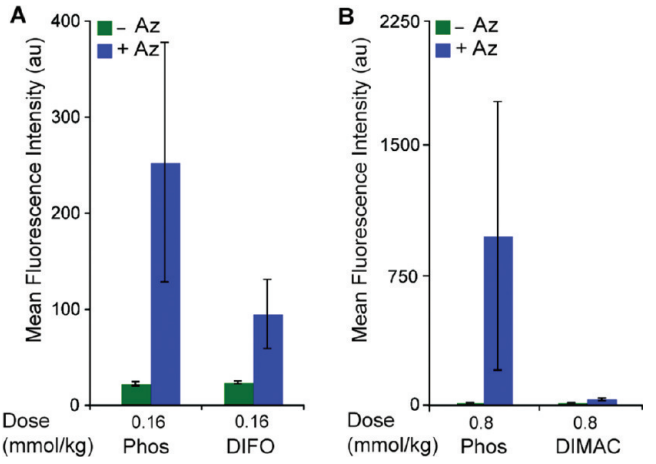

FIGURE 13. The Staudinger ligation is the superior reaction for labeling cell-surface azide-labeled glycoproteins in mice. (A,B) Mice were injected once daily with (blue bars) or without (green bars) Ac_4ManNAz for 7 d. On the eighth day (A) Phos-FLAG or DIFO-FLAG or (B) Phos-FLAG or DIMAC-FLAG was injected. After 3 h, the mice were sacrificed, and their splenocytes were isolated, incubated with FITC-anti-FLAG, and analyzed by flow cytometry. Au = arbitrary units.

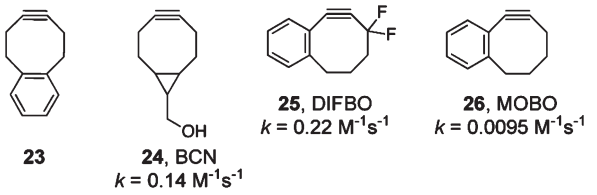

FIGURE 14. Cyclooctynes of recent theoretical and experimental interest.

serum protein murine serum albumin (MSA), likely resulting in sequestration from tissue-resident azides.

To realize the full potential of reactive cyclooctynes, we sought to improve their solubilities and pharmacokinetic properties. The more hydrophilic dimethoxyazacyclooctyne (DIMAC, **22**, Figure 9) was designed with this purpose in mind.[42] DIMAC was considerably less reactive with azides than DIFO ($k = 0.0030\ M^{-1}\ s^{-1}$). However, DIMAC was far more water-soluble, which minimized nonspecific protein binding. Still, in mice, DIMAC's improved solubility properties did not compensate for its sluggish reaction kinetics (Figure 13B).[41] Further optimization of the cyclooctyne reagents remains necessary to obtain the optimal balance of reactivity and pharmacokinetic properties. Analogs of BARAC are promising in this regard, because they are very reactive and also bind MSA at reduced levels compared with DIFO.[12] The evaluation of BARAC conjugates as *in vivo* imaging reagents is an important next step.

In addition to capturing the attention of biologists, Cu-free click chemistry has stimulated interest among the current generation of physical organic chemists, particularly theorists. Several groups have sought to explain the physical basis of the rate enhancement of cyclooctynes versus linear alkynes in the cycloaddition reaction, as well as the effects of fluorination, aryl ring fusions, and other modifications on reaction kinetics. Using density functional theory (DFT), Houk and co-workers concluded that the bent alkyne angles within cyclooctyne increase the rate of the cycloaddition due to a minimization of the distortion required to reach the transition state.[43] Goddard and co-workers have also studied Cu-free click chemistry through DFT calculations and proposed that a monobenzocyclooctyne with one fused aryl ring would yield an optimal balance between strain enhancement and minimization of steric hindrance.[44] Ideally, this aryl ring would be fused at the 5,6 positions of the cyclooctyne (**23**, Figure 14), but **23** was previously shown to be unstable.[45] However, the notion that cyclooctyne can be further activated by modifications distal to the reactive site was recently realized by Van Delft and co-workers. They demonstrated that bicyclononyne **24** (BCN) has reactivity similar to DIBAC/ADIBO ($k \approx 0.1\ M^{-1}\ s^{-1}$) due to a combination of strain effects from the fused cyclopropyl

group and reduced steric hindrance surrounding the alkyne.[46]

We sought to combine the rate-enhancing modifications embodied in DIBO and DIFO (fused aryl rings and fluorination, respectively) by synthesizing difluorobenzocyclooctyne **25** (DIFBO). While the compound reacted rapidly with azides ($k = 0.22\ M^{-1}\ s^{-1}$), it was unstable and prone to oligomerization in concentrated solution.[47] Nonetheless, DIFBO taught us about the relative contribution of its rate-enhancing modifications. For comparative purposes, we prepared monobenzocyclooctyne **26** (MOBO) and measured its second-order rate constant to be $0.0095\ M^{-1}\ s^{-1}$.[47] Thus, DIFBO was 20-fold more reactive than MOBO, while MOBO was only 8 times more reactive than OCT. These results demonstrate that the electronic effects of propargylic fluorination are a major contributor to rate enhancement and should facilitate further optimization of cyclooctynes with regard to their balance of reactivity and stability.

Theoretical and mechanistic work has contributed important insights into the design of modified cyclooctynes with enhanced reactivity. However, Cu-free click chemistry is not the only bioorthogonal reaction to benefit from the skills of physical organic chemists. For example, a recent addition to the bioorthogonal chemistry compendium, the tetrazine ligation, was optimized to achieve an impressive rate constant of $22\,000\ M^{-1}\ s^{-1}$ with guidance from theoretical work.[48] This inverse-demand Diels–Alder reaction between tetrazines and *trans*-cyclooctenes is the fastest bioorthogonal reaction known to date. These examples highlight the opportunities for classically trained theoreticians and experimentalists in this new area of chemical biology.

Conclusion

Bioorthogonal chemistry has evolved to be a rather unusual field in that it brings together traditional mechanistic chemistry, reaction methodology, cell biology, and biomedicine. It is not surprising then that contributors to this special issue come from backgrounds as divergent as theoretical chemistry and clinical imaging. In our own work summarized above, we were compelled to characterize fleeting reaction intermediates, develop new synthetic routes, hunt down unwanted side products on proteins and cells, track probes during cellular internalization, disintegrate embryos to map reaction product distribution, and monitor the health and well-being of laboratory animals in which we performed bioorthogonal chemistries. Collaborators were critical for some of this work, because no single lab can properly master such a breadth of experimental approaches.

Now in its second decade, the field of bioorthogonal chemistry offers several lessons. First, the above examples, as well as many others in this special issue, confirm that chemical reactions can indeed be designed to perform in environs as demanding, and also as intriguing, as living systems. Success in this endeavor requires keen insight into promising reaction prototypes (and perhaps invention of new ones looking forward) and diligence in mechanistic optimization. Second, biologists are eager to embrace tools from chemistry, but they must be made accessible and straightforward to execute. Fortunately, several commercial suppliers now offer azide (or alkyne)-labeled sugars, amino acids, lipids, and other biomolecular substrates, as well as complementary probes for detection or enrichment. Such "kits" enable the use of bioorthogonal chemistry by non-experts, which is essential for widespread adoption of the technology outside of chemistry circles. The success of these commercial kits is no doubt related to the fact that bioorthogonal chemistry is intrinsically low-tech; after all, the reagents should find each other and react no matter the complexity of their surroundings. An ideal bioorthogonal chemical reaction should translate seamlessly from flask to fish.

A final lesson pertains to the importance of reaction discovery as the foundation of bioorthogonal chemistry. The handful of prototype reactions on which current bioorthogonal transformations are based were discovered long before the chemistry/biology interface was a fashionable venue for research. Staudinger, Huisgen, and Wittig could not foresee that their discoveries would someday lead to methods for *in vivo* biomolecule imaging. Likewise, contemporary studies of fundamental chemical reactivity can have an unforeseen impact in biology and beyond. Such explorations should be encouraged even if specific applications are not yet on the horizon. After all, a sector of reaction space that is newly charted today could produce a prototype for bioorthogonal reaction development tomorrow.

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FOOTNOTES

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