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Bioorthogonal Chemistry: Recent Progress and Future Directions

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Abstract

The ability to use covalent chemistry to label biomolecules selectively in their native habitats has greatly enhanced our understanding of biomolecular dynamics and function beyond what is possible with the genetic tools alone. To attain the exquisite selectivity essential in the covalent approach in a complex biological environment, a “bottom-up” two-step strategy has achieved many successes recently. In this approach, a bioorthogonal chemical functionality is built into the basic life’s building blocks—amino acids, nucleosides, lipids, and sugars—as well as secondary metabolites; after their incorporation into the biomolecules, an array of biophysical probes are selectively appended to the chemically tagged biomolecules via a suitable bioorthogonal reaction. While much has been accomplished in the expansion of unnatural building blocks carrying unique chemical moieties for all major classes of biomolecules, the dearth of robust bioorthogonal reactions has limited both the scope and utility of this promising approach. Here we summarize the recent progress in the development of bioorthogonal reactions and their applications in various biological systems. A major emphasis has been placed on the mechanistic and kinetic studies of these reactions with the hope that continuous improvements can be made with each reaction in the future. In view of the gap between the capabilities of the current repertoire of bioorthogonal reactions and the unmet needs of potential biological problems, we also strive to project the future directions of this rapidly developing field.

1. Introduction

The use of covalent chemistry to modify biomolecules in vitro in order to gain insights into their structure and function has long been appreciated by the biophysics and biochemistry community, both for enzymes¹ and for nucleic acids.² In the post-genomic era, there has been an increasing desire to study the structure, dynamics, and function of biomolecules either individually or as a family in their native environments in living cells and organisms. The classic residue-specific biomolecular modification chemistry became inadequate because of the presence of identical residues in other biomolecules in living systems. To overcome this limitation, one successful and widely employed strategy has been the genetic tagging of a fluorescent protein to the target protein. However, other classes of biomolecules, such as nucleic acids, lipids and glycans, and certain protein regulatory processes, such as posttranslational modifications, are not amenable to genetic tagging. To this end, bioorthogonal chemistry has emerged as a general strategy for the study of biomolecular dynamics and function in living systems.^{3–6} Compared to the noncovalent binding based approach, bioorthogonal chemistry relies upon a specific, covalent attachment of a probe molecule to the biomolecule of interest. As a result, it offers several unique advantages: (1) it is applicable to all classes of biomolecules, including proteins, nucleic

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acids, lipids, and glycans; (2) it is extremely versatile, with the choice of probe molecules limited only by the imagination of a researcher; (3) it is highly scalable, suitable for functional annotation of individual biomolecules in living cells as well as a class of biomolecules in a genome-wide functional profiling. Because of these unique features, bioorthogonal chemistry has been exploited successfully in a multitude of protein functional studies, ranging from visualizing protein expression, tracking protein localization, measuring protein activity and turnover, to identifying protein interaction partners in living systems.

Two successive steps are required for the implementation of bioorthogonal chemistry: (1) incorporation of a bioorthogonal reporter into the biomolecule of interest by either native or the engineered biosynthetic pathway; (2) bioorthogonal reaction between the bioorthogonal reporter and the cognate, externally introduced chemical probe (Figure 1). While a plethora of methods have been developed to address the first step, e.g., amber codon suppression mutagenesis,^{7–9} expressed protein ligation,^{10–15} metabolic engineering,^{16–18} and tagging-via-substrate,^{19–24} very few bioorthogonal reactions are known to date that permit the covalent bond formations between the bioorthogonal reporter and the small-molecule probe. This can be explained by numerous constraints encountered during the development of bioorthogonal reactions, some of which are: (1) reactions need to take place in the physiological conditions in water at neutral pH; (2) reactions need to be robust with high yields and fast rates at relatively low biomolecule concentrations; (3) reactants must not cross-react with the abundant biological electrophiles and nucleophiles inside cells, and should only react with the externally introduced reaction partners—a property referred to as *bioorthogonality*; (4) reactants need to be stable both thermally and metabolically inside cells before the reaction, and non-toxic to living systems; and (5) reaction products need to be stable in the physiological conditions so that the functional measurement can be carried out.

Since bioorthogonal reactions are invariably bimolecular reactions in nature (with the exception of the metal-catalyzed reactions), the conjugate formation can be quantified by the following equation: $[conjugate] = k_2[biomolecule] \times [reagent] \times t$, where k_2 is the second-order rate constant and t is the treatment time. For labeling of a target biomolecule, the yield of bioconjugation is proportional to the reagent concentration as well as the second-order rate constant k_2 (Figure 2a). Because excess reagents may cause side effects on cellular physiology, it would be more desirable if the rate constants of the bioorthogonal reactions, which typically range from 0.001 to 1,000 M⁻¹s⁻¹ (see discussions in the follows), are high. In general, higher rate constants result in faster and more efficient labeling with the reduced reagent use. By contrast, for labeling of a target biomolecule whose antibodies have been developed (Figure 2b), the antibody-antigen complex formation is proportional to association constants, k_{ass} , which are typically in the range of 0.1 ~ 3 × 10⁶ M⁻¹s⁻¹,²⁵ and significantly faster than the second-order rate constants of known bioorthogonal reactions. Therefore, the antibody-based approach shall be the method of choice for labeling biomolecules in fixed cells because of its faster kinetics and hence higher sensitivity. For the applications in live cells and organisms, however, bioorthogonal chemistry approach would be preferred because the cell-permeable small-molecule reagents are used.

Despite these challenges, a number of bioorthogonal reactions have been developed successfully that showed excellent biocompatibility and selectivity in living systems. In this Feature Article, we organize some of these reactions based on the representative reactant pairs, and briefly describe their utilities in cellular and organismal biology. While it is convenient for our discussion to separate bioorthogonal chemistry into two parts—incorporation and reaction, these two parts are in fact intertwined as the constraints placed upon the biosynthetic incorporation will dictate the substrate structures available for

the reaction development. Since a more inclusive review of this subject was published elsewhere recently (see ref. 6), we decided to focus our discussion on the key attributes of each reaction, with a particular emphasis on the reaction mechanism and kinetics studies. We hope the insights gained from the studies of these reactions will help shed light to the development of new robust bioorthogonal reactions in the future.

2. Bioorthogonal reactions

2.1 Aldehyde/ketone-hydrazine (hydrazide)/alkoxyamine pair

Aldehyde and ketone functionalities are attractive bioorthogonal chemical reporters because: (1) because of their small size, they can be readily incorporated into biomolecules via the biosynthetic machineries;^{16, 26} and (2) they are virtually inert towards other endogenous functional groups at the physiological condition. At the acidic condition (pH = 5 ~ 6), the amine group attacks the protonated carbonyl group to form a reversible Schiff base where equilibrium usually favors the free carbonyl form. When reactive amines such as hydrazines (or hydrazides) and alkoxyamines are used, however, the equilibrium favors the imine forms due to the α -effect,²⁷ which gives rise to the stable hydrazone and oxime adducts, respectively (Scheme 1a).^{28–30}

The mutual reactivity between aldehyde and hydrazide in living cells was first exploited by Rideout in 1986 for the *in situ* drug assembly inside cancer cells.³¹ In this seminal study, human erythrocytes were exposed to a mixture of decanal and *N*-amino-*N'*-1-octylguanidine (AOG) at 28 μ M each in phosphate buffered saline (PBS) at 37 °C, leading to the *in situ* formation of cytotoxic hydrazones which killed the cancer cells after 80 min. The pseudo-first-order rate constants between decanal and AOG were measured to be in the range of $5.6 \pm 1.6 \times 10^{-4} \text{ s}^{-1}$ to $9.8 \pm 1.0 \times 10^{-4} \text{ s}^{-1}$, depending on the medium conditions. More than 10 years later, Bertozzi and co-workers elegantly demonstrated the capacity of this chemistry for cell surface remodeling by treating Jurkat cells displaying a surface ketone reporter in the form of *N*-levulinoylmannosamine (ManLev) modified sialic acids with a biotin-hydrazide reagent which exhibited an apparent ligation rate constant, k_{lig} , of $0.033 \pm 0.001 \text{ M}^{-1}\text{s}^{-1}$ (Scheme 1a).^{16, 32} In general, this aldehyde/ketone-based bioorthogonal reaction is best suited for extracellular applications^{16, 23, 33, 34} because: (1) the reaction requires an optimal pH of 5 ~ 6, incompatible with normal intracellular environment; (2) the presence of abundant biogenic electrophiles inside the cells such as sugars, pyruvate, and oxaloacetate interfere with this reaction; and (3) high reagent concentrations (2–5 mM) are typically required in order to compensate for the low reaction rates.³²

The limitations of acidic pH and slow kinetics were recently addressed by Dawson and co-workers through the use of aniline as a nucleophilic catalyst. Their initial studies in 2006 showed that aniline significantly accelerates both the hydrazone³⁵ and oxime ligations³⁶ by forming *in situ* a highly reactive electrophile, the protonated aniline Schiff base intermediate, which then undergoes rapid transimination to form the hydrazone/oxime (Scheme 1b). In the presence of 100 mM aniline at neutral pH, they found that the hydrazone formation between 10 μ M benzaldehyde and 10 μ M 6-hydrazinopyridyl-modified peptide proceeded rapidly with a rate constant of $170 \pm 10 \text{ M}^{-1}\text{s}^{-1}$ and an overall 80% yield. On the other hand, the aniline-catalyzed oxime ligation between benzaldehyde and an aminooxyacetyl-modified peptide (100 μ M each) gave a lower reaction rate with a rate constant of $8.2 \pm 1.0 \text{ M}^{-1}\text{s}^{-1}$; however, the oxime adducts appeared to be hydrolytically more stable than hydrazones. Both chemoselective ligations were successfully applied to biomolecular labeling.^{37, 38} In one example, they reported a greater than 95% labeling yield when 30 μ M of a 6-hydrazinopyridyl-modified human serum albumin was treated with 216 μ M *p*-¹⁹F-benzaldehyde in the presence of 100 mM aniline in 0.1 M sodium phosphate, pH = 7.0, for 30 min at room temperature.³⁷ In another example, they reported a robust, non-

disruptive labeling method, dubbed “PAL methodology”, to visualize the sialylated glycoproteins on live cell surfaces where the aldehyde functionality was generated through a mild oxidation procedure followed by the aniline-catalyzed oxime ligation with an aminooxy-modified biotin tag (Scheme 1b).³⁸

2.2 Azide-alkyne pair

The 1,3-dipolar cycloaddition reaction between azides and acetylenes to yield triazoles was first described by Huisgen more than forty-five years ago.³⁹ Since the reaction requires heating in order to obtain the triazoles in appreciable amounts, it was considered to be incompatible with the biological systems. In 2002, a dramatic rate acceleration was uncovered independently by the Sharpless group and the Meldal group when a catalytic amount of Cu^I salt was used in the reaction (Scheme 2).^{40, 41} Based on the extensive density functional theory calculations carried out by Fokin, Sharpless and co-workers,⁴² this rate acceleration is attributed to the stepwise formation of the unusual six-membered Cu^{III} metallacycle estimated to have a dramatically reduced activation barrier of 14.9 kcal/mol (versus 25.7~26.0 kcal/mol for the uncatalyzed reaction and 18.7 kcal/mol when ligand is water, Scheme 2b). Despite the lack of precedence, the proposed mechanism (Scheme 2b) agreed well with the experimental data. For example, it was observed that the reaction proceeds much faster in aqueous solution, which can be explained by the relative ease in displacing a water ligand from the Cu^I-center (an exothermic process with $\Delta H = 11.7$ kcal/mol) relative to an acetonitrile ligand during the formation of the Cu^I-acetylide (Scheme 2b). This may also explain why the Cu^I-catalyzed azide-alkyne 1,3-dipolar cycloaddition reaction (CuAAC), now widely known as “click chemistry”, proceeds extremely efficiently at the physiological conditions.

Because of its fast reaction kinetics and excellent functional group tolerance, click chemistry has been widely employed in many biological studies, e.g., tagging of a variety of biomolecules,^{42–44} virus surface remodeling,⁴² nucleic acid immobilization⁴⁵ and tracking,^{46, 47} selective protein modification,⁴⁸ activity-based protein profiling,⁴⁹ and lipid labeling in living cells and live animals.^{50, 51} The main advantage of click chemistry is its fast rate; the reaction between azides and alkynes proceeds at least 25 times faster than Staudinger ligation between azides and triarylphosphines in the cell lysates.⁵² Therefore, click chemistry is extremely valuable in situations involving the manipulation of a very small quantity of biomolecules.⁵³ However, the need for a copper catalyst, which is toxic to cells,⁵⁴ may preclude its wider use in cellular and organismal applications. For example, mammalian cells can survive for 1 hour after the treatment of <500 μM copper(I) salt,⁶ but began to die when 1 mM copper(I) salt was used.⁴⁹ The copper(I) induced cytotoxicity may be related to the imbalance of copper homeostasis, the altered cell division, and/or the disruption of lipid metabolism.⁵⁵

To circumvent the cytotoxicity associated with the copper salt, several metal-free azide-alkyne cycloaddition reactions have been developed.⁵⁶ One approach was reported by Bertozzi and co-workers whereby they harnessed the ring strain present in cyclooctyne (ca. 18 kcal/mol)⁵⁷ based from the earlier work of Wittig and Krebs in 1961.⁵⁸ The angle strain of the triple bond (twisted out of plane by 19°), together with the ring strain present in cyclooctyne, contribute to a significantly faster rate (Scheme 3a).⁵⁹ The second-order rate constant for the cycloaddition reaction between a cyclooctyne derivative and benzyl azide in aqueous CD₃CN was determined to be 0.0012 M⁻¹s⁻¹, lower than that of a typical Staudinger ligation (0.0025 M⁻¹s⁻¹).^{52, 60} Despite its initial slow kinetics, this earlier version of the strain-promoted cycloaddition reaction was successfully employed to label glycoproteins both *in vitro* and on cell surfaces without observable cytotoxicity.⁶¹ Further optimization led to the development of difluorocyclooctyne (DIFO) reagents (Scheme 3b) that showed an increased reaction rate ($k_2 = 0.076$ M⁻¹s⁻¹).⁶² Subsequent B3LYP density

functional theory calculations carried out by Houk and co-workers suggested that the charge-transfer interactions contribute principally to the rate enhancement afforded by the fluorine substitution (the activation barrier is lowered to 6.0 kcal/mol from 16.2 kcal/mol for the reaction between phenyl azide and acetylene).^{63, 64} For simple cyclooctynes, the rate acceleration is primarily due to the reduction by 8 kcal/mol in distortion energies for both the 1,3-dipole and the alkyne, the major energy barriers leading to the transition state geometries.^{65, 66} Because of the improved reaction kinetics, the DIFO reagents have allowed the dynamic *in vivo* imaging of both CHO cells⁶² and the developing zebrafish.⁶⁷ An improved synthesis of the second-generation DIFO reagents which showed similar reaction kinetics was also reported recently.⁶⁸ The main disadvantage of the DIFO reagents is their demanding synthesis; it takes 12 steps to prepare the first-generation DIFO reagents with an overall yield of ~1%⁶² and 8 steps to synthesize the second-generation DIFO reagents with a total yield of 28%.⁶⁸

An alternative strained cyclooctyne derivative was reported by Boons and co-workers in which two benzene rings were fused to cyclooctyne to increase its reactivity (Scheme 3c).⁶⁹ The second-order rate constant for the cycloaddition reaction between dibenzocyclooctyne and benzyl azide in acetonitrile–H₂O (4:1) was determined to be 2.3 M⁻¹s⁻¹, approximately three orders of magnitude faster than that of simple cyclooctyne. This strained alkyne-derived probe was then used to visualize the metabolically labeled, azide-containing glycoproteins on the surfaces of live cells. Compared to the DIFO reagents, the major advantage of the dibenzocyclooctyne-based reagents appear to be their synthetic accessibility and the potential of additional rate enhancement through substituent effect on the benzene rings.

A related metal-free triazole formation reaction was reported by Cornelissen and co-workers in which alkynes are hidden in the form of oxa-norbornadienes.⁷⁰ Through a tandem 1,3-dipolar cycloaddition—retro-Diels-Alder reaction sequence, triazole adducts were formed with the second-order rate constants of 1.9 ~ 24 × 10⁻⁴ M⁻¹s⁻¹ at 37 °C as measured by NMR in the deuterated solvents. This strain-promoted reaction was then successfully employed to label an oxa-norbornadiene functionalized hen egg white lysozyme (HEWL) with 3-azido-7-hydroxycoumarin in a sodium acetate buffer (pH 5.5). However, the Michael addition products were observed when the electron-deficient oxa-norbornadiene was incubated with a pool of excess amount of 20 naturally occurring amino acids.

2.3 Azide-phosphine pair

One of the classic reactions involving organic azides is Staudinger reaction in which azides are converted to primary amines upon treatment with phosphines.⁷¹ The reaction begins with the attack on the distal nitrogen by the phosphorus atom to form a phosphazide intermediate which converts to the aza-ylide intermediate following the loss of N₂. In the presence of water, aza-ylide hydrolyzes spontaneously to yield the products.^{60, 72}

Recognizing the mutual reactivity of azide and phosphine, Bertozzi and co-workers cleverly designed a phosphine reagent such that the aza-ylide intermediate can be trapped by an adjacent electrophilic carbonyl group which upon hydrolysis forms a stable amide bond (Scheme 4a).⁷³ This modified Staudinger reaction is now referred to as “Staudinger ligation” because of its capacity in covalently linking two molecules together.⁷⁴ Kinetic analysis of the Staudinger ligation of triphenylphosphine and benzyl azide in CD₃CN in 5% H₂O revealed that the apparent second-order rate constant was 0.0025 ± 0.0002 M⁻¹s⁻¹, with the rate-limiting step to be the formation of the phosphazide intermediate.⁶⁰

Shortly after the original report, modifications termed “traceless” Staudinger ligation were reported in which the ligation products were devoid of phosphine oxide motif.^{75, 76} Taking cues from the native chemical ligation, Raines and co-workers described a ligation method

between a thioester and azide using phosphinothiol where the ligated product was linked by an amide bond. In a parallel study, Bertozzi and co-workers designed several phosphine reagents with a cleavable linker connecting the acyl group and the phosphine such that once the aza-ylide intermediate is formed, attack of the aza-ylide nitrogen on the carbonyl displaces the linker and the attached phosphonium group. Hydrolysis of the rearranged adduct produces an amide bonded ligation product and liberates a phosphine oxide (Scheme 4b).⁷⁶

Staudinger ligation has proven to be a practical tool for biomolecular manipulation with high chemoselectivity under a very mild reaction condition. In their seminal work, Bertozzi and co-workers engineered the Jurkat cell surfaces to carry an azide functionality, and subsequently modified the cell surfaces with a water-soluble biotin-containing phosphine reagent.⁷³ Later on, mice were fed with daily doses of 300 mg Kg⁻¹ of an azide modified mannosamine (ManNAz) for 7 days before euthanasia. The metabolic labeling of splenocytes (cells rich in sialosides) with the azide functionality in living animals was demonstrated with the Staudinger ligation using a FLAG-modified phosphine reagent both *ex vivo* and *in vivo*.¹⁸ Other applications include the enrichment of glycoprotein subtypes in the cell lysates,^{77, 78} addition of new functionality to recombinant proteins,⁷⁹ site-specific fluorescent labeling of proteins,⁸⁰ detection of active proteasome in living cells,⁸¹ FRET-based live-cell imaging using a fluorogenic phosphine reagent,⁸² site-selective protein immobilization,^{83, 84} and detection of protein fatty acylation.⁸⁵ Oxidation of phosphines by air or metabolic enzymes is by far the major disadvantage of this reaction, which can be overcome through the use of a large excess of phosphine reagents. Other potential side reactions may include the reduction of azides by the cytosolic glutathione and the interaction of phosphines with disulfide bonds present in proteins.

2.4 Tetrazole-alkene pair

For a bioorthogonal reaction to be broadly useful in studying biological dynamics in living system, three attributes are critical: 1) high bioorthogonality; 2) fast reaction kinetics; and 3) rapid inducibility. While the importance of excellent bioorthogonality and fast reaction kinetics is well recognized, the importance of inducibility has not been widely appreciated. In rapid response to environmental cues, many biological processes such as gene transcription and protein posttranslational modifications demonstrate a high degree of *temporality* or *inducibility*. In studying these dynamic processes, it would be advantageous if we can control the initiation as well as the extent of the bioorthogonal reactions with a spatiotemporal resolution such that a kinetic perturbation can be applied to a biological system. To this end, our group has endeavored to develop the photoinducible bioorthogonal reaction, also known as “photoclick chemistry”, that may provide a time-resolved chemical tool to the study of biological processes.

More than four decades ago, Huisgen and co-workers reported a photoactivated 1,3-dipolar cycloaddition reaction between 2,5-diphenyltetrazole and methyl crotonate.⁸⁶ A concerted reaction mechanism was proposed whereby the diaryltetrazole undergoes a facile cycloreversion upon photoirradiation to release N₂ and generate *in situ* a nitrile imine dipole which then cyclizes spontaneously with the alkene dipolarophile to afford the pyrazoline cycloadduct (Scheme 5). Attracted by this novel mode of substrate activation, our group successfully identified a milder photoactivation procedure with the use of a handheld low-powered UV lamp that allows the cycloaddition reaction to proceed with excellent solvent compatibility including water, functional group tolerance, regioselectivity, and yield.⁸⁷

Our subsequent studies demonstrated that this photoinduced cycloaddition reaction can be employed to modify the tetrazole-containing proteins in biological media.⁸⁸ In a kinetic study between a tetrazole peptide and acrylamide in PBS buffer at pH 7.5, we found that

under 302-nm photoirradiation the nitrile imine intermediate was generated rapidly ($k_1 = 0.14 \text{ s}^{-1}$) and that the subsequent cycloaddition with acrylamide proceeded very efficiently with a second-order rate constant equal to $11.0 \text{ M}^{-1}\text{s}^{-1}$, significantly faster than the Staudinger ligation and the strain-promoted azide-alkyne cycloaddition. Following this kinetic study, the bioorthogonality of the reaction was evaluated in both residue-specific and site-specific protein modifications. In the former, diphenyltetrazole was introduced into lysozyme via acylation of the surface lysines. The resulting tetrazole-lysozyme conjugate was irradiated with a 302-nm handheld UV light for 2 min in the presence of 50 equiv of acrylamide in PBS buffer. The pyrazoline adduct was formed with greater than 90% conversion based on the LC-MS analysis. It is noteworthy that the pyrazoline cycloadducts were fluorescent, allowing us to use in-gel fluorescence analysis to tract specific cycloadduct formation. In the latter, diphenyltetrazole was introduced into the C-terminus of enhanced green fluorescent protein (EGFP) via the intein-based chemical ligation. The tetrazole-containing EGFP (EGFP-Tet; $10 \mu\text{M}$ in 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.5) was then reacted with 1.2 mM of a lipid dipolarophile via irradiation under 302 nm for 1 min. The in-gel fluorescence analysis revealed the presence of a fluorescent band, consistent with formation of the fluorescent pyrazoline cycloadduct (Figure 3). The conversion from the tetrazole-EGFP to pyrazoline-EGFP was estimated to be 52% based on the LC-MS analysis.

The utility of this photoinduced cycloaddition reaction in selectively labeling proteins *in vivo* was subsequently demonstrated with *E. coli* cells overexpressing an alkene-containing Z-domain protein (alkene-Z).⁸⁹ By employing an *E. coli* strain that charges *O*-allyltyrosine site-specifically into the Z-domain protein, BL21(DE3) cells expressing either wild-type Z (wt-Z) or alkene-Z were suspended in PBS buffer containing 5% glycerol and treated with $100 \mu\text{M}$ tetrazole **1**. After incubation at 37°C for 30 min, the cell suspension was photoirradiated at 302 nm for 4 min followed by an overnight incubation at 4°C . In the cyan fluorescent protein (CFP) channel of a fluorescent microscope which captures the fluorescent light emitted by the pyrazoline cycloadduct, only the alkene-Z expressing bacteria showed strong fluorescence while the wild-type-Z expressing bacteria did not, indicating that this reaction is highly selective toward the exogenously introduced terminal alkene group in intact bacterial cells (Figure 4).

A significant observation was that the cycloaddition reaction between tetrazole **1** and *O*-allyltyrosine proceeded much slower than the reaction involving acrylamide as a dipolarophile ($k_2 = 0.00202 \pm 0.00007 \text{ M}^{-1}\text{s}^{-1}$ vs. $0.15 \text{ M}^{-1}\text{s}^{-1}$ for acrylamide), which can be explained by the significantly higher LUMO (Lowest Unoccupied Molecular Orbital) energy of *O*-allyltyrosine relative to acrylamide. In an effort to optimize the tetrazole reactivity toward the unactivated terminal alkenes, we systematically tuned the HOMO (Highest Occupied Molecular Orbital) energies of the nitrile imine dipoles by introducing various substituents onto the aryl rings. We found that electron-donating groups led to higher HOMO energies, which in turn gave rise to faster cycloaddition rates.⁹⁰ One simple tetrazole, 2-(*p*-methoxyphenyl)-5-phenyltetrazole, showed a robust reaction with *O*-allyltyrosine with a k_2 value of $0.95 \text{ M}^{-1}\text{s}^{-1}$, 475-fold faster than that of tetrazole **1**. Gratifyingly, with the improved reaction kinetics it has become possible to use this reaction to label the alkene-encoded proteins in bacteria in less than one minute.⁹⁰

A critical question during our investigation regarding the tetrazole reactivity was about the real electronic structure of the photogenerated nitrile imine dipoles.⁹¹ To answer this question, we used the photocrystallography technique and briefly exposed a series of diaryltetrazole crystals to the 325 nm He-Cd laser beam at 90 K (Figure 5a). To our satisfaction, one of the crystals showed a discrete photodifference map (Figure 5b) which upon least-squares refinement gave a 13% yield of a bent nitrile imine product (Figure 5c).⁹²

Additional water-quenching studies suggested that this bent geometry can be ascribed to the 1,3-dipolar form, the major electronic structure possibly responsible for the excellent cycloaddition reactivity in the aqueous medium.

Since short-wavelength UV light invariably causes photo damage to the exposed cells, as a first step, we designed the long-wavelength photoactivatable diaryltetrazoles that undergo ring-opening at 365 nm to generate the reactive nitrile imine dipoles. We found that by placing an auxochromic or conjugative substituent on the *N*-phenyl ring, diaryltetrazoles exhibited good to excellent photoreactivity at 365 nm and that the resulting nitrile imines also showed excellent reactivity towards the electron-deficient and conjugated alkenes.⁹³ Based on this finding, we recently found that one of the 365-nm photoactivatable tetrazoles, 2-(*p*-aminophenyl)-5-phenyltetrazole, can undergo a two-photon (700 nm) induced cycloaddition reaction with the homoallylglycine (HAG)-modified proteome *in vitro*. This tetrazole also allowed us to image the newly synthesized, HAG-encoded proteins in a spatiotemporal controlled manner in mammalian cells.⁹⁴

Compared to other bioorthogonal reactions, the major advantage of photoclick chemistry is its light inducibility. Since tetrazoles become activated only upon UV irradiation, the use of light allows a spatiotemporal control over the reaction initiation. Additional advantages of this bioorthogonal reaction include the mild reaction conditions (with incident photons to be the only reagents), easy access of tetrazole reagents (only 2 steps are required for their syntheses),⁹⁵ and convenient fluorescent monitoring due to the formation of the fluorescent pyrazoline cycloadduct. While the applications in live cells so far have focused on the labeling of the biosynthetically incorporated terminal alkenes because of their pre-existence, it is also possible to contemplate the genetic incorporation of the tetrazole-derived building blocks. Indeed, we have reported the synthesis of a series of tetrazole amino acids and found that some of them showed excellent reactivity toward alkenes upon photoactivation,⁹⁶ and very recently we were able to incorporate one of these tetrazole amino acids into proteins site-selectively in *E. coli*.⁹⁷ With the development of the genetically encoded tetrazole functionality in proteins, it should be possible to use light to regulate the localization and function of the target protein via the photoinduced modification, e.g., lipidation, with an exquisite spatiotemporal control.

2.5 Tetrazine-alkene pair

Diels-Alder reaction is a highly selective transformation that is known to proceed faster in water than in organic solvents due to the hydrophobic effect.⁹⁸ As a result, it is not surprising that Diels-Alder reaction has been employed previously in bioconjugation.^{99–102} In 2008, Fox and co-workers reported an unusually fast bioorthogonal reaction based on the inverse-electron-demand hetero-Diels-Alder reaction between *s*-tetrazine and *trans*-cyclooctene.¹⁰³ In this reaction, tetrazine acts as a voracious diene to produce the dihydropyrazine cycloadduct along with N₂ as the only byproduct (Scheme 6a).¹⁰⁴ They found that the reaction reached completion in 40 min at 25 °C and the apparent second-order rate constant for the cycloaddition reaction between 3,6-di-(2-pyridyl)-*s*-tetrazine and *trans*-cyclooctene was $2,000 \pm 400 \text{ M}^{-1}\text{s}^{-1}$ measured in a 9:1 methanol-water mixture, at least two orders of magnitude faster than all other bioorthogonal reactions known to date. The suitability of this reaction in modifying proteins was illustrated by reacting 3,6-di-(2-pyridyl)-*s*-tetrazine (30 μM) with a pre-modified thioredoxin (15 μM) carrying a single copy of *trans*-cyclooctene in an acetate buffer, pH = 6.0. An estimated 100% conversion was achieved in just 5 min as measured by ESI-MS. At about the same time, Hilderbrand and co-workers independently demonstrated the bioorthogonality of this reaction through the use of a tetrazine-derived fluorescent probe in modifying a norbornene-conjugated antibody both in serum and in live cells (Scheme 6b).¹⁰⁵ The second-order rate constants of this particular reactant pairs were determined to be 1.9 and $1.6 \text{ M}^{-1}\text{s}^{-1}$ in aqueous buffer and in 100% fetal

bovine serum (FBS) at 20 °C, respectively. Recently, the Hilderbrand group reported an even faster tetrazine ligation in which a serum-stable fluorophore-tethered tetrazine (500 nM) was used to selectively image a *trans*-cyclooctene-labeled cancer cells (Scheme 6b).¹⁰⁶ Using a succinimidyl ester analog, *trans*-cyclooctene was first attached to anti-EGFR antibody, which specifically binds to green fluorescent protein (GFP) positive A549 lung cancer cell line that showed an elevated level of EGFR. These pre-targeted cancer cells can then be selectively imaged via tetrazine ligation using the fluorophore-linked tetrazine reagent. In a model reaction between 1 mM of tetrazine and 1 mM of *trans*-cyclooctene in PBS buffer, a conversion of greater than 95% was obtained after the mixture was stirred at room temperature for only 10 min. A nominal second-order rate constant of $6,000 \pm 200 \text{ M}^{-1}\text{s}^{-1}$ was also derived by incubating the polystyrene-bead immobilized *trans*-cyclooctene-modified antibody with various concentrations of VT680 dye-modified tetrazine and measuring the on-bead fluorescence development over a period of 60 min.

Because of its very fast kinetics, the tetrazine ligation may be particularly useful in cases where rapid reactions are essential for tracking fast biological events as well as for labeling of biomolecules of low abundance. While this reaction is clearly suitable for *in vitro* experiments, however, it remains unclear how tetrazines or the strained alkenes can be genetically encoded into biomolecules in living system so that this robust bioorthogonal reaction can be applied seamlessly to *in vivo* applications.

2.6 Alkene homo-pair

Olefin metathesis is one of the most powerful organic reactions for the construction of new carbon-carbon bonds. The suitability of cross-metathesis in modifying proteins containing an allyl sulfide group in the biological buffer was recently demonstrated by Davis and co-workers (Scheme 7).¹⁰⁷ In their study, a small panel of alkenes were screened and *S*-allylcysteine (Sac) was found to be the most efficient substrate for the cross-metathesis reaction with allyl alcohol using the Hoveyda-Grubbs second-generation catalyst.¹⁰⁸ By introducing Sac on the surface of a single cysteine mutant of serine protease subtilisin *Bacillus lentus* (SBL), a cross-metathesis reaction between allyl alcohol and allyl sulfide-containing SBL can be carried out with 50–90% conversion in the presence of 20 equiv of catalyst, 80–160 mM MgCl_2 , and 30% *tert*-butanol at pH 8.0 (Scheme 7a). The enhanced reactivity of allyl sulfide was believed to be the result of sulfur coordination to the ruthenium center, thereby effectively bringing the two alkenes close in proximity for the cross-metathesis reaction (Scheme 7b). A multitude of site-specific SBL modifications such as glycosylation and PEGylation were subsequently demonstrated with this reaction. Importantly, there was no loss of protease activity with SBL after the modifications, indicating that the reaction condition was indeed very mild. In the same paper, they also demonstrated that Sac can be metabolically incorporated into a methionine mutant of *Sulfolobus solfataricus* β -glycosidase, raising an interesting possibility for future studies to use Sac as a metabolic alkene tag for cross-metathesis reactions *in vivo*. For cellular applications, however, it remains to be investigated whether the ruthenium catalyst is toxic to cells and whether it can permeate the cell membrane, a prerequisite for intracellular applications.

2.7 Arene-Alkene/Alkyne/Arene pair

The palladium-catalyzed cross-coupling reactions are amongst the most powerful and versatile organic transformations known today.¹⁰⁹ With excellent functional group tolerance and the advent of aqueous cross-coupling conditions,^{110, 111} it is not surprising that these reactions have been exploited for selective functionalization of proteins in the biological buffer. The first example was reported by Yokoyama and co-workers¹¹² in 2006 where they genetically engineered a Ras protein to carry an iodophenylalanine amino acid, which then

serves as an orthogonal functionality for selective conjugation with an alkene-tethered biotin via the Mizoruki-Heck reaction (Scheme 8a). The reaction was carried out using a water-soluble Pd-TPPTS (triphenylphosphine-3,3',3''-trisulfonate) catalyst in a *N*-tris(hydroxymethyl)methyl-3-aminopropanesulfonate (TAPS) medium (pH 8.3) containing 80 mM MgCl₂ at 5 °C. They noted that addition of 12% DMSO was necessary to achieve a low yield of 2% after 50 hr incubation, presumably by disrupting non-specific sequestering of the Pd catalyst by the protein substrates. A year later, the same group reported a related site-specific biotinylation via the Sonogashira reaction¹¹³ where they treated the iodophenylalanine-encoded Ras protein with a propargylic biotin (15 mM) at 6 °C for 80 min in a complex medium containing 1.7 mM Pd(OAc)₂, 8.3 mM TPPTS, 0.7 mM CuOTf, DMSO (2.3 M, 18% v/v), 0.2 mM sodium ascorbate, Triton X-100 (0.4% v/v), and 90 mM TAPS (pH 8.3) to afford the coupled product in 25% yield (Scheme 8b). In 2008, Schultz and co-workers demonstrated the Suzuki cross-coupling between a genetically incorporated *p*-boronophenylalanine and an aryl iodide fluorescent probe in a basic buffered solutions (pH 8.5) using the Pd⁰-dibenzylidene acetone (Pd-DBA) catalyst in 20 mM 3-(4-(2-hydroxyethyl)-1-piperazinyl)-propanesulfonic acid (EPPS) buffer at 70 °C with approximately 30% yield (Scheme 8c).¹¹⁴ It is unclear whether these cross-coupling reactions are compatible with the biological system as the reaction procedures involve very high catalyst loading, slightly basic pH, and high (or low) temperature and produce the products in relatively low yields.

Recently, Davis and co-workers reported a water-soluble, active palladium catalyst for the Suzuki-Miyaura cross-coupling which gave rise to an excellent yield with protein substrates.¹¹⁵ The catalyst is a sodium salt of 2-amino-4,6-dihydropyrimidine which forms a water-soluble complex with Pd. This new catalyst was found to greatly accelerate the cross-coupling reaction between a chemically installed *p*-iodobenzyl cysteine (Pic) at a subtilisin *Bacillus lentus* (SBL) mutant S156C and various aryl and alkenyl boronic acids; the reactions typically reached completion in 30 min (1 h for a synthetic glycoprotein) at 37 °C with greater than 95% conversion (Scheme 8d). With the discovery of this robust catalyst, it clearly makes the cross-coupling reaction an attractive method for protein modification because the genetic incorporation of iodophenylalanine has been reported.¹¹⁶ Like other metal catalysts, the toxicity and cell-permeability of this water-soluble palladium catalyst needs to be investigated before any intracellular applications can be contemplated. Moreover, there is a concern about the bioorthogonality of boronic acids as they are known to have strong affinity towards the polysaccharides abundant in living systems.¹¹⁷

3. Future directions

While tremendous progress has been made in bioorthogonal chemistry in the past few years, there are still many significant challenges facing the field, particularly from the cellular and organismal applications perspective. For example, unlike antibody-based detection/labeling, most bioorthogonal reactions require high concentration of reagents and catalysts (ranging from μM to hundreds of mM) in order to achieve appreciable reaction yields and/or detectable signals. To this end, three directions may be worthwhile to pursue in the future: 1) improve the rate of the existing reactions using the physical organic principles that have been successfully employed, such as the ring-strain effect, the fluorine effect, and the HOMO-lifting effect; 2) discover new chemical reactivity in water, particularly with under-utilized elements in the periodic table such as phosphorus and sulfur; and 3) exploit the local environment of the bioorthogonal functionality in order to increase reaction efficiency.¹¹⁸ Besides fluorescent probes, it should also be possible in the future to engineer other detection modality such as magnetic resonance imaging (MRI) contrast agents as the reaction partners for whole animal studies. For the photoinduced bioorthogonal reactions, the spatiotemporal control of biological processes in cells and tissues can be realized if the

two-photon-activatable reagents can be developed in the future. In addition to monitoring biological processes in living systems, bioorthogonal chemistry may also be employed in the future to either mimic¹¹⁹ or augment native biomolecular functions beyond what is known in nature—an important goal of synthetic biology.¹²⁰

4. Conclusion

Bioorthogonal chemistry has come of age. A number of water-compatible organic transformations such as nucleophilic carbonyl addition, 1,3-dipolar cycloaddition reactions, Diels-Alder reactions, olefin cross-metathesis reactions, and the palladium-catalyzed cross-coupling reactions have been optimized for use with biomolecular substrates in biological systems. Compared to conventional organic reaction development, a larger set of parameters need to be considered, such as bioorthogonality, reaction rate, inducibility, and reactant encodability, in addition to reaction yields and selectivity. It is noteworthy that in optimizing these reactions, the same physical organic principles that govern the small-molecule substrates have proven to work equally well with macromolecular substrates.

For live cell applications, the ability to genetically encode reactants employed in the bioorthogonal reactions is of paramount importance. For this reason, bioorthogonal reactions involving functionalization of small substrates such as azides, terminal alkynes, and simple alkenes are particularly promising. On the other hand, pericyclic reactions such as 1,3-dipolar cycloaddition and Diels-Alder reaction were most frequently exploited in the bioorthogonal reaction development because there are no reactive intermediates involved in these reactions that can be intercepted by biological nucleophiles and electrophiles. While each reaction has its own strengths and drawbacks, the dexterous use of these reactions in combination to functionalize biomolecules *in vivo* could further enhance the utility beyond what is possible with their own.¹²¹ With the rapid progress of bioorthogonal reaction development, the future of bioorthogonal chemistry as a tool for the study of biomolecular function *in vivo* is bright indeed.

Biographies

Reyna Koreen V. Lim was born in Gamu Isabela, Philippines in 1982. She obtained her B.S. in Chemistry from the University of the Philippines Diliman in 2003 where she also worked as a chemistry instructor before coming to the State University of New York at Buffalo in 2007. She is currently a Ph.D. student under Professor Qing Lin and her research focuses on the development of a 2*H*-azirine-based photoinduced 1,3-dipolar cycloaddition as a potential bioorthogonal reaction.

Professor Qing Lin received his B.S. in Chemistry from University of Science and Technology of China in 1994, and his Ph.D. in Organic Chemistry from Yale University in 2000 under Professor Andrew Hamilton. He then worked in Professor Peter Schultz's lab at Scripps Research Institute as a Damon Runyon Postdoctoral Fellow. After a brief stay in industry, he joined the faculty of the State University of New York at Buffalo in 2005 where he is now an Assistant Professor of Chemistry. His research interests include the development of (i) photoinducible bioorthogonal reactions, and (ii) enabling chemistries for peptide-based therapeutics.

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Figure 1.

Schematic representation of bioorthogonal chemistry approach for labeling of a targeted biomolecule with a small-molecule probe: Blue rectangle: biomolecular target; yellow circle, bioorthogonal chemical reporter; red rectangle, cognate reaction partner of the reporter; green star, small-molecule probe.

Figure 2.
(a) Bioorthogonal chemistry and (b) antibody-based approaches toward biomolecular labeling with a small-molecule probe.

Figure 3.

Photoinduced lipidation of EGFP carrying a tetrazole motif at its C-terminus: (a) Scheme for a photoinduced lipidation by a lipid dipolarophile. (b) Fluorescence imaging (top panel, $\lambda_{\text{ex}} = 365 \text{ nm}$) and Coomassie Blue staining (bottom panel) of EGFP-Tet and EGFP upon photoirradiation in the presence or absence of the lipid dipolarophile. Duration of 1-min 302-nm UV irradiation was applied to the samples.

Figure 4.

Selective labeling of alkene-Z by tetrazole in *E. coli* cells: (1) reaction scheme; (2) CFP channel (top row) and DIC (Differential Interference Contrast) channel (bottom row) images of bacteria expressing either alkene-Z (left) or wt-Z (right) proteins after treatment of 100 μ M tetrazole **1**.

Figure 5.

Observing a bent nitrile imine structure in the solid state: (a) scheme for the photocrystallography; (b) photodifference map based on the $F_o(\text{after}) - F_o(\text{before})$. Blue, 2.0; light blue, 1.0; orange, -1.0; red, -2.0 $e/\text{\AA}^3$. Only one half of the map is shown because of the 2-fold symmetry; (c) ORTEP representation of the geometry-refined nitrile imine structure.

Scheme 1.

Acid-catalyzed (a) and aniline-catalyzed (b) condensations of aldehyde/ketone with reactive amine nucleophiles.

Scheme 2.

Scheme 3.

Scheme 4.

Scheme 5.

Scheme 6.

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Scheme 8.