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# Click Chemistry and Bioorthogonal Reactions: Unprecedented Selectivity in the Labeling of Biological Molecules

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ABSTRACT: In recent years, a number of bioorthogonal reactions have been developed, exemplified by click chemistry, that enable the efficient formation of a specific product, even within a highly complex chemical environment. While the exquisite selectivity and reliability of these transformations have led to their broad application in diverse research areas, they have proven to be particularly beneficial to biological studies. In this regard, the ability to rationally incorporate reactive tags onto a biomolecular target and subsequently achieve high selectivity in tag derivatization within a complex biological sample has revolutionized the toolbox that is available for addressing fundamental issues. Herein, an introduction to the impact of click chemistry and other bioorthogonal reactions on the study of biological systems is presented. This includes discussion of the philosophy behind click chemistry, the details and benefits of bioorthogonal reactions that have been developed, and examples of recent innovative approaches that have effectively exploited these transformations to study cellular processes. For the latter, the impacts of bioorthogonal reactions on drug design (i.e., in situ combinatorial drug design), biomolecule labeling and detection (site-specific derivatization of proteins, viruses, sugars, DNA, RNA, and lipids), and the recent strategy of activity-based protein profiling are highlighted.

The ability to achieve high selectivity in the modification of a molecule that exists within a complex sample represents an extremely advantageous skill for studies conducted in a broad range of chemical and biological systems. In particular, the selective derivatization of biomolecules long presented a significant barrier due to the vast array of functionality present in biological systems, rendering side reactions and nonspecific labeling all but unavoidable. Recently, a series of chemical reactions that are orthogonal to functional groups present within biological systems have revolutionized this field through the unprecedented selectivity they exhibit in the tagging of biological targets within complex samples.

A prime example of the advantages of orthogonal reactivity is presented by a family of reactions collectively termed "click chemistry" (*1*). Since the inception of the click chemistry philosophy, these reactions have found application to a remarkably diverse range of research areas, including materials science (*2*, *3*) and supramolecular chemistry (*4*). The attributes of these transformations render them particularly advantageous for achieving the selective coupling of molecules within a complex biological environment. This ability has opened the door to a range of applications in chemical biology that have greatly enhanced the efficiency of biological studies aimed at understanding natural systems. This current topic article provides an introduction to the profound effects that click chemistry and other bioorthogonal reactions have had on state-of-the-art biological applications, including in situ drug development, biomolecule labeling and imaging, and activity-based protein profiling (ABPP).[1]

## 1. STRATEGY OF CLICK CHEMISTRY AND BIOORTHOGONAL REACTIVITY

The philosophy of click chemistry, as laid out by Sharpless, Finn, and Kolb in 2001, envisioned an innovative approach to chemical synthesis in which molecules are efficiently synthesized from a core group of highly effective reactions that exhibit several specific attributes (*1*). In particular, the reactions needed to proceed rapidly in high yields under ambient conditions to a single desired product. This potent combination required reactions with a high thermodynamic driving force that nevertheless were orthogonal to other functional groups that may be present, termed being "spring-loaded" for a single trajectory. It is for this reason that the azide group is exploited for many click reactions, as this group possesses a high intrinsic reactivity yet is quite

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[1]Abbreviations: ABPP, activity-based protein profiling; DIFO, difluorinated cyclooctyne; FRET, Förster resonance energy transfer; Fuc-T, fucosyltransferase; GDP, guanosine diphosphate; tRNA, transfer ribonucleic acid; BONCAT, bioorthogonal noncanonical amino acid tagging; PEG, polyethylene glycol; CPMV, cowpea mosaic virus; MRI, magnetic resonance imaging; HPLC, high-performance liquid chromatography; MS, mass spectrometry; FPP, farnesyl pyrophosphate; PA, phosphatidic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; MudPIT, multidimensional protein identification technology; TOP, tandem orthogonal proteolysis; TEV, tobacco etch virus; HDAC, histone deacetylase; PC, phosphatidylcholine.


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selective in the manner in which it reacts and is stable to water and unreactive in biological samples.

Reactions that meet the click chemistry requirements are effective for selective labeling of biomolecules as they are high-yielding under biological conditions and are bioorthogonal since the precursors do not react with common functional groups present in biological molecules. Furthermore, the azide moiety is yet again beneficial due to its diminutive size, rendering its introduction into a biomolecule target as only a minor structural perturbation. In addition to reactions that are technically classified using the term click chemistry, other transformations are also highly effective for bioorthogonal labeling, such as the Staudinger ligation. Below, the primary reactions that have been employed for such studies are introduced.

*1.1. Azide−Alkyne Cycloaddition.* The prototypical click chemistry reaction, the azide−alkyne cycloaddition (Figure 1A), involves a modification to the triazole-forming 1,3-dipolar cycloaddition originally described by Huisgen (*5*). In 2002, both Sharpless and co-workers (*6*) and Meldal and co-workers (*7*) independently reported the dramatic improvement of this process through the use of a copper(I) catalyst. Since these seminal reports, the numerous advantages of the copper-catalyzed azide−alkyne cycloaddition have been uncovered, including the rapid rate of reaction, improved chemo- and regioselectivity, reaction scope, and water compatibility (*8*). The reaction rate is also increased if the alkyne and azide reactive partners are brought into proximity, such as by receptor binding, which has been useful for applications including in situ combinatorial drug development (see section 2). Finally, as with other cycloadditions, the reaction rate is enhanced when the reactive groups are polarized, and thus, it is common to include an electron-withdrawing group proximal to the alkyne. Since the inception of this copper-catalyzed [3 + 2] cycloaddition, several studies have been performed to elucidate the reaction mechanism (*9*−*11*) and optimize reaction conditions. For example, the addition of a ligand that chelates copper is known to enhance the reaction rate and stabilize the metal (*12*−*16*). A modification that is particularly useful for achieving high resolution in biological labeling is the development of fluorogenic reactants, of which multiple versions have been reported (*17*−*21*).

*1.2. Copper-Free Azide−Alkyne Cycloaddition.* While the copper-catalyzed azide−alkyne cycloaddition is primarily effective for in vitro biological studies, the reliance on the copper catalyst is detrimental to in vivo studies. This is primarily due to the need to deliver this catalyst to the reaction site, and the toxicity of the metal at concentrations employed for labeling (*22*). To overcome these obstacles, the copper-free modification was reported by Bertozzi and co-workers (*23*), in which the alkyne is incorporated within a strained cyclooctyne system, which increases its intrinsic reactivity and circumvents the requirement for copper catalyst. Subsequently, a difluorinated cyclooctyne (DIFO, Figure 1B) (*24*) was developed that exhibited greatly enhanced reactivity and was effective for bioorthogonal imaging of azide-tagged sugars in living organisms (*25*). An analogue with increased hydrophilicity has also been reported (*26*), as well as a modified approach developed by Boons and co-workers employing a dibenzocyclooctynol derivative (*27*). Finally, the mechanism of this reaction has been studied using theoretical methods (*28*).

*1.3. Staudinger Ligation.* The Staudinger ligation, reported just prior to the copper-catalyzed azide cycloaddition (*29*), is another bioorthogonal reaction that is highly effective for in vivo labeling (*30*). This reaction was devised as a modification to the Staudinger reduction reaction (*31*) by capturing the aza−ylide intermediate through intramolecular addition to an electrophile, typically an ester, appended to the phosphine reagent. While the original reaction product contained a phosphine oxide moiety, a modified approach, termed the "traceless Staudinger ligation", was quickly reported in which the phosphine is released, resulting in a simple amide bond linkage (*32*−*35*). In these original publications, multiple phosphine reagents bearing different groups for aza−ylide capture were reported, one of which is depicted in Figure 1C. Since this time, the mechanism of the

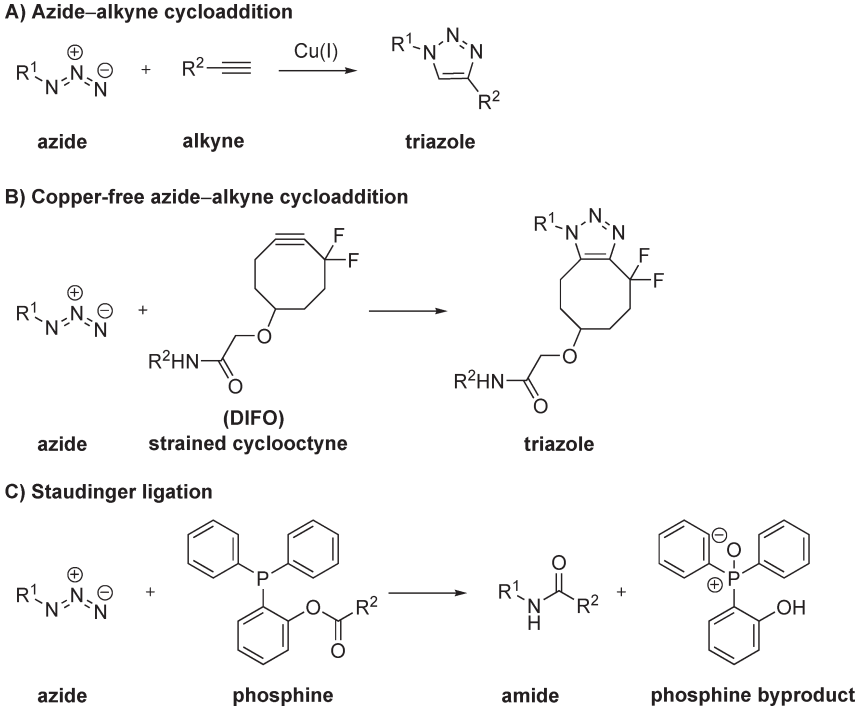


FIGURE 1: Bioorthogonal reactions commonly employed for labeling of biological molecules.

reaction and other details have been probed in depth (*36*−*39*), and a number of iterations have been reported, such as fluorogenic versions (*40*), including a FRET-based system (*41*), as well as reagents with increased water solubility (*42*).

*1.4. Other Reactions*. While the previous reactions have revolutionized the labeling of molecules for biological study, other reactions have also been employed for bioconjugation (*1*, *43*). Briefly, examples, including the addition of nucleophiles such as hydrazides to carbonyl groups (*44*), the reaction of a thiol with a maleimide functionality (*45*), the Diels−Alder cycloaddition (*46*), and the thiol−ene reaction (*47*), have also been successfully harnessed for bioconjugation. While these reactions can be effective, they are not as general and advantageous as the aforementioned reactions since many are not truly bioorthogonal or suffer from other drawbacks.

*1.5. Comparison of Bioorthogonal Reactions and Conditions*. Since multiple bioorthogonal reactions have now been devised, a key decision when initiating studies involves the choice of reaction and conditions to be employed. While these reactions exhibit impressive selectivity in biological samples, issues such as background labeling and toxicity can still be problematic and can be dependent upon subtle reaction details. For example, in early ABPP studies (see section 3.6), Cravatt and co-workers found that high background using a rhodamine−alkyne conjugate to label an azide-tagged phenyl sulfonate probe was ameliorated by reversing the reactive tags, instead using an alkynyl probe and rhodamine−azide conjugate (*48*). Systematic studies implicated the rhodamine−alkyne conjugate as the culprit in nonspecific proteome labeling. In addition, Wong and co-workers (*19*), as well as Wu, Bertozzi, and co-workers (*49*), found alkynyl sugars to be substantially better for labeling studies compared to the corresponding azido sugars. In this case, the discrepancy was attributed to the increased toxicity of the azido sugar in cells. These reports indicate that subtle details are quite important for optimizing labeling studies. While in both of these situations the alkynyl probe proved to be more effective, the basis for the enhanced efficacy appears to be different in each case. Thus, when studies are performed using a new system, attempting both combinations of probe labeling protocols seems prudent.

In addition, the choice of bioorthogonal reaction can also be important. Recently, a comparative study of the efficacy of these reactions was reported, in which the optimal approach was found to be specific to the particular application and structures employed for analysis (*50*). For proteomic applications, particularly using cell extracts, the azide−alkyne cycloaddition is the most accessible and efficient approach. Studies involving live cells and organisms generally require the use of either the copper-free azide−alkyne cycloaddition or the Staudinger ligation. However, these approaches are generally less accessible due to the synthesis required to access the strained alkyne and phosphine reagents, respectively. Since the application of these latter reactions in living organisms represents a relatively recent advancement, time will ultimately tell how generally applicable each of these approaches will prove to be.

## 2. IMPACTS OF CLICK CHEMISTRY ON DRUG DEVELOPMENT

From the outset, one of the primary goals of the click chemistry philosophy was to simplify the methods by which molecules are constructed and thus streamline the process of synthesizing and identifying molecules with important biological properties. As such, click chemistry, and in particular the azide−alkyne cycloaddition, has had a profound effect on the design and development of novel compounds for therapeutic applications, a topic that has been the focus of recent reviews (*43*, *51*, *52*).

In addition to the bioorthogonal nature of click chemistry, the fact that the azide−alkyne cycloaddition is catalyzed by reactant proximity has also proven to be beneficial for innovative methods of drug development. This is particularly the case for in situ fragment-based drug design, an approach that employs an enzyme target to catalyze the synthesis of an inhibitor best suited to bind to that enzyme (*53*). In this strategy, upon incubation of a library of prospective alkyne and azide reactants with an enzyme target, the reactants that bind the enzyme most effectively are thus brought into proximity, leading to their selective reaction to generate a potent ligand (Figure 2A). Product identification is then used to identify the structure with the highest affinity for one or more binding domains on the receptor target. Since the proximity effect is used to drive the reaction, the copper catalyst is not implemented in this strategy. This concept is especially efficacious for generating bisubstrate or multivalent inhibitors, which are often otherwise difficult to design.

In situ fragment-based drug design represented one of the early applications of the azide−alkyne cycloaddition reported by Sharpless and co-workers, with the initial study producing an extremely potent (77 fM) inhibitor of acetylcholinesterase (Figure 2B) (*53*−*55*). This enzyme was known to be inhibited by a tacrine motif at the active site, as well as by a phenanthradinium moiety at a proximal peripheral domain. To develop a potent inhibitor that simultaneously bound both sites, a series of alkyne- and azide-tagged phenanthridinium and tacrine analogues, exemplified by **2a**−**e** and **3a**−**c**, respectively, were incubated with the enzyme, and the subsequent major product was identified as inhibitor **1**. Since this pioneering report, in situ drug development via click chemistry has been extended to the selective generation of potent inhibitors of carbonic anhydrase (*56*) and HIV-1 protease (*57*), the latter of which is active against a multi-drug-resistant HIV-1 protease variant (*58*).

Beyond in situ techniques, the simplicity and reliability of click reactions have led to an explosion in their application in combinatorial drug development. Furthermore, the azide−alkyne cycloaddition again proves to be fruitful as the product of this reaction, the triazole, possesses a number of properties that are favorable in drug design (*52*). Finally, the high yield, selectivity, and mild conditions often allow products to be directly screened for activity without purification. This was exhibited in the development of a potent and selective inhibitor of α-1,3-fucosyltransferase (Fuc-T), a key enzyme in the biosynthesis of cell-surface glycans, by Wong and co-workers (*59*). In the study, GDP-alkyne **4** (Figure 3) was used as the core structure that was reacted with a library of 85 azide-containing reactants of the type **5a**−**e**. This was performed in a high-throughput fashion, as the reactions and evaluation of the resulting products for Fuc-T inhibition were both directly achieved in microplates. This approach allowed for rapid identification of inhibitor **6**, which possesses high potency and selectivity for Fuc-T VI.

## 3. LABELING AND IMAGING OF BIOMOLECULES USING BIOORTHOGONAL REACTIONS

The bioorthogonal nature of click chemistry components lends these reactions as invaluable tools for the selective labeling and

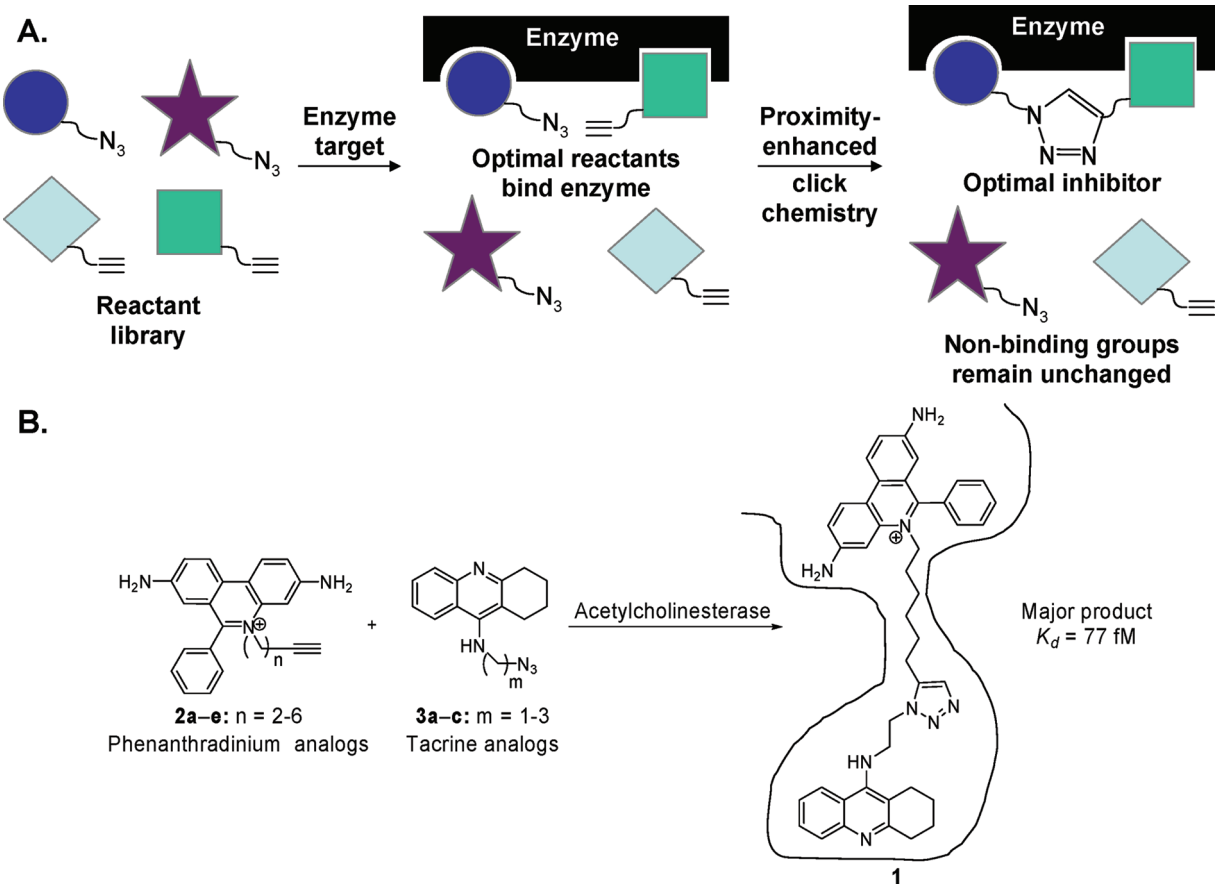


FIGURE 2: In situ combinatorial drug development. (A) General schematic for templated inhibitor development. Binding of reactants by the target enzyme leads to catalytic formation of the optimal inhibitor. (B) Application of this approach to the development of a potent bisubstrate inhibitor of acetylcholinesterase.

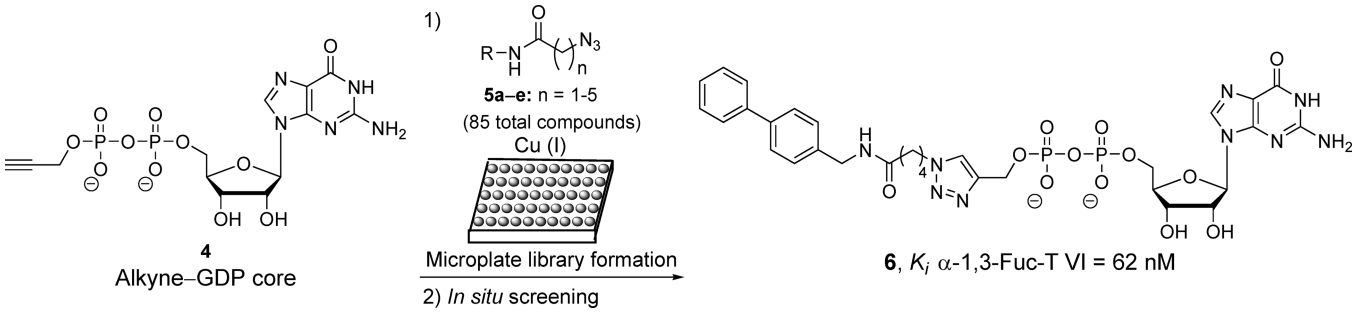


FIGURE 3: Direct screening of click chemistry products to identify a potent Fuc-T inhibitor.

detection of biological molecules in complex samples such as cellular extracts and ultimately in vivo. Such studies are necessary to understand the complex nuances of spatial and temporal aspects of biomolecule localization and function within the cell. The Staudinger ligation and azide−alkyne cycloadditions have both proven to be effective for these challenging tasks and have now been employed for selective derivatization of a wide range of biomolecules, including proteins, viruses, sugars, DNA, RNA, and lipids. Each of these is briefly presented below along with a discussion of representative studies.

*3.1. Introduction of Unnatural Amino Acids Bearing Reactive Tags into Proteins.* While azide or alkyne tags are often easily introduced onto small molecules via chemical synthesis, the site-specific introduction of these labels onto more complicated structures, particularly proteins, presents a significant challenge. One approach developed by Tirrell and co-workers exploited the inability of certain tRNA synthetases employed for protein biosynthesis to differentiate between natural amino acids and analogues with closely related structures (*60*). This allows for global replacement of methionine with homopropargylglycine (**7**, Figure 4A) (*61*−*63*) or azidohomoalanine (**8**) (*63*), as well as introduction of alkynylphenylalanine (**9**) and azidophenylalanine (**10**) (*64*) in place of phenylalanine when the natural amino acids are in low abundance. In fact, click chemistry was used to improve the efficiency of this method by screening the extent of unnatural amino acid incorporation by mutant tRNA synthetases through postlabeling (*65*).

The aforementioned method is effective for global replacement of a particular residue with a structurally similar analogue. However, many applications would benefit from the ability to perform site-specific incorporation of a single unnatural residue and to introduce unnatural amino acids that deviate substantially from the natural residue. This challenge was overcome by Schultz and co-workers, who developed a powerful technique

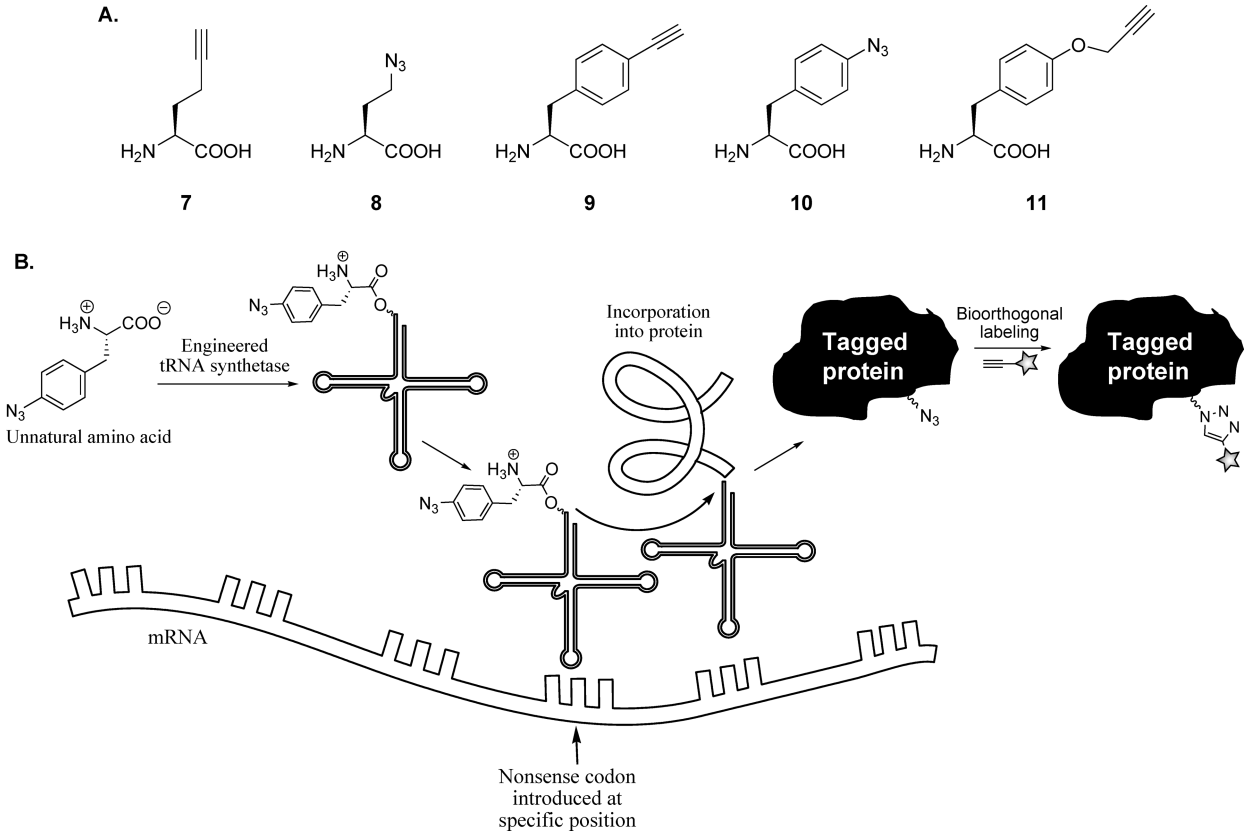


FIGURE 4: Incorporation of unnatural amino acids into proteins. (A) Noncanonical amino acids that have been introduced into proteins. (B) Schematic for unnatural amino acid mutagenesis using a genetically engineered tRNA synthetase.

for site-specific installation of unnatural amino acids by reengineering protein synthesis (*66*−*69*). In this method, an orthogonal tRNA/mutant tRNA synthetase pair is exploited to introduce a noncanonical amino acid at a prescribed location (Figure 4B). This was initially performed in *Escherichia coli* (*70*) and then subsequently expanded to eukaryotic (*71*) and mammalian(*72*) cells by harnessing the amber codon in concert with suppressor tRNA synthetases that were genetically engineered to accept unnatural amino acids as substrates. Using this strategy, an impressive array of amino acid derivatives bearing various reactive or reporter groups (*69*, *73*−*75*), including **10** and **11** bearing azide and alkyne tags, respectively (*72*, *76*−*78*), have been site-specifically incorporated into protein targets, the latter of which can be subsequently modified via click chemistry.

The combination of unnatural amino acid mutagenesis and bioorthogonal chemistry provides an exciting tool for site-specific derivatization of proteins for various applications. Initially, both the Staudinger ligation (*79*, *80*) and the azide−alkyne cycloaddition (*81*, *82*) were shown to be effective for postmodification of proteins presenting azido or alkynyl residues. Since these findings, various site-specific derivatizations of protein structures have been achieved for different purposes. In one study, a single azido residue at Trp33 of human superoxide dismutase-1, an enzyme that combats reactive oxygen species in the cell, was derivatized with a polyethylene glycol (PEG) chain in an effort to engineer protein properties for therapeutic gain (*83*). Schuman, Tirrell, and co-workers developed an approach to the detection of newly synthesized proteins through click chemistry-based postlabeling that was termed bioorthogonal noncanonical amino acid tagging (BONCAT) (*84*, *85*). The ability to detect newly synthesized proteins allows for spatial and temporal detection of protein biosynthesis that will be invaluable for studying variations in responses to different stimuli. Finally, in another inspiring application reported by Schultz and co-workers, a phage display technique for selection among proteins containing unnatural amino acids was reported (*86*). This diversification strategy led to the identification of an antibody containing a sulfotyrosine residue that binds gp120, the HIV-1 viral coat protein, more effectively than similar known antibodies.

*3.2. Labeling of Viral Surfaces*. An early example of the derivatization of biomolecules via the azide−alkyne cycloaddition was that of cowpea mosaic virus (CPMV) reported by Finn and co-workers (*87*). Viral surfaces represent beneficial scaffolds as their highly ordered and rigid structures lead to defined presentation of groups appended to the viral backbone. In this original study, 60 azide or alkyne groups were introduced onto either lysine or cysteine residues in a predictable manner, followed by selective modification of each of these sites via click chemistry. Since then, the derivatization of CPMV via click chemistry has been applied to the development of multivalent conjugates by introducing ligands, including sugars, peptides, PEG, and proteins (*14*, *88*). In addition, this strategy was used to derivatize CPMV with a gadolinium−cyclen contrast agent for MRI (*89*). More recently, Wang and co-workers have extended viral click chemistry to the tobacco mosaic virus (*90*).

*3.3. Incorporation of Labeled Probes onto Proteins via Post-Translational Modification*. The sheer importance and complexity of the post-translational modification of proteins (*91*), which involves the addition of a range of moieties, including alkyl, acyl, sulfate, phosphate, lipid, and sugar groups, has generated intense interest in this field. The immense scope of

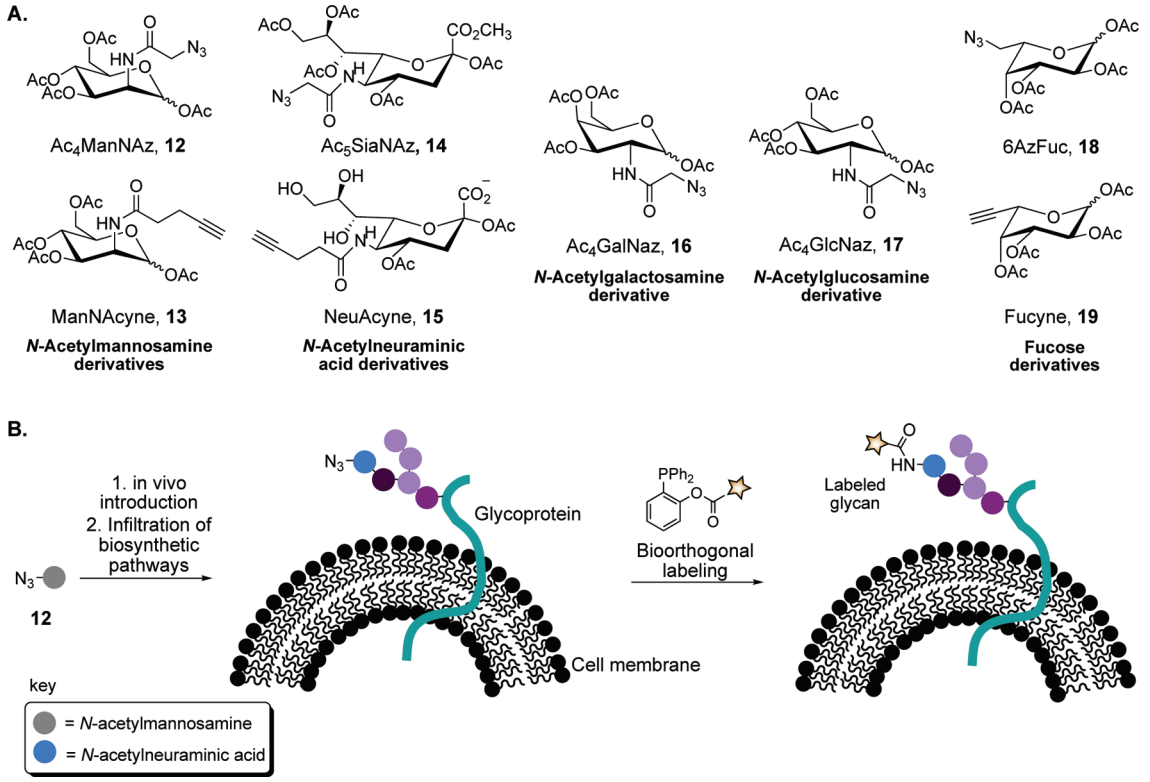


FIGURE 5: Metabolic labeling of cell-surface glycans using bioorthogonal chemistry. (A) Azide and alkyne-derivatized sugars that successfully infiltrate biosynthetic pathways. (B) Schematic for the incorporation of simple sugar derivatives onto glycoproteins via metabolic pathways, followed by selective labeling through bioorthogonal chemistry.

such modifications and their regulatory effects on protein function presents a task much more convoluted than unraveling genomes and will likely keep chemical biologists occupied for quite some time. Elucidating protein glycosylation presents a particularly daunting yet rewarding challenge because of the massive number of sugar structures that are present in living systems and the important roles that glycans play in cellular processes such as immune responses, inflammation, cancer metastasis, and cell-surface recognition, which leads to bacterial and viral cell entry (*92*−*94*). Due to this significance, the function of protein glycosylation has naturally been approached using bioorthogonal chemistry, which has provided important insights into these complex systems (*95*−*97*).

The labeling of sugar moieties incorporated onto glycoproteins was originally spurred by the observation that sugars with slight structural modifications could act as substrates in metabolic pathways (*98*, *99*). While Bertozzi and co-workers initially utilized ketone-bearing sugar analogues for incorporation into sialic acid biosynthesis and selective labeling, (*44*) this strategy was quickly extended in the initial report of the Staudinger ligation (*29*). In this subsequent study, an *N*-acetylmannosamine derivative (ManNAz) containing an azidoacetyl group was developed. Since the azide group represents a small structural perturbation, ManNAz is effectively incorporated into sugar biosynthetic pathways and is converted to sialic acid, a key glycan presented on cell surfaces. Following cellular incubation, infiltration of sialic acid biosynthesis, and presentation on the cell surface, the resulting azide-tagged glycans were selectively modified using a biotin−PEG−phosphine reagent and labeled with an avidin−fluorescein conjugate to detect glycosylation (Figure 5B). Flow cytometry was also used to correlate emission to the extent of labeled molecules in the sample. Later, a peracetylated derivative ($Ac_4$ManNaz, **12**, Figure 5A) (*100*) was introduced to enhance membrane penetration, after which the acetyl groups are hydrolyzed to unleash ManNaz within the cell. Alkynylmannosamine (ManNAcyne, **13**) (*19*) and different neuraminic acid (**14** and **15**) (*19*, *96*, *97*, *101*) analogues have also been successful for labeling sialylated glycoproteins. In fact, a report by Wong and co-workers that **13** yielded increased labeling and decreased background in cells (*19*) was recently followed up by Wu, Bertozzi, and co-workers, who showed this alkyne to be substantially more effective than **12** for labeling studies across six different cell lines and in mice (*49*).

Following initial reports, metabolic labeling of glycans has been advanced through multiple avenues. The efficacy of the copper-free azide−alkyne cycloaddition for bioorthogonal labeling was first exhibited through the use of the $Ac_4$ManNaz approach, followed by staining with a biotin−cyclooctyne conjugate (*23*). In another study, the substrate specificities of glycosyltransferases involved in mucin-type O-linked glycoprotein biosynthesis were probed using an approach that is amenable to high-throughput screening. Here, the incorporation of a substrate analogue, UDP-GalNaz, onto biotinylated peptides was detected via the Staudinger ligation in microplates. Labeling of mucin-type O-linked glycoproteins was also achieved using *N*-acetylgalactosamine derivative **16** ($Ac_4$GalNAz) (*102*). Zhao and co-workers employed this approach for the incorporation of an azide-tagged *N*-acetylglucosmaine analogue ($Ac_4$GlcNAz, **17**) into glycoprotein biosynthesis (*103*). Later, proteins labeled with this sugar were detected and isolated, and nano-HPLC−MS/MS was used to identify 10 known and 41 putative modified proteins (*104*). Wong, Cravatt, and co-workers have since expanded upon MS-based analysis of glycan labeling toward comprehensive glycoprotein identification and glycan mapping (*105*).

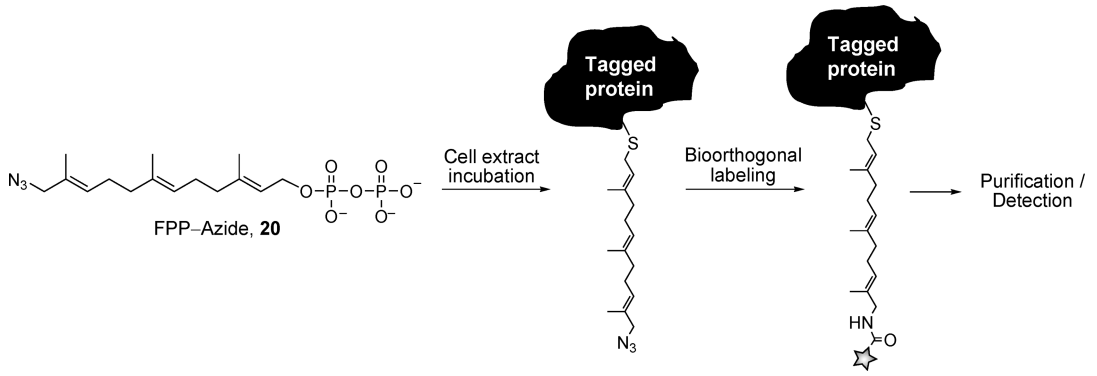


FIGURE 6: Labeling of proteins via post-translational addition of tagged lipid tails.

Fucose has generated interest in labeling studies because of the presence of this motif in important oligosaccharides such as sialyl Lewis$^X$ (*92*). Wong and co-workers imaged the incorporation of fucose into glycoproteins using both azide- and alkyne-tagged GDP-fucose as modified fucosyltransferase (Fuc-T) substrates (*20*). In this case, detection was advanced through the use of fluorogenic reactants that were developed for the study. In addition, peracetylated azide- and alkyne-tagged fucose derivatives **18** and **19** were also effective for labeling fucosylated glycoproteins (*19*, *106*.) In recent years, methods for optical glycan imaging have been enhanced by using improved labeling reagents and detection strategies (*25*, *107*, *108*). Finally, while early studies were performed in cell culture, technological advancements have led to glycan labeling in living organisms, including mice (*109*, *110*) and zebrafish (*111*), the latter of which was greatly enhanced through the use of later generation, copper-free azide−alkyne cycloaddition reagents (i.e., DIFO). These studies clearly illustrate the robust properties of this approach and its utility to glycobiology.

Bioorthogonal chemistry has been beneficial for studying carbohydrates using approaches other than the hijacking of metabolic pathways. For example, Wong and co-workers have employed click chemistry for convenient immobilization of carbohydrates in the development of platforms for microarray analysis of protein−glycan binding interactions (*96*, *112*, *113*). This strategy was later implemented to characterize the structural requirements for recognition of the tumor antigen Globo H by the antibodies MBr1 and VK-9, including direct analysis of serum from breast cancer patients (*114*). Furthermore, click chemistry has also allowed for facile access to a range of synthetic derivatives of complex glycans, such as multivalent conjugates, a topic that has recently been reviewed (*115*).

Post-translational lipidation is another important modification that has been exploited and studied using selective labeling. The introduction of saturated (palmitoyl and myristoyl) and unsaturated (farnesyl and geranylgeranyl) lipid tails is vital as it regulates both protein function and subcellular localization. For example, the cycling of Ras GTPases, key signaling proteins in which mutation is commonly linked to cancer, between the Golgi apparatus and plasma membrane is directly controlled by the extent of lipidation (*116*). In an early study, Zhao and co-workers employed an azide-tagged farnesyl pyrophosphate derivative (FPP-azide, **20**, Figure 6) to label proteins that are post-translationally lipidated by this moiety (*117*). Model studies showed that the azido-farnesyl group was effectively incorporated into certain (Ras and Hdj-2) but not all (Rap1) protein targets. Next, the probe was introduced into cell lysates for the selective labeling, purification, and identification of farnesylated proteins. Proteins that were modified by the labeled lipid chain were then biotinylated via the Staudinger ligation and subsequently detected and purified to identify 18 farnesylated proteins, some of which were not previously known.

The use of labeled substrates has been further extended for the study of post-translationally lipidated proteins, including probes corresponding to saturated lipid chains (*118*−*121*). Also, the lipidation of proteins with azide-tagged substrates has been employed as a strategy for site-specific covalent immobilization of proteins onto surfaces (*122*). Recently, Ting and co-workers implemented the enzyme lipoic acid ligase (LplA) from *E. coli* for selective azide labeling of protein targets (*123*). This enzyme accepts azidooctanoic acid as a substrate for incorporation onto lysine, and the authors identified a 22-amino acid sequence that could be acylated by LplA for introduction of this group. The azide handle can thus be selectively introduced onto protein targets that contain this peptide sequence appended to the N- or C-terminus.

*3.4. Labeling of Nucleotides for Imaging DNA and RNA*. The labeling of both DNA and RNA using bioorthogonal chemistry has been successfully performed and exploited for studies (*124*). Initially, Ju and co-workers synthetically introduced an azide tag to the 5′-end of single-stranded DNA, which was then labeled with a fluorescent tag and analyzed by Sanger dideoxy fragmentation (*125*). DNA alkylation has also been probed through click derivatization of an alkynyl nucleotide cofactor analogue (*126*). Carell and co-workers reported the synthesis of oligonucleotides containing alkynyl−nucloetide conjugates, which were successfully postsynthetically derivatized (*127*). Finally, Salic and co-workers recently reported the in vivo labeling of both DNA and RNA. In these studies, both deoxyuridine (**21**, Figure 7A) (*128*) and uridine (**22**) (*129*) nucleotide analogues were developed containing a propargyl group introduced onto the uracil moiety. These nucleotide derivatives were effectively incorporated into DNA and RNA biosynthesis, respectively, and subsequently labeled with fluorescent tags via click chemistry to achieve optical detection of DNA and RNA (Figure 7B). Studies were successful both in cultured cells and then in mice.

*3.5. Derivatization of Lipid Probes*. While probes can be designed for rational entry into biosynthetic pathways, such compounds have also been employed for direct analysis of the activities of small molecules within the cell. A recent example reported by Schultz and co-workers achieved the selective labeling and optical detection of phosphatidic acid (PA) probes bearing reactive tags (*130*). Despite its relatively simple structure, PA is an important signaling phospholipid (*131*) that regulates

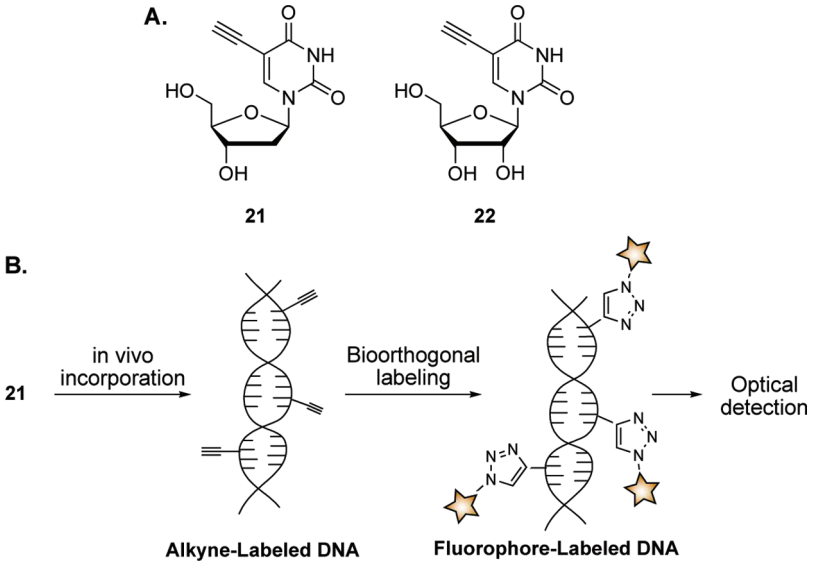


FIGURE 7: In vivo labeling of DNA and RNA. (A) Alkyne-tagged deoxynucleotide (**21**) and nucleotide (**22**) analogues that have been successfully incorporated into DNA and RNA, respectively. (B) Schematic for selective click chemistry-based postlabeling of DNA containing alkynyl−nucleotide analogues.

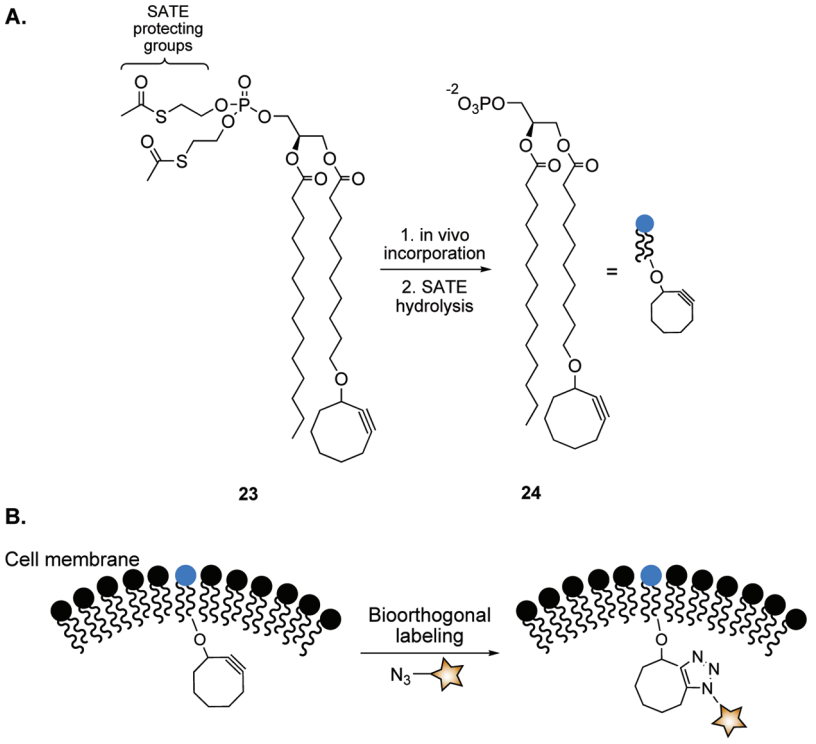


FIGURE 8: Strategies for in vivo imaging of lipid probes. (A) Tagged PA analogue **23** contains a hydrolyzable headgroup that assists in membrane penetration and a cyclooctyne tag for subsequent labeling. (B) Schematic for the selective in vivo labeling of lipid probe **24** through bioorthogonal chemistry.

the function of many proteins, such as Raf-1 kinase (*132*). In this study, PA probes including **23** (Figure 8A), containing a cyclooctyne moiety embedded within a lipid acyl chain for reaction via copper-free click chemistry, were designed and synthesized. These compounds also included *S*-acetyl-2-thioethyl (SATE) protecting groups on the headgroup that enhance membrane permeability and are enzymatically cleaved to reveal the phosphate moiety of **24** following cell entry. Compound **23** was incubated with RAW macrophages and treated with a fluorogenic coumarin−azide conjugate for optical visualization of the probe in living cells (Figure 8B).

*3.6. Activity-BasedProtein Profiling*. A probe-based approach that has revolutionized the study of biological systems is that of activity-based protein profiling (ABPP) (*133*−*135*). This technique entails the mechanism-based collective labeling, detection, identification, and characterization of enzymatic targets based on their activities, providing an extremely efficient approach to enzymatic profiling. Since the enzymes are labeled on the basis of activity in this method, ABPP also overcomes a significant impediment to traditional proteomic methods, which detect proteins on the basis of abundance and thus do not account for variations in activity caused

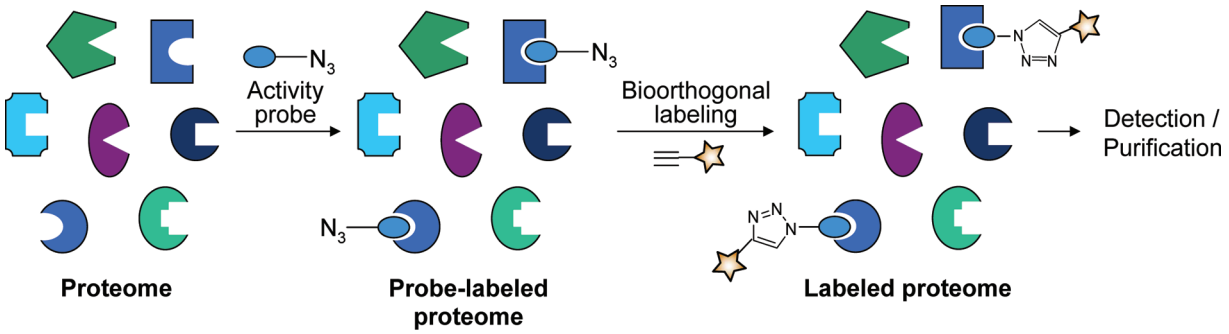


FIGURE 9: Schematic for the strategy of ABPP using click chemistry postlabeling. Proteins are labeled by activity probes through reaction at enzyme active sites. The resulting probe-labeled proteins are then selectively tagged and manipulated via click chemistry.

by regulatory events, and in particular post-translational modification.

In ABPP, a probe, often based on a substrate analogue or inhibitor of a particular enzyme class, is designed that contains: 1) a reactive moiety by which the probe is selectively cross-linked to enzymes with which it interacts and 2) a secondary tag by which the resulting enzyme−protein conjugates are selectively labeled for analysis. In an original design of ABPP, the probe consisted of biotin conjugated to a fluorophosphonate-based irreversible inhibitor of serine hydrolases (*136*). The biotin secondary handle was used to purify cross-linked adducts of these enzymes from in vitro experiments via affinity chromatography. However, it was recognized that the physicochemical properties of biotin are likely disadvantageous when it is introduced onto a small molecule probe. As a result, a second-generation approach entailed the introduction of an azide moiety as a latent secondary handle that could be converted via click chemistry after cross-linking to introduce a fluorophore or biotin tag for subsequent analysis (Figure 9) (*137*). This use of bioorthogonal labeling also allowed ABPP to leap into the realm of in vivo studies using COS cells and then mice. In ABPP, the specific protocols employed for probe labeling are important as they affect the sensitivity of analysis (see section 1.5 for details) (*48*). In addition, the choice of reactive group on an activity probe has been shown to control the specificity of labeling studies, as alteration of the reactive electrophile used to cross-link to enzymes leads to the modification of different amino acid residues on target proteins (*138*).

As is general for applications employing bioorthogonal reactions, ABPP is reliant upon effective analytical techniques for detecting labeled proteins. Initially, studies were performed using traditional SDS−PAGE separation followed by on-gel detection of labeled probes via fluorescence imaging or biotin labeling. ABPP has since been advanced using high-content mass spectrometric-based proteomic methods (*139*). Multidimensional protein identification technology (MudPIT) was initially exploited, which entails trypsin digestion analysis of enriched biotinylated proteins to identify probe-labeled targets (*140*). However, a drawback of MudPIT is that is does not allow for identification of the site at which probe labeling occurs since the peptide fragment that is modified remains conjugated to streptavidin beads used for enrichment. To address this issue, Cravatt and co-workers have devised tandem-orthogonal proteolysis (TOP-ABPP) (*141*) in which both an affinity handle and a TEV protease cleavage site are introduced onto probe-labeled proteins via click chemistry postlabeling. Tandem digests with trypsin and TEV protease are then used to simultaneously identify both enzymatic targets and the site at which they are modified by the probe, respectively.

Since its inception, ABPP has been extensively implemented to probe enzyme classes, including hydrolases, proteases, kinases, phosphatases, glycosidases, histone deacetylases (HDACs), and cytochrome p450s, for which studies have recently been reviewed in detail (*133*). A number of interesting insights and applications have arisen from this work. For example, early studies employing analogues of known irreversible enzyme inhibitors led to the identification of previously unknown targets in addition to those that were accepted. This opened up ABPP as a strategy for identifying additional drug targets that may lead to side effects (*142*−*144*). In addition, comparisons of the enzymatic profiles of normal cells with those associated with a particular condition have led to the identification of proteomic variations in cells corresponding to cancer (*48*, *145*−*147*), malaria (*148*, *149*), and obesity (*150*).

Another exciting application of ABPP utilized a chemical genetics screen of a library of protein-reactive small molecules bearing a latent azide or alkyne handle. Following the observance of a desired property by a library member, ABPP was then used to identify the specific protein target that was affected. Cravatt, Sorensen, and co-workers applied this strategy by using a library of compounds containing spiroepoxy reactive groups in which a library member that inhibited proliferation of breast cancer cells was found to target phosphoglycerate mutase 1 (*151*). In addition, ABPP has been employed for inhibitor development using a competition assay in which the ability of compounds to block the activity probes from labeling their known targets was detected (*152*, *153*). Finally, dynamic imaging has been achieved using activity probes in which fluorescence is quenched until the probe labels an enzyme active site, at which point emission is activated (*154*, *155*).

While ABPP was devised to profile the catalytic activities of enzymes, this general bifunctional probe strategy has found broad application to other biological processes such as receptor−ligand binding interactions. Since these studies do not necessarily target a catalytic domain on a protein target, the reactive tag that is introduced is often a photoaffinity tag (*156*), by which the probe is selectively cross-linked to cognate receptors following binding. Once again, azide and alkyne groups have both been employed as secondary tags, which can be derivatized via bioorthogonal chemistry after cross-linking for the analysis of adducts. Pieters and co-workers reported an initial approach of this type (*157*), in which a bifunctional disaccharide probe (**25**, Figure 10A) was devised and cross-linked to galectin-1, and the resulting adduct was analyzed via azide derivatization with a rhodamine−alkyne reagent for optical detection (Figure 10B). This was later extended to use multivalent carbohydrate probe **26** (*158*)

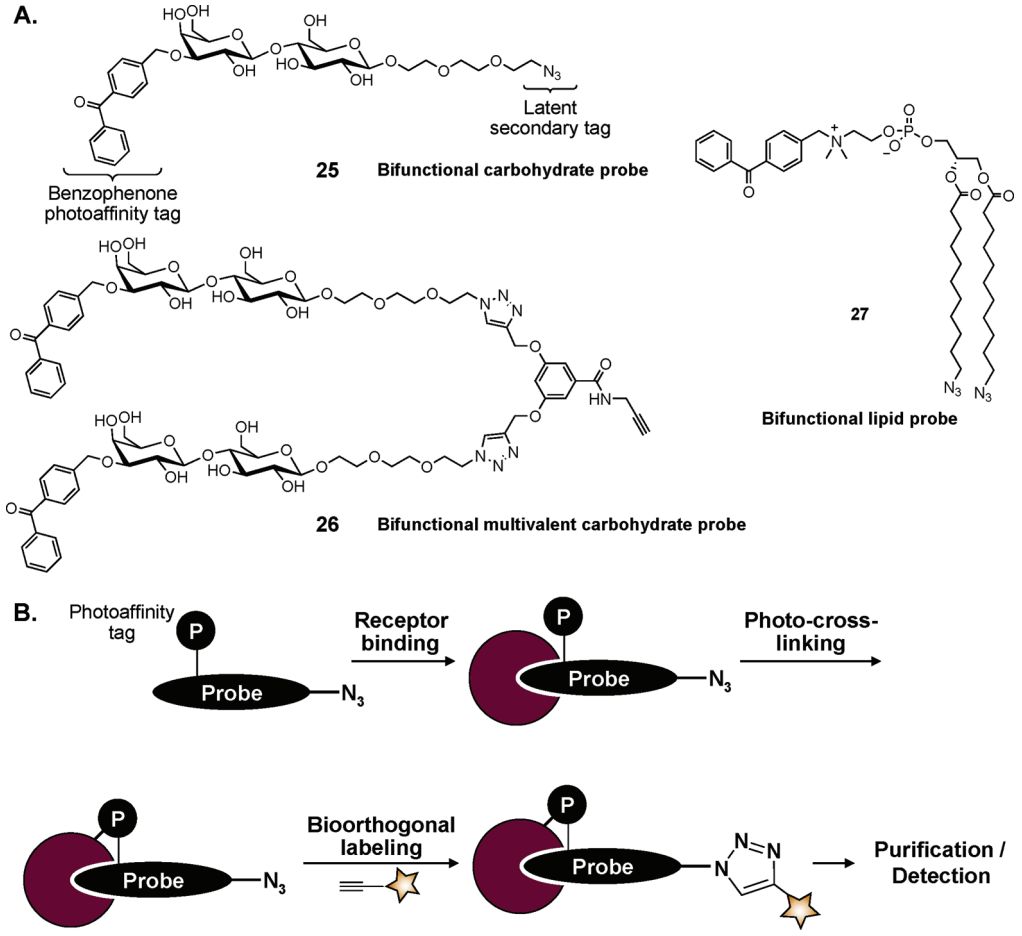


FIGURE 10: Bifunctional probe strategy for identification and characterization of cognate receptors. (A) Bifunctional probes that have been developed to selectively tag receptors, including sugar (**25**), multivalent sugar (**26**), and phospholipid (**27**) derivatives. (B) Schematic for the bifunctional ligand probe strategy. Following binding to a receptor target, probes are photolyzed to cross-link to bound proteins, and the resulting adducts can be selectively tagged and manipulated via bioorthogonal chemistry.

and peptidic probes (*159*) to target the cancer marker galectin-3 in cell lysates. More recently, a bifunctional probe corresponding to phosphatidylcholine (PC, **27**) was employed to selectively purify, identify, and characterize receptors that interact with this lipid from a cell extract (*160*). Cross-linked receptors included proteins that were previously known and not known to interact with PC.

## 4. CONCLUSIONS

Although the onset of click chemistry and bioorthogonal reactions represents a recent advancement, the advantages of these technologies have rapidly become apparent, and they have become entrenched in the fabric of chemical biology. As is illustrated by the examples discussed herein, the exquisite reliability, selectivity, and modularity of bioorthogonal chemistry render it an invaluable chemical tool for probing and perturbing biological systems. As such, it is clear that these reactions will act as enabling technologies that will spur the course of future research. The scope of fundamental biological questions that can now be addressed through application and extension of this methodology is quite broad. In particular, since these techniques allow real-time detection in vivo, they will be invaluable for elucidating the spatial and temporal aspects of biomolecule function, which are critical to the manner in which biological systems are precisely managed. Since bioorthogonal chemistry advances our capabilities in performing chemical biology, creativity is required on the part of researchers to devise innovative and clever ways to exploit this chemistry to maximize its potential. Judging from the initial efforts in this area, researchers in the field will be up to this task.

The advancement of bioorthogonal chemistry will also require the improvement of technological capabilities in other aspects of chemical biology. A primary beneficial aspect of synthetic chemistry is the precise control over chemical structure that can be achieved, much of which can be lost in a biological setting, where the heterogeneity drastically complicates the understanding of what is present and is being measured. A primary issue in the deployment of probes is that, following cell entry, these compounds are subject to numerous chemical or enzymatic modifications. As such, precise control over the activities of derivatized probes will be crucial for harnessing the full potential of bioorthogonal labeling approaches. This can be achieved by either blocking or rationally directing the pathways by which probes are altered within biological systems, examples of which are illustrated in the applications discussed in this manuscript. Chemical biologists will represent the front line in these efforts to design probes and understand pathways so as to devise the most effective strategy for studying a particular system. Finally, control can also be exerted by expanding our analytical capabilities to precisely measure and detect labeled molecules within complex biological systems. In these ways, advancement of the infrastructure that is complementary to bioorthogonal chemistry will allow for the full potential of this strategy to be realized.

## NOTE ADDED AFTER ASAP PUBLICATION

After this paper was published ASAP June 1, 2009, a correction was made to Figure 10; the corrected version was reposted June 24, 2009.

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