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Practical Considerations, Challenges, and Limitations of Bioconjugation via Azide-Alkyne Cycloaddition

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Abstract

Interrogating biological systems is often limited by access to biological probes. The emergence of “click chemistry” has revolutionized bioconjugate chemistry by providing facile reaction conditions amenable to both biologic molecules and small molecule probes such as fluorophores, toxins, or therapeutics. One particularly popular version is the copper-catalyzed azide-alkyne cycloaddition (AAC) reaction, which has spawned new alternatives such as the strain-promoted azide-alkyne cycloaddition reaction, among others. This focused review highlights practical approaches to AAC reactions for the synthesis of peptide or protein bioconjugates and contrasts current challenges and limitations in light of recent advances in the field. The conical success of antibody drug conjugates has expanded the toolbox of linkers and payloads to facilitate practical applications of bioconjugation to create novel therapeutics and biologic probes. The AAC reaction in particular is poised to enable a large set of functionalized molecules as a combinatorial approach to high-throughput bioconjugate generation, screening, and honing of lead compounds.

Graphical Abstract

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Conflict of Interest Disclosure

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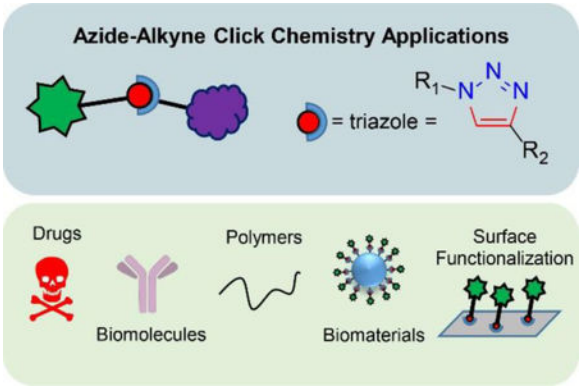

Introduction

In the field of chemical biology, scientists design and create molecules that exert an effect on, or reveal new information about biological systems. Unfortunately, the limiting factor is often the efficient and convenient synthesis of biological probes. Prompted by nature's ability to generate vast biological diversity from a limited number of monomers, Sharpless and co-workers, in a 2001 review,[1] proposed the development of a set of powerful, selective, and reliable reactions for coupling molecular fragments under mild reaction conditions. He termed the foundation of this approach "click chemistry". The unique features of click chemistry provide a toolbox for efficient coupling methodologies for the synthesis of a variety of conjugates (Figure 1). Thus, through the advent of click chemistry, bioorthogonal reactions have emerged as highly specific tools that allow for investigation of biological systems.

Of all the bioorthogonal click reactions that have been developed, the most widely applied is the copper-catalyzed azide-alkyne cycloaddition reaction (CuAAC). Since its inception, researchers from diverse disciplines have utilized this highly efficient coupling reaction for the synthesis of conjugates with various architectures and functional groups. In order to improve upon the CuAAC reaction, the strain-promoted azide-alkyne cycloaddition reaction (SPAAC) was introduced, which mitigated several disadvantages of the CuAAC. Several other click reactions are present in the literature, such as Diels-Alder,[2] Staudinger ligation,[3, 4] thiol-Michael addition,[5, 6] and oxime ligation reactions,[7, 8] to name a few. For a more comprehensive review regarding click chemistry, there are a number of reviews which provide excellent background on the implementation of these chemistries.[9–14] However, the practical challenges and limitations in terms of relevant physiochemical properties of the conjugate are often overlooked. In this review, we focus on the use of azide-alkyne cycloaddition (AAC) reactions for the synthesis of bioconjugates, including their history, reaction conditions, methods for installation of reactive handles, and utilization in peptide or protein bioconjugates, with a particular emphasis on practical examples as well as challenges and limitations with this approach. Finally, we conclude with a section outlining the trends of non-AAC linkers, which can be extrapolated in future implementation of AAC chemistry in bioconjugation.

Azide-Alkyne Cycloaddition Reaction

The formation of 1,2,3-triazoles via AAC was first studied by Huisgen in the 1960's (Table 1).[15] This heterocycle is an attractive bioisosteric replacement for an amide due to its stability toward common biological stresses including enzymatic degradation, oxidizing or reducing conditions, and pH. Specifically, a 1,4-substituted triazole is similar to a *E*-amide in both electronic properties and topology, exhibiting spacing of 5.0 Å as compared to 3.9 Å in the *E*-amide bond[16] and a dipole moment of 5 Debye as compared to 4 Debye in the amide.[17] Further, a 1,5-substitued triazole and *Z*-amide are geometrically analogous in that they both exhibit a bond distance of 2.4 Å.[18] Despite the appeal of the triazole ring, however, the lack of regioselectivity of the original Huisgen reaction, as well as the need for elevated temperature and pressure, limited its utility.

In 2002, the Sharpless[19] and Meldal[20] groups independently reported that the use of catalytic amounts of Cu^{1+} led to the formation of regiospecific 1,4-substituted triazoles under mild reaction conditions and at rates 10^6-10^7 greater than the mixture of 1,4- and 1,5-substituted products in the absence of Cu. Moreover, this reaction occurs over a wide range of temperatures (0–160°C), in a variety of solvents (including water), and over a wide range of pH values (4–12).[16, 19, 21–23] The initial surge in the application of the CuAAC reaction, however, rapidly exposed drawbacks primarily associated with the use of Cu. The active catalytic species necessary for the CuAAC reaction is Cu^{1+}, which is readily oxidized to the more stable Cu^{2+} in solution. Therefore, Cu^{1+} is typically formed *in situ* via the action of Cu^{2+} and excess reducing agent. The most commonly employed reducing agent is sodium ascorbate in a 3- to 10-fold excess,[16] but other reducing agents, including hydrazine[24] and hydroxylamine[6] have been successfully used. Unfortunately, sodium ascorbate and Cu^{1+} have been shown to promote the oxidation of histidine and arginine residues.[25] These unintended side reactions have led to the introduction of Cu-stabilizing ligands (Table 1) to both limit degradation of these amino acids, as well as accelerate the rate of the CuAAC reaction.[26, 27] Additionally, the toxic effect of Cu on cells limits its use in cell based assays where long-term viability is a concern.

To alleviate the need for Cu, reducing agents, and accelerating ligands, Bertozzi and co-workers developed the SPAAC reaction in 2004 (Table 1).[27] This modification enables the reaction to proceed efficiently in the absence of a catalyst due to the high degree of ring strain on the cyclooctyne ring (18 kcal/mol), allowing for mild reaction conditions and relatively fast reaction times.[28] Despite these advantages, however, the SPAAC approach lacks regiospecificity of the reaction product, forming a mixture of 1,4-substituted products. Initially, the aqueous solubility of the cyclooctyne reagents were of concern, but recent developments have seen the installation of solubilizing moieties such as polyethylene glycol (PEG) or sulfonate groups in the linker attached to the ring. Furthermore, the cost of the strained cyclooctyne reagents is considerably higher than their terminal alkyne counterparts. Fortunately, alternate synthetic routes[29] are making SPAAC reagents more accessible and less cost-prohibitive to employ. Consequently, a number of strained alkyne moieties have been developed and shown to function as a coupling partner in the SPAAC reaction, with varying reaction rates.[30] Photolabile "caged" cyclooctyne variants present an important added functionality to the reaction, revealing the reactive strained alkyne group under

exposure to 350 nm light and enabling spatially-controlled conjugation (Figure 3A).[31] This approach is of particular interest in surface functionalization applications where spatiotemporal control of conjugation is critical.[31, 32]

The success of the CuAAC reaction further highlighted the need for regioselective formation of 1,5-substituted triazoles. Since catalytic transformations of alkynes via the action of ruthenium (Ru) complexes have been established,[33–35] various Ru catalysts were evaluated and shown to be capable of catalyzing the regioselective formation of 1,5-substituted triazoles.[18, 36, 37] Ru catalysis is advantageous over Cu in that it allows the reaction between internal alkynes and azides.[18] Despite this benefit, steric and electronic considerations of the 1,5-triazole become important. First, the 1,5-triazole mimics the *Z*-amide bond, which is sterically unfavorable compared to the *E*-amide bond due to repulsion of the groups flanking the amide moiety. In addition, the value of the triazole as an amide bond surrogate relies on the oxygen lone pair, the acidic N-H bond and the polarized carbonyl carbon. The polarization of the 1,5-triazole, however, is such that the electrophilic carbonyl carbon is replaced by a negatively polarized nitrogen atom.[18] Therefore, despite the ability of Ru to catalyze reactions between internal alkynes and azides, the dissimilar electronic properties and unfavorable steric interactions compared to the naturally-occurring amide bond limits the implementation of Ru in bioconjugation approaches.[38]

The facile synthesis of the 1,4-substituted triazole ring, in addition to its stability and chemical properties has been a significant driver for its implementation in bioconjugation applications. The history of AAC reactions, as well as the reaction conditions necessary to yield the triazole moiety provide important background information. However, in order to exploit the utility of the triazole ring, one must first understand various ways to introduce reactive handles to molecules of interest, which will undergo subsequent AAC reactions.

Installation of Azide and Alkyne Functionalities on Intact Biomolecules

There are two common approaches to functionalize biomolecules with azide or alkyne handles: *N*-hydroxysuccinimide (NHS) mediated amide bond formation using an amine and carboxylic acid, or reaction of a thiol with a substituted maleimide (Figure 2). NHS esters are among the most popular compounds used to functionalize biomolecules due to their aqueous compatibility, commercial availability, and ability to selectively target primary amines present on lysine residues or the N-terminus (Figure 2A). For biomolecules, reaction conditions generally employ aqueous buffers at pH 7–9. At a pH closer to 9, the reaction proceeds more efficiently due to a higher degree of amine deprotonation. However, at elevated pH, hydrolysis of the activated ester also occurs at a greater rate. On the contrary, at neutral pH, the reaction between primary amines and NHS esters proceeds at a slower rate, yet is favored over hydrolysis of the NHS ester. Thus, a pH range between 7–9 is commonly employed to balance reaction rate and extent of functionalization while also considering the solubility and stability of the biomolecule. Reactions with NHS esters are typically performed at room temperature over 1–2 hours. However, reactions involving sensitive biomolecules can proceed at 4°C overnight.

Due to the difference in acidity between the α-amino group of the N-terminus (pK_a ~ 8) and the ε-amino group of lysine residues (pK_a ~ 10), selective N-terminal functionalization has been shown at pH values below 7, with pH 6.3 being ideal.[39] Performing the reaction in the absence of amine-containing buffers such as tris or glycine is essential, however, these can be useful as quenching buffers to ensure no additional reactive NHS ester is available after achieving the desired degree of functionalization. When possible, NHS ester reagent solutions should be prepared in anhydrous organic solvents to limit hydrolysis prior to initiating the reaction, provided that all reaction components remain compatible. Unfortunately, NHS ester reagents are typically not stable for more than a few hours in solution, even when prepared in anhydrous solvents.

For small molecules which do not necessitate an aqueous reaction environment, NHS ester reactions in anhydrous organic solvents such as *N,N*-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), acetonitrile (MeCN), and dichloromethane (CH_2Cl_2) are ideal. This generally results in higher yields and better selectivity for amino functional groups due to a reduction in aqueous hydrolysis. In the absence of amine functionality, NHS esters can also modify other nucleophilic groups in a molecule such as deprotonated hydroxyl or thiol moieties. Alternatively, carboxylic acids can be functionalized through *in situ* formation of the activated ester using NHS and an appropriate coupling reagent such as 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) or *N,N'*-dicyclohexylcarbodiimide (DCC).

The reaction of a thiol group, such as those present on cysteine residues, with a substituted maleimide represents another commonly utilized approach for functionalization of biomolecules. This reaction yields a thioether bond via a Michael addition reaction (Figure 2C). It is important to note that thioethers have been shown to undergo thiol exchange reactions, as well as to convert back to the starting thiol and substituted maleimide.[40] Similar to the NHS ester, maleimide reactions are pH-controlled in aqueous media, generally in the pH range of 6–8. At lower pH values, the reaction proceeds at a slower rate, but favors thiol functionalization over hydrolysis. At higher pH values, the reaction proceeds at a faster rate, but hydrolysis of the maleimide is of greater concern. As with NHS esters, reaction buffers should avoid the use of thiols such as dithiothreitol (DTT) or β-mercaptoethanol (BME), but are useful post-reaction to quench any remaining maleimide after achieving the desired conjugation levels. Mechanistic investigations of the thiol-maleimide reaction have shown a significant dependence on the solvent, initiator (base), and thiol used, and the reaction mechanism is dependent on these three factors.[41]

In summary, functionalizing intact biomolecules using NHS ester or maleimide chemistry represents one of many ways in which azides and alkynes can be introduced. There are a variety of commercially available heterobifunctional linkers containing NHS esters or maleimides, which can be conjugated to amines or thiol groups present on the molecule of interest adding the desired azide or alkyne functionality. Specifically, at the end of this review, we highlight the importance of the structure of these linkers, as they have been shown to modulate a variety of processes *in vitro* and *in vivo*.

Functionalization During Peptide or Protein Synthesis

Selective and reproducible modification of larger protein molecules has proven to be challenging with NHS ester or maleimide chemistries, since the surface-exposed lysine and cysteine residues targeted can be abundant on larger proteins.[42, 43] To circumnavigate this challenge, unnatural amino acids (UAAs) bearing azide or alkyne functionalities (Figure 3C) have been employed (Table 2A).[44–47] Installation of an azide or alkyne handle on a peptide or protein is most commonly achieved using UAAs in either solid-phase peptide synthesis (SPPS) or by metabolic labeling during expression in cell systems, respectively. In particular, functionalization of biologics can be further divided into site-specific and residue-specific. Site-specific functionalization refers to the modification of a single amino acid, whereas residue-specific functionalization allows for partial or quantitative replacement of a particular amino acid. AAC is also used for peptide conjugation in complex biomaterials like nanoparticles, micelles, hydrogels, and functionalized surfaces for a diverse number of applications, such as investigations of cell behavior or targeted drug delivery. For information regarding these applications, Tang *et al.*[14] provides a review of click chemistry reactions for peptide conjugates with examples outside the scope of this review.

Residue-Specific Functionalization

In residue-specific labeling, UAAs are included in cell growth medium and incorporated into the primary sequence of the expressing protein. In a review, Budisa *et al.*[48] outlines the biology of the metabolic labeling methods as well as some issues that arise with the incorporation of UAAs. A major disadvantage to residue-specific labeling is that the UAAs may be incorporated at multiple sites heterogeneously, resulting in altered physical or chemical properties which impacts binding dynamics or solubility.[49] Residue-specific functionalization through metabolic labeling is more common for larger biologics due to the relative ease of implementation when compared to site-specific functionalization. Metabolic labeling is becoming an increasingly popular technique to measure metabolic activity when used in conjunction with a functionalized fluorophore, since the modified biomolecules incorporate into biosynthetic pathways in the same manner as their unmodified counterparts.

Site-Specific Functionalization

For smaller peptides, site-specific functionalization using SPPS is relatively simple. A wide range of UAAs are commercially available with both azide and alkyne functional groups and protected versions of these compounds (Figure 3B) can be used to enable selective incorporation of the desired handle during SPPS.[50] Peptides bearing azide and alkyne handles also present an attractive way to make cyclic peptides, which have literature precedence[51–53] (Table 2A). Typically, disulfide bonds are used for cyclization reactions, but are susceptible to oxidizing/reducing environments and disulfide exchange. Cyclization via the triazole ring, however, enhances stability compared to the disulfide bond. In addition, there is evidence that using a triazole bridge to create a dipeptide results in increased solubility over naturally self-assembling peptides, while also allowing for post-assembly modifications.[53] In another case, a double triazole bridge was used to induce secondary and tertiary structure and stabilize helical peptides.[54] Additionally, exploiting the Cu- and Ru-catalyzed variants of the cycloaddition reaction can have a dramatic effect on activity as a

result of geometric alterations of the binding pocket.[52] For biomolecules which cannot be constructed via SPPS, site-specific functionalization becomes more complex, but can be achieved by site-specific incorporation of UAAs or by exploiting post-translational modifications. An UAA is incorporated site-specifically into a protein by using an engineered tRNA unique for the target codon in the proteins' sequence.

Unlike residue-specific modification, site-specific incorporation methods generally avoid drastic changes in physical and chemical properties of the resulting protein.[55] Additionally, site-specific functionalization tends to yield a more homogenous product, simplifying purification and characterization. Site-specific installation of click reactive handles has been utilized for many functions such as fatty acid conjugation,[55] conjugation of an antibody to a drug,[46, 56] or for fluorophore labeling of a protein.[47]

Heterobifunctional Linkers

In the event that preservation of side chain binding interactions is required to maintain activity, heterobifunctional reagents can also be useful to install N-terminal linkers as the final step of SPPS. Since these linkers do not contain a primary amine functionality, the need for protection and deprotection is eliminated. The heterobifunctional linker reacts in the same manner as an amino acid during the SPPS process, which requires a carboxylic acid functionality, in addition to the azide or alkyne functional group of interest (Figure 3D). In addition to SPPS, heterobifunctional azide or alkyne linkers have been utilized to modify reactive functional groups on various platforms, such as polymer surfaces,[57] glass slides,[32] and magnetic beads.[58, 59] Successful click conjugation using this approach has been shown with a variety of different molecules,[60–63] and enables the selective orientation and spacing of the compound of interest (Figure 3D). When choosing a linker for an AAC reaction, one must consider physical and chemical properties such as solubility and flexibility. In an effort to conjugate peptides on a surface for diagnostic screening, Gori *et al.*[62] found that the linker type affects binding efficiency to the platform but also binding efficiency to antibody samples in a diagnostic test. Others have shown including PEG in the linker can improve water solubility and alleviate steric effects between the two molecules, but the benefit can be limited as the PEG chain increases in length.[64, 65] Branched heterobifunctional linkers have also been employed in attempts to control conjugation ratios and spatial separation.[66] These examples illustrate how the properties of the linker must be carefully considered and optimized for each application. A large variety of heterobifunctional linkers are commercially available with different of solubilizing moieties like PEG and sulfate groups. PEGylated forms of the heterobifunctional linkers are available in various lengths, which permits precise spacing of the reactive handle.

Post-Translation Modifications

For proteins expressed in cell systems, exploiting post-translational glycosylation becomes another attractive approach for site-specific conjugation. For instance, acetylated versions of modified sugars may be added to growth medium, and following internalization, non-specific hydrolases or esterases remove the acetate groups and release the sugar mimic bearing an azide or alkyne functional group (Figure 3E). These sugars incorporate into glycosylated proteins, permitting subsequent conjugation either *in vitro* or following protein

isolation (Table 2C).[67–72] For proteins which undergo post-translational lipidation, prenylation, or farnesylation, alkyne-modified variants of fatty acids[73–78] (Figure 3F*i*) or isoprenoid precursors[79–81] (Figure 3F*ii*) can incorporate into biosynthetic pathways in the same manner as their unmodified counterparts. This enables a method of site-specific incorporation of a reactive handle, or to directly measure metabolic activity when combined with an azide- or alkyne-modified fluorophore (Table 2C).

Nucleic Acids and Other Anchors for Live Cell Bioconjugation

While primarily used for imaging applications, azide- and alkyne-modified nucleic acids (Figure 3G*i-ii*) and other membrane components (Figure 3G*iii-iv*) have been used to install a reactive handle in actively synthesized DNA,[82–84] RNA,[85] or cell membrane components[86] (Table 2D). Following installation of the reactive handle, conjugating molecules that enhance targeting, alter binding[87] or modify expression provide significant potential for therapeutic use. Neef *et al.*[82] suggested that the structure of the unnatural nucleic acid in DNA labeling made a difference in incorporation efficiency and in toxicity, but was correlated to the particular cell type used. Additionally, when used in conjunction with a fluorescent probe bearing an azide or alkyne moiety, these molecules are useful as probes of cellular metabolic activity (Table 2D).

Payload Molecules for Detecting or Modulating Biomolecule Function

With the significant interest and development surrounding the AAC reaction since its inception, a number of molecules have emerged in the literature which enable facile access to key functionalities (Figure 3H-I). Most commonly, fluorescent tags installed on a biomolecule permit visualization inside a cell and/or quantitation using techniques such as flow cytometry. Sivakumar *et al.*[98] developed a pro-fluorescent coumarin molecule (coumarin-N_3, Figure 3H), having negligible fluorescence in the unconjugated form, and becoming fluorescent after the click reaction extends the conjugated system.[99] This molecule has been useful in optimizing reaction conditions and for studying kinetics of the click reaction.[25, 26] A number of radiolabels (Figure 3I) have also been described in the literature, enabling access to both targeted radiotherapy and *in vivo* visualization and quantitation techniques not possible with fluorescent labels, such as positron emission tomography (PET)[94] and single photon emission computed tomography (SPECT).[100] PEGylation reagents (Figure 3J) can also be utilized to modify clearance or alter biodistribution of a molecule by making significant changes to its molecular weight (MW). Van Geel *et al.*[65] observed that click conjugation can be improved with increased PEG chain length, up to a length of twelve. In the biopharmaceutical field, click chemistry is an attractive option for antibody-drug conjugates (ADCs). ADCs on the market and in clinical trials are commonly formed by reaction of a thiol and alkyl maleimide, which is a slowly reversible process that lacks selectively and produces a heterogeneous mixture of products.[101] The main factors that improve the therapeutic index of ADCs are site-specific modification and stability.[102] More recently, researchers are exploring click chemistry for conjugation of payloads to antibodies (Table 2E). Multiple groups have shown success by incorporating an azide or alkyne functionalized UAA into an antibody for subsequent reaction with an azide or alkyne functionalized payload[46, 47, 56] using a variety of linker

types including a cleavable linker.[47] Commonly used cytotoxic drugs in ADCs, such as the auristatins and maytansinoids, have been functionalized with handles for click conjugation (Figure 4). Twenty-two of the ADCs currently in clinical trials or on the market incorporate monomethyl auristatin E (MMAE) or monomethyl auristatin F (MMAF).[101] Similarly, the maytansinoids are used in 13 ADCs either in the clinic or commercially-approved.[101] Heterobifunctional linkers (Figure 3D) have also been used to functionalize the payload molecule.[63, 101] The incorporation of azide-UAAs into a monoclonal antibody (mAb) allows for site-specific conjugation of modified payloads,[46, 47, 56] but requires careful optimization for each payload, linker, and mAb.[56] Another approach which does not alter the primary sequence exploits post-translational modifications by attaching click handles on glycans of the heavy chain.[26, 65, 92, 93] More information on payload conjugation in click chemistry and in ADCs can be found in Meyer *et al.*.[100] Beck *et al.*.[101] and Akkapeddi *et al.*[102]

Challenges Associated with Bioconjugates

Synthetic and Analytical Considerations

Bioconjugates can exploit the benefits of multiple molecules with different properties, but their complexity often brings new challenges with respect to the synthesis and analysis of these constructs. Synthetic complications typically center on solubility or stability of the parent molecules or resulting conjugate. Proteins and other biomolecules are generally more sensitive than the payload to temperature and other environmental factors such as organic solvents, thereby limiting the approaches available to the traditional synthetic chemist.[6,25] In addition to reaction condition considerations, analytical hurdles reside around heterogeneity, deconvolution of data, and limited sample quantities due to high costs of proteins, which requires adaption of methods and techniques to comply with these demands. A summary of the challenges, drawbacks, and solutions is provided in Table 3.

Solubility of the biomolecule and payload are required for success of the conjugation reaction. Payload molecules with minimal aqueous solubility require assistance from an organic solvent to ensure the conjugation reaction can proceed. Typically, DMSO, DMF, and MeCN are utilized in these situations. However, one must also bear in mind the effect of organics on the biomolecules. Biomolecules with higher order structure can be conformationally altered in the presence of organic solvents and may have limited solubility as the organic concentration increases. Therefore, minimizing the overall organic solvent concentrations used in the reaction is often essential.[6,103] As covered previously, NHS ester and related chemistries which target surface-exposed lysines or other charged amino acids can have a great impact on the overall solubility of the product as these amino acids will lack a charge following conjugation, thereby decreasing solubility of the bioconjugate. To negate some of the deleterious effects of conjugation on solubility, linkers which have solubilizing moieties can either counteract or enhance solubility as desired in the conjugate.

Importantly, a thorough understanding of the stability and degradation mechanisms of the parent molecules will guide the chemist in selecting appropriate reaction conditions while maintaining the integrity of the functional entities involved. These studies must assess both the physical and chemical stability of the molecules, since mechanisms leading to instabilities in small molecules are different than those seen for larger biomolecules.

Proteins have a frequent propensity to aggregate in solution, a phenomenon which can be reversible or irreversible. Aggregation can be especially problematic during synthesis, leading to precipitation and lower yields if not properly controlled. Aggregation during a chemical reaction can generally be limited by excipient addition, modification of the reaction environment (pH or ionic strength), decreasing reaction temperature, and limiting the amount of time the biomolecule is in solution. Any aggregates that form during the reaction should be separated from the product prior to final isolation, typically by filtration, centrifugation, or other size exclusion methods. Analytical techniques employed for studying aggregation in biomolecules include size exclusion chromatography (SEC), gel electrophoresis, or various spectrophotometric and light scattering particle sizing methods appropriate for the aggregate size involved. Liquid chromatography coupled with tandem mass spectrometry (LC-MS/MS), often preceded by enzymatic digestion, is a powerful technique for understanding chemical degradation products in biomolecules. Small molecules are generally more robust than biomolecules and the degradation products can be more easily understood using LC-MS and NMR techniques. When designing bioconjugates that utilize the AAC reaction, the chemical and physical stability of the biomolecule, linker, payload, and any intermediates or catalysts must be taken into consideration and monitored appropriately.

Purification of bioconjugates constructed via the AAC reaction often require additional considerations. When employing the CuAAC variant, residual Cu is undesirable in final products due to the toxic and oxidative effects in living systems. Fortunately, removal through common techniques appropriate for the compounds involved is generally adequate. For small molecules, Cu binding resins such as Cuprisorb or chelators such as ethylenediaminetetraacetic acid (EDTA) are added following the reaction, which can also serve to quench the reaction.[6] However, metal binding resins such as Cuprisorb have a tendency to bind biomolecules and should be avoided during purification. Purification for peptides and proteins is typically completed by chromatographic techniques such as reverse phase high pressure liquid chromatography (HPLC), or through size exclusion methods such as dialysis, where appropriate. Following purification, inductively coupled plasma mass spectrometry (ICP-MS) is a useful technique for assessing residual Cu levels. Fortunately, Cu-free approaches such as SPAAC can be utilized to avoid problems associated with residual Cu.

Limiting side reactions with amino acid residues of biomolecules is a necessity to maintain activity in the final conjugate. CuAAC reactions employing the common Cu^{2+} and sodium ascorbate catalyst system are known to lead to the formation of reactive oxygen species, which can degrade amino acids such as histidine, arginine, cysteine, and methionine.[6] Cu-chelating ligands such as THPTA or TBTA and side chain surrogates, such as aminoguanidine (surrogate for arginine) can assist in limiting degradation. In addition, cyclooctyne compounds are known to react with reduced cysteine residues through a thiol-yne mechanism. However, pre-incubation with iodoacetamide has been shown to mitigate this side reaction.[6] In order to verify the structure of the chemically altered biomolecule, analytical methods such as reverse phase HPLC, LC-MS/MS, and peptide mapping can be utilized.[104]

The CuAAC and SPAAC reactions have been employed extensively in construction of hydrogels due to high yields, mild conditions (aqueous solutions, physiological pH, ambient temperature), and minimal formation of byproducts.[105–108] An added complication of bioconjugation to polymers is the tendency for intra- or inter-molecular entanglement, limiting the accessibility to reactive sites. Reduction of this phenomenon can occur through the addition of organic solvents known to denature the polymer,[6] addition of heat to induce thermal unfolding, or altering pH or ionic environment to disrupt specific interactions causing entanglement.[109] In other cases, limited reactive site availability is due to the viscosity of high MW polymers, requiring more dilute reaction conditions. In general, conjugation efficiency will decrease as MW increases due to reduced collisions with appropriate geometry and sufficient energy to react, especially for polymers such as hyaluronic acid which are self-associating or have secondary structure.[110]

Lessons Learned from Other Bioconjugation Chemistries: Linker Stability

Another critical component of bioconjugates is the stability of the linker, since this can also have a dramatic impact on the efficacy of the bioconjugate. Both cleavable and non-cleavable linker strategies have been used in the ADC field as well as in other bioconjugates. Zimmerman and coworkers[46] utilized the incorporation of the azide UAA *p*-azidomethyl-L-phenylalanine (pAzF) into trastuzumab (Tmab), conjugated via SPAAC chemistry to DBCO-PEG-MMAF. The resultant ADCs were highly potent when tested in *in vitro* cell cytotoxicity assays (Figure 4). The authors proposed that their ADCs were released during lysosomal degradation, which has been observed in other non-cleavable Tmab-maytansinoid ADCs. Some ADCs with non-cleavable linkers are active without cleavage and release of the drug, while the mechanisms of non-cleavable ADCs can be more complicated and must be determined experimentally.[46, 111, 112] Cleavable linkers typically exploit environmental factors present in the cellular environment where payload release is desired, involving pH, oxidizing/reducing conditions, or the presence of a relevant enzyme. While this approach is attractive in theory, and may be necessary for successful action of the payload, the mechanisms that release the payload may not be specific to only the desired target location.[113] For example, pH-sensitive linkers present in the bioconjugate can encounter acidic microenvironments during trafficking to the target location, and could release the payload in unintended locations throughout the body, yielding adverse side effects for the patient.[113–115] The site of conjugation of a payload onto the antibody can affect the ADCs properties such as stability, immunogenicity, antigen binding, and pharmacokinetics. Careful optimization of conjugation site can lead to ADCs with desirable properties over ADCs made via random conjugation.[56, 116]

Some ADCs suffer from the ‘bystander effect’, which is when the conjugated toxin is released from the antibody following internalization in antigen-positive cells, crosses the cellular membrane as a neutral species, and kills surrounding cells that may have lower expression of cell-surface antigen. In these cases, an understanding of the drug release mechanism is essential, since treatment with an ADC may not be sufficient to eliminate all of the solid tumor, because cell populations with limited surface antigen expression will also be exposed to the toxin. Brentuximab vedotin utilizes a cathepsin cleavable linker to conjugate MMAE to the antibody, which results in the release of this toxin in a neutral form

capable of crossing biological membranes of adjacent cells not targeted by the ADC. Conversely, when the structural analogue, MMAF, is conjugated to an antibody and released in active form as a charged species, diffusion across membranes is limited, and the bystander effect is reduced. Payloads attached using cleavable linkers, such as those present in Tmab duocarmazine[117] are more prone to the bystander effect[118] than those containing non-cleavable linkages, such as the thioether linkage in Tmab emtansine. This underlines the importance of a thorough understanding of the stability of the linker and payload release, since the bystander effect provides an added mechanism to tune therapeutic specificity that must be accounted for in the design of ADCs with cleavable linkers. Jain and coworkers[119, 120] have published a more detailed review on ADCs and associated linkers.

Lessons Learned from Other Bioconjugation Chemistries: Biological Consequence of the Linker

To date, comparative studies focused on the impact of different AAC linkers on biodistribution are largely absent from the literature. Fortunately, studies have been conducted utilizing alternative bioconjugation chemistries, and the trends for those studies can be extrapolated to provide useful insight into the design of bioconjugates that utilize AAC chemistry. Tmab emtansine is an ADC used to treat patients with HER2-postitive metastatic breast cancer, and it is composed of an anti-HER2 antibody Tmab linked via a thioether linkage to the maytansinoid DM1 (Figure 5*i*).[121] The thioether-linked 5*i* has been reported to be slightly more efficacious in mouse models than the disulfide-linked 5*ii*,[113] contrary to other maytansinoid-containing ADCs where the disulfide-linked drugs showed greater efficacy.[113, 122–124] Erickson *et al.*[125] compared how the thioether and disulfide linkages in 5*i* and 5*ii* affected the mechanism of action and anticancer activity. In Tmab-sensitive breast cancer cell lines, both ADCs showed similar potency in terms of cell viability, but in Tmab-insensitive cell lines, 5*i* showed slightly greater potency than 5*ii*, though the cause was not fully understood. The clearance of 5*i* in plasma (Figure 5B) and in tumor cells (Figure 5C) was approximately two-fold slower than 5*ii*, most likely a result of improved linker stability. The payload delivery to tumors, however, was similar for both bioconjugates (Figure 5D). While the linker had an impact on pharmacokinetics (PK) and the rate of metabolism, both ADCs were successful in payload delivery and antitumor activity. Thus, much remains to be learned regarding the implications of linker design.

Efflux pumps, such as multidrug resistance (MDR) proteins, target molecules for export out of cells. These pumps are more effective at transporting hydrophobic molecules than hydrophilic molecules. In addition, there is a strong correlation between MDR expression and poor clinical response.[126] Specifically, studies have shown that Tmab linked to DM1 via a hydrophilic PEGylated linker is more efficacious towards MDR^+ cells than those containing the more hydrophobic maleimidomethyl cyclohexane-1-carboxylate (MCC) linker.[127] Again, the relative hydrophobicity of the linker contributed to differences in activity as well as PK properties of the molecules.

In the context of ADCs, most drugs conjugated to the antibody are relatively hydrophobic and have minimal aqueous solubility, while the antibody typically has good aqueous solubility. Therefore, the resulting conjugate generally has intermediate solubility, often

proportional to the MW contribution of the parent compounds in the overall conjugate. This can provide a significant benefit to the drug, since hydrophobic drug molecules are more likely to enter cells via passive diffusion. On the other hand, ADCs often exploit the active transport mechanisms used to internalize antibodies. Therefore, an increase in drug-to-antibody ratios (DAR) of ADCs leads to increased potency *in vitro*. but highly loaded ADCs often suffer from more rapid *in vivo* clearance.[128] Multiple studies report that by limiting the average DAR to the range of 2–4, potency can be maximized without negatively impacting *in vivo* performance.[128–130] Methods have been developed to allow for DAR values of up to 8 by utilizing native cysteine residues on human IgG1 (Figure 6A). Using conventional hydrophobic drug-linker chemistries to make ADCs with a DAR of 8 resulted in fast plasma clearance.[128–130] Lyon *et al.*[131] reported that by reducing hydrophobicity of ADCs through linker design, a DAR of 8 could indeed be achieved without accelerating plasma clearance. The authors conjugated 8 MMAF (6*v*) to an anti-CD70 antibody h1F6, using the protease cleavable linker 6*iv*. PK studies showed faster clearance of the h1F6–6*iv* than the unconjugated h1F6 (Figure 6C). They hypothesized that the difference in clearance maybe due to the loss of interchain disulfide bonds, leading to destabilized h1F6. However, the clearance of 6*i* conjugated to h1F6 and unconjugated h1F6 used as a control was similar. Therefore, the authors implicated linker hydrophobicity as the cause of accelerated clearance of h1F6–6*iv*. The relative hydrophobicity of h1F6 and conjugates was determined by hydrophobic interaction chromatography (HIC), revealing the following trend of increasing hydrophobicity h1F6<6*ii*<6*iii*<6*iv* (Figure 6D). When a modified hydrophilic MMAF version (6*ii*) was conjugated directly to h1F6 with no linker, it had similar hydrophobicity to the parent antibody and both showed comparable plasma clearance *in vivo*. Similar *in vitro* potency was observed for h1F6–6*iv* and h1F6–6*iii*, however there was a >4-fold increase in *in vivo* activity for h1F6–6*iii* due to slower clearance (Figure 6C) of the more hydrophilic h1F6–6*iii*. Since h1F6–6*iii* is less hydrophobic than h1F6–6*iv*, it can be suggested that the reduction in hydrophobicity leads to improved plasma clearance of ADCs with high DAR of 8.

Solubility plays an important role in many physiological processes from biodistribution and clearance to immune recognition and response. Insoluble antigens are often processed by macrophages, while dendritic cells tend to process soluble materials.[132] Therefore, modification of the solubility of biomolecules through conjugation can perturb native function, altering recognition, trafficking, or uptake events. An understanding of aggregation propensity, with the associated decrease in solubility, is of critical importance in protein therapeutics. Aggregated proteins have the potential to induce an undesired immune response and lead to potentially serious adverse events for the patient.[133] In 2014, the FDA issued guidance on immunogenicity assessment as a key parameter in the development of therapeutic protein products. Recent studies have also shown that anti-drug antibodies formed upon repeat administration of biologic treatments such as rituximab have led to an immunogenic response in the patient.[134, 135]

Conclusions and Future Directions

Bioconjugates combining the specificity of biomolecules with the potency of toxins or small molecules represent a rapidly growing field. Unfortunately, convenient and efficient

synthesis of such biological probes is often a critical factor impeding research. In this review, we have summarized synthetic methods, challenges, and recent advances for AAC reactions applied to the synthesis of bioconjugates.

As the field continues to emerge, careful attention should be given to key parameters such as solubility and stability of reactants, which are often mismatched in terms of their physiochemical properties. In addition, variations in product solubility, size, charge, and stability should be more systematically investigated, since these features directly affect pharmacokinetics, pharmacodynamics, and safety.

Given the significant clinical advancement of ADCs, a broad expansion of the toolbox of available linkers, antibodies, and payloads will continue to advance practical applications of bioconjugation to create novel therapeutics and biological probes. The AAC reaction in particular is poised to enable a large set of functionalized molecules as a combinatorial approach to high-throughput bioconjugate generation and screening.

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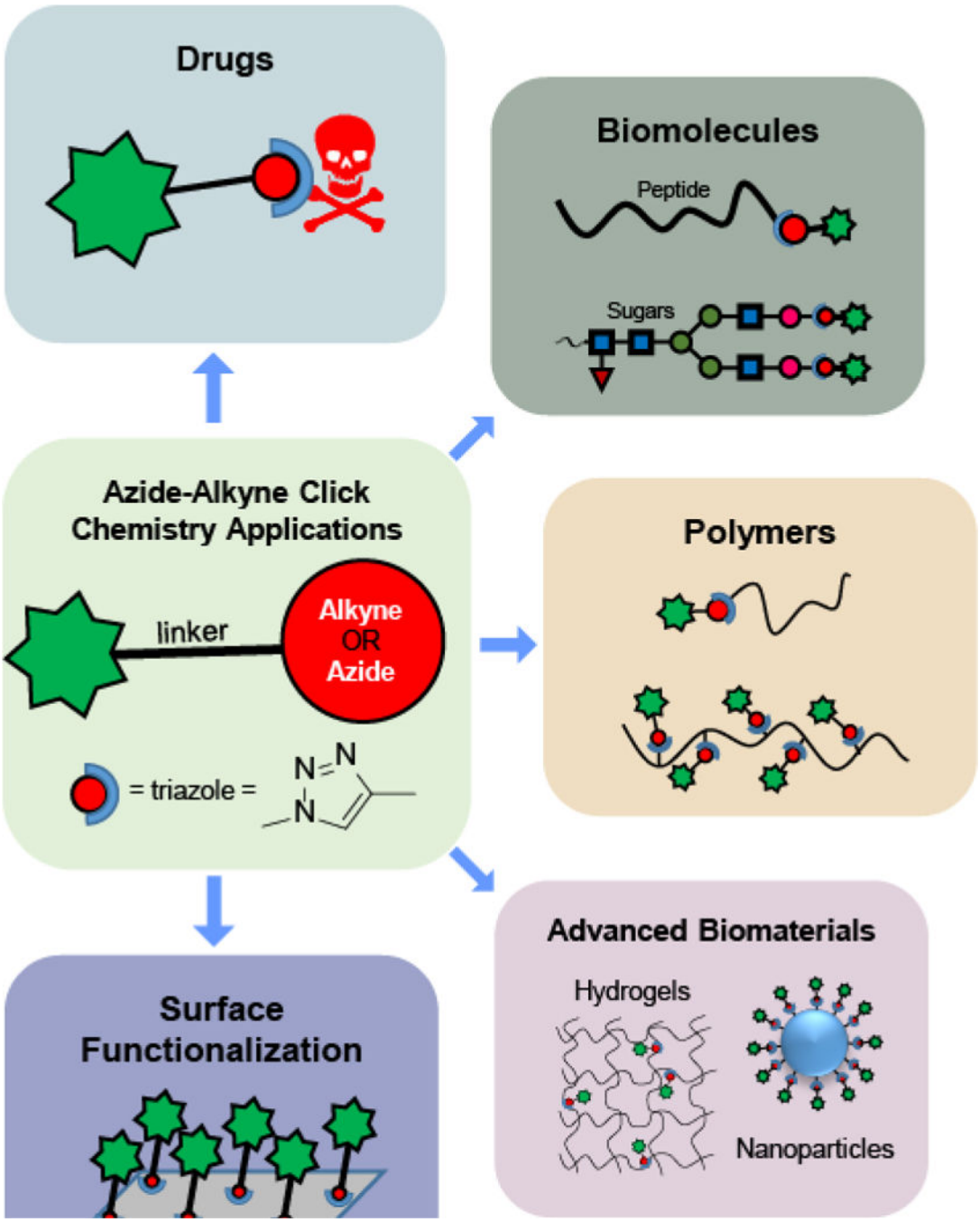


Figure 1:
Toolbox of Azide-Alkyne Click Chemistry.

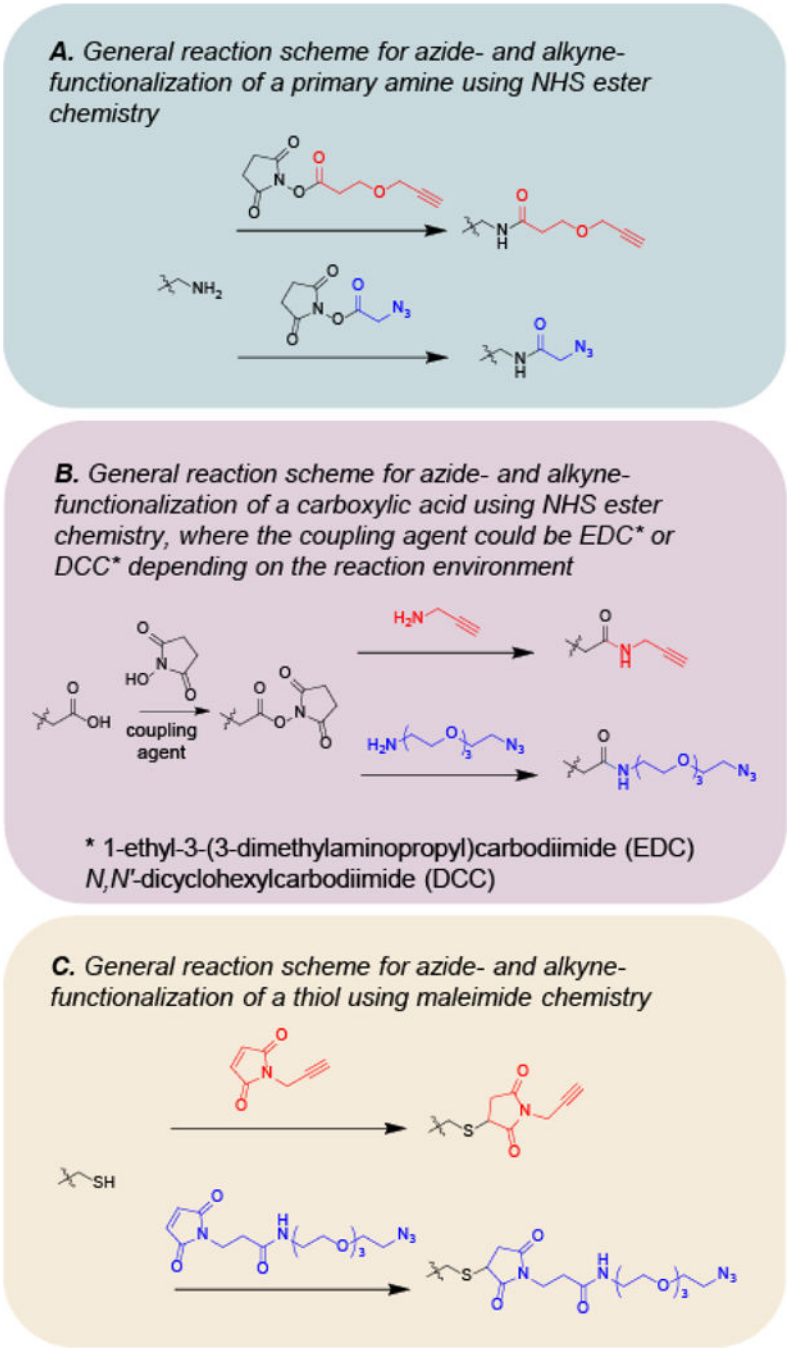


Figure 2:
General reaction schemes to functionalize biomolecules with azides and alkynes.

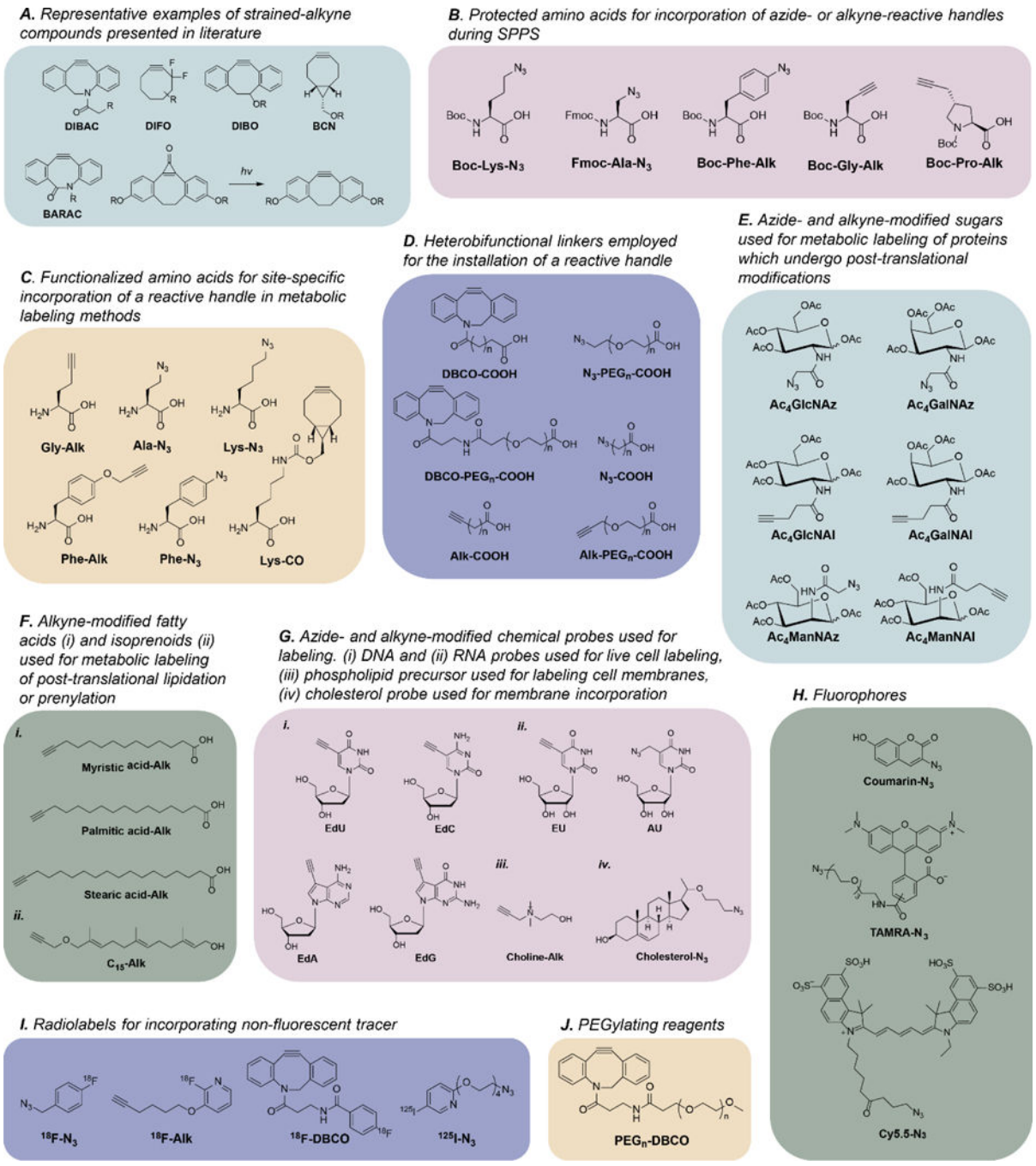


Figure 3.

Commercially-available materials for biomolecule functionalization via azide- or alkyne-reactive handles.

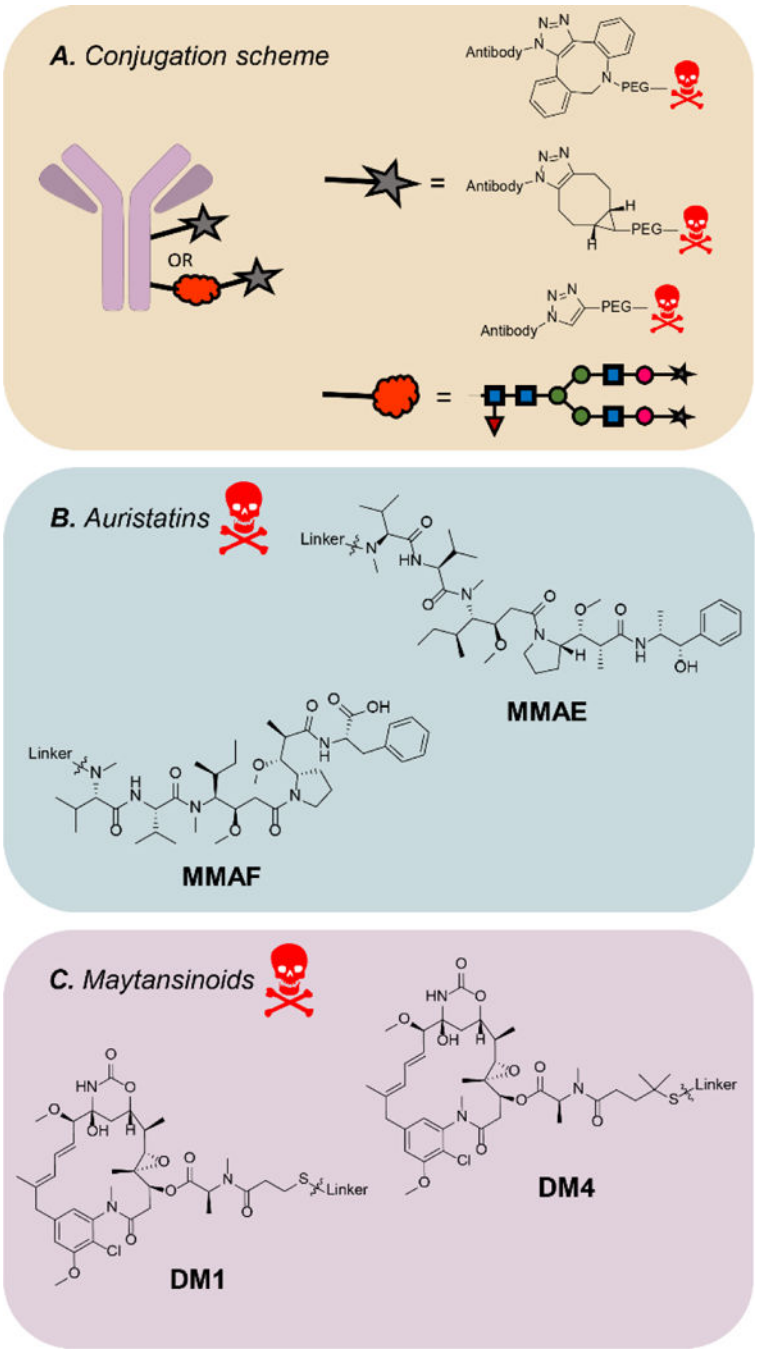


Figure 4.
General conjugation in the construction of ADCs.

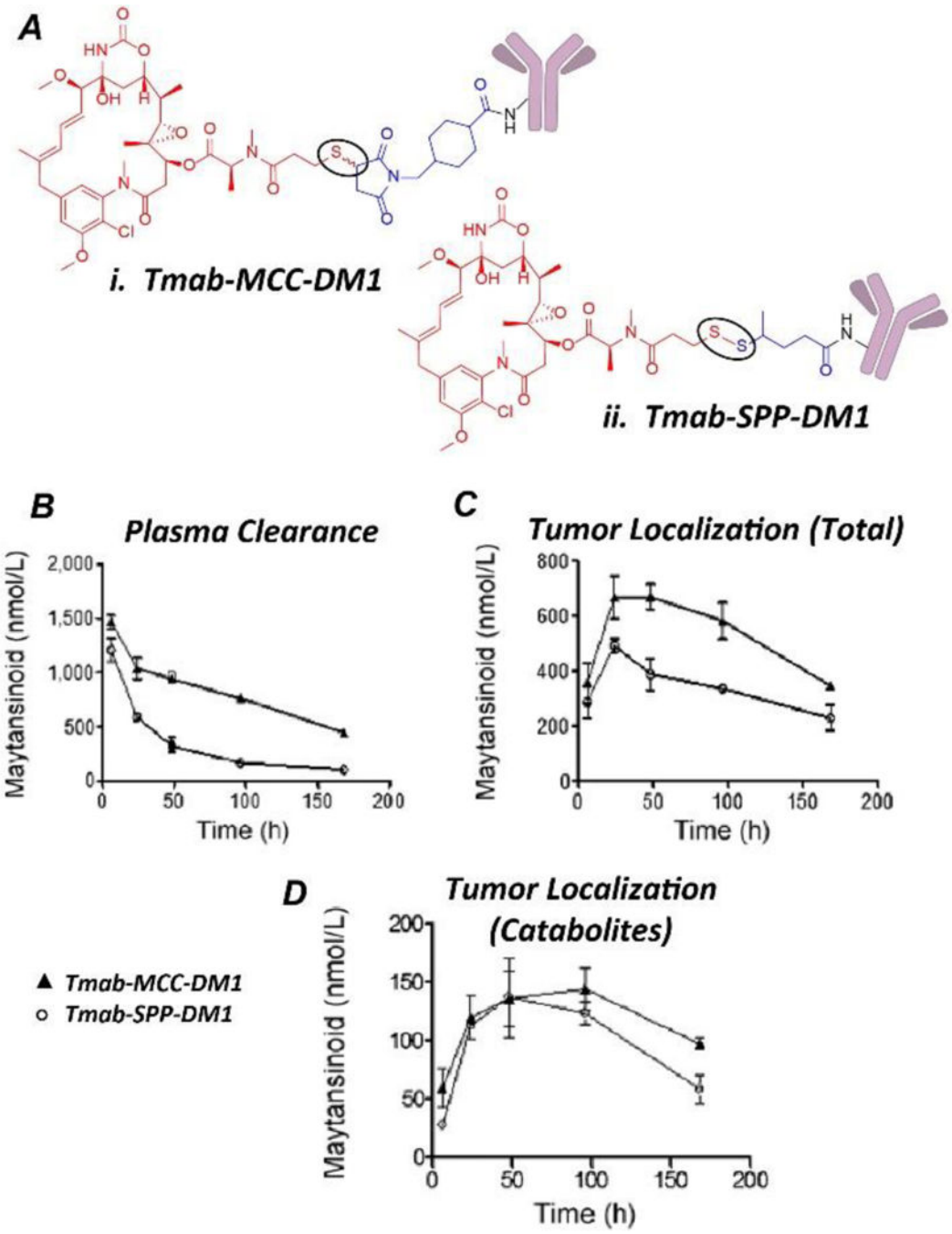


Figure 5:

A) Parent linker compounds: maleimidomethyl cyclohexane-1-carboxylate (MCC) and N-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP). Tmab-MCC-DM1 (Ai) and Tmab-SPP-DM1 (Aii) structures, with DM1 in red, linkers in blue, and thioether and disulfide bonds circled. B) Plasma clearance of ADCs, C) Accumulation of ADCs in tumors, D) Accumulation of ADC catabolites in tumors. (Figure adapted from Erickson et al.,[124] permission pending.)

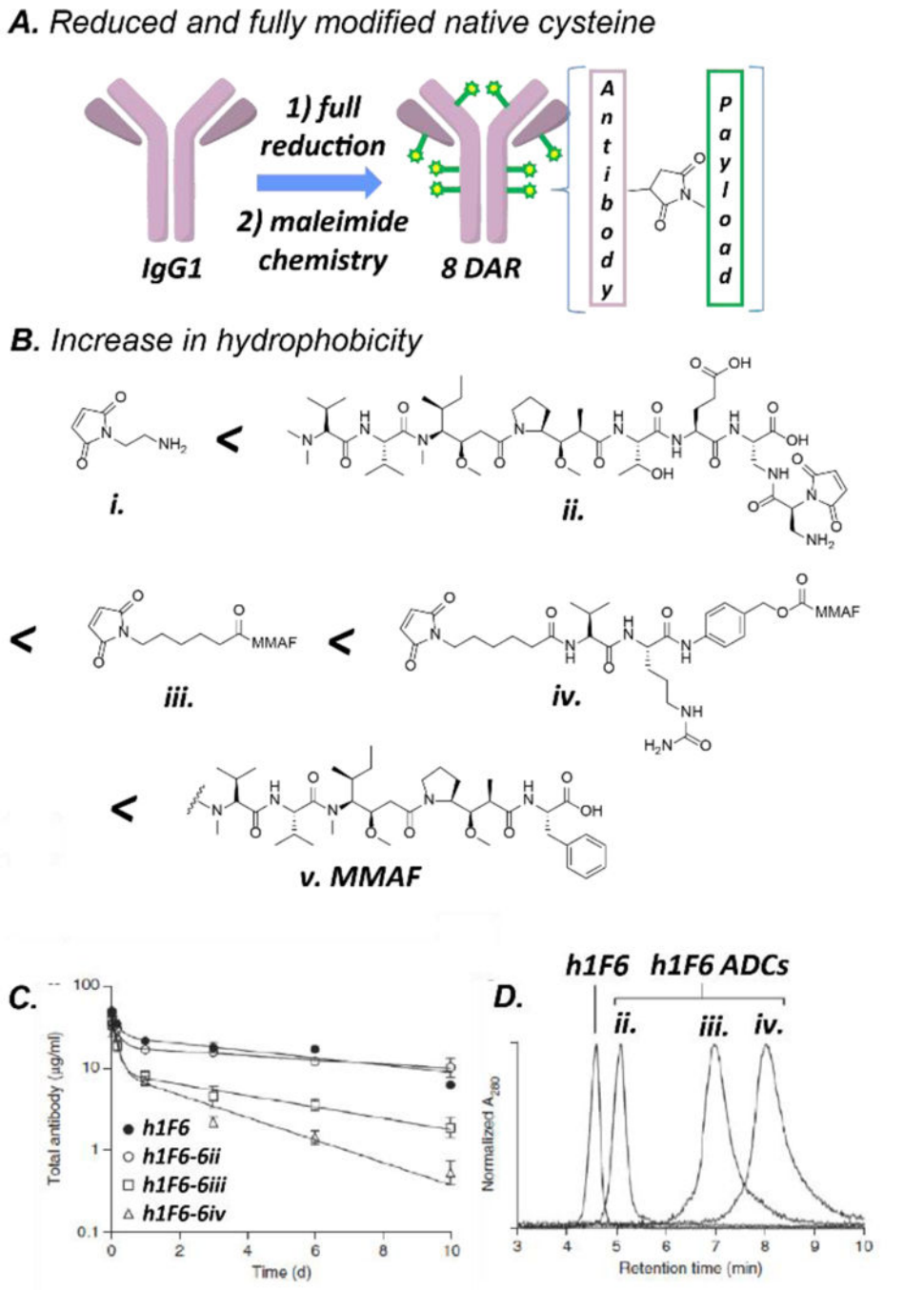


Figure 6:

A) Modification of native cysteine residues to make homogenous ADCs with DAR of 8, B) Rank-order of increasing hydrophobicity of molecules conjugated to h1F6 C) Plasma clearance of h1F6 and h1F6 ADCs in mice, D) Determination of hydrophobicity using hydrophobic interaction chromatography. (Figure adapted from Lyon et al.,[130] permission pending)

Table 1.

Summary of various types of AAC reactions and their limitations

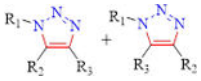
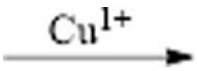
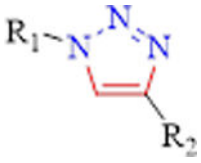
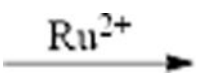
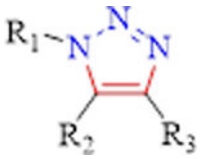
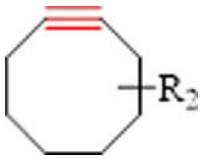
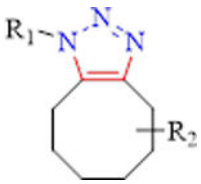
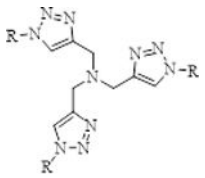
Reaction Type	Reagent 1	Reagent 2	Catalyst	Product	Limitations
Huisgen AAC	R_1-N_3				>100°C Days-Weeks Not regiospecific
CuAAC	R_1-N_3				Requires catalyst, reducing agent, and stabilizing agent*
RuAAC	R_1-N_3				Requires catalyst Forms 1,5-regioisomer
SPAAC	R_1-N_3				Not regiospecific Cyclooctyne reagents more expensive
*** Examples of Cu-stabilizing reagents commonly employed in the CuAAC reaction**				R = benzyl (**TBTA**) R = *tert*-butyl (**TTTA**) R = $CH_2CH_2CH_2OH$ (**THPTA**)	

Table 2.

Specific examples of click chemistry in peptides, proteins, and biological systems.

Modification	Functional Group	Purpose/Description	Reference
A. Peptide and Protein Synthesis			
SPPS	Azidolysine	• Epitopes of Gp120 synthesized with AA mimics for conjugation to a TAC scaffold • Synthetic vaccines for HIV	50
SPPS	Azidolysine and Propargylglycine	• Screened epitope binding to analytes for vaccine design • CuAAC and RuAAC utilized to create macrocyclic peptides	51
SPPS	Azidoalanine, Azidohomoalanine, and Propargylamine	• Created macrocyclic peptides by mimicking disulfide bridges with triazole bridge ○ Strategy to overcome oxidative difficulties in oxidative folding of cysteine rich peptides *in vitro*, increased solubility and stability	52, 53
SPPS	Azidoalanine	• Double azide-alkyne cycloadditions mimicked monomeric helix folding-induction of secondary and tertiary structure for increased stability	54
Metabolic	*p*-azido-L-phenylalanine, BCNK, and AZK	• UAAs incorporated into protein expression in *E. coli* to make protein-protein conjugates	88
Metabolic	Tyrosine-Alk derivatives	• Labeled proteins expressed in *E. coli* for imaging, protein dynamic studies, and synthesis of therapeutic agents • Labeled GFP with a variety of reactive handles and observed that spatial configuration and size of the UAA handle is vital for reactivity	45
Metabolic	*p*-azidomethyl-L-phenylalanine (pAMF)	• Analyzed cell-free based expression of UAA proteins for SPAAC for antibody-drug conjugates • Type of UAA affected conjugation rate	46
Metabolic	Propargylglycine	• Site-specific incorporated UAAs into fibroblast growth factor 2 (FGF2) and reacted with fluorophores or cytotoxic drug, MMAE	47
Metabolic	*p*-ethynylphenylalanine	• Site-specific incorporation of an UAA into a protein for subsequent conjugation to fatty acids in an effort to prolong *in vivo* half-life	55
Metabolic	*p*-azido-L-phenylalanine, *p*-ethynylphenylalanine	• Explored differences in incorporation efficiency as related to the type of UAA in a site-specific approach to functionalizing model protein	89
B. Heterobifunctional Linkers			
	Homopropargyl, DBCO, or Azide linkers (Figure 3D)	• Conjugated peptides to polymer backbones • Type of linker, length, and orientation affected reaction efficiency and binding profiles • Conjugation to fluorophores	60–64
C. Post Translational Modifications			
Glycosylation	Ac_4ManNAz, Fuc-Alk, GalNAz, ManNAz, GlcNAz, Ac_4GlcNAz, Ac_5SiaNAz	• Incorporated functionalized sugars into cell systems • Labeled sugars that can selectively tag cancer cells[67] • Tracked transplanted cells[68] • Monitored human mesenchymal stem cell differentiation[71] • First report of glycosylation of ubiquitin ligase NEDD4–1[72]	67–72, 90, 91
Lipidation	Alkynyl-fatty acid (Alk-FA), Alk-16, C_{15}-Alk (Figure 3F), C_{15}-DH-Alk, Farnesyl Alkyne, azide- or alkyne-farnesyl diphosphate derivatives	• Labeled bacterial lipoproteins[73] • Labeled fatty-acylated proteins in mammalian cells[75] • Labeled palmitoylated proteins in human cells[74] • C_{15}-Alk and derivatives incorporated into cells, mimicking isoprenoid moieties for labeling of prenylated proteins	73, 75–81
D. Nucleic Acids and Other Anchors for Live Cell Bioconjugation			

A. Peptide and Protein Synthesis			
Modification	**Functional Group**	**Purpose/Description**	**Reference**
DNA	EdA, EdG (Figure 3G), variety of azide- and alkyne-modified nucleotides	• Labeled 3'-terminal of DNA using modified nucleotides clicked to fluorophores or biotin ○ Type of handle affected click reaction[84] • Metabolically labeled genomic DNA in HeLa cells and zebrafish embryos using nucleoside analogues ○ Structure of analogue affected incorporation efficiency and toxicity[82]	82, 83
RNA	Azide-modified UTP analogues	• CuAAC and SPAAC utilized to label RNA using UTP analogues • Specifically labeled DNA and RNA simultaneously	85
Phospholipids	Propargylcholine	• Biosynthetically incorporated propargylcholine to label choline-containing phospholipids	86
E. Payloads in Biomolecule Conjugation			
UAA Modification	Click-modified toxin	• Incorporated UAA into FGF2 in order to click to fluorophores or cytotoxic drug, MMAE[47] • Cell-free based expression of UAA proteins for conjugation to MMAE[46] Alkyne-AF or Alkyne-PBD clicked to IgG. Conjugation position must be optimized for each payload and linker[56]	46, 47, 56
Sugar Modification	BCN/DBCO or alkyne-modified payloads	• Made homogenous glycoforms of anti-CD22 with approximately four azide groups, then clicked on DIBO-DOX[92] • Used pyrene as a payload mimic, and clicked DBCO or BCN-pyrene onto modified glycans in CHO IgG[65] • Radiolabeled heavy chain N-linked glycan mAbs with ^{89}Zr and the chelator desferrioxamine[93]	26, 65, 92, 93
Sugar Modification	Radiolabeling	• Incorporated F^{18}-N_3 in SPAAC labeling for PET[94] • Sugar modification on IgG radiolabeled via click chemistry with ^{89}Zr and the chelator desferrioxamine[93]	93, 94
Alternative Methods of ADC Construction	DBCO or BCN payload	• MTGase mediated conjugation of branched DBCO linkers of varying lengths on mAbs conjugated to MMAF for more control over specificity and DAR[66] • Labeled both the mAb and the payload (via the linker) with two different fluorophores for pre- or post-cell incubation labeling[95] • 96-well plate method utilized for cysteine-based conjugation of payload to antibodies towards more site-specific conjuguation[96] • Utilized click handle to functionally re-bridge two pairs of disulfide bonds on IgG for specific DAR of two[97]	66, 95–97

Table 3.

Challenges, drawbacks, and potential solutions to bioconjugates formed via AAC (partially adapted from information in Presolski et al.[6])

Challenge	Cause	Potential Solutions
Required assistance from organic solvents (DMSO, DMF, MeCN) to solubilize biomolecule(s) or linker(s)	Poor water solubility of payload molecules	• Use water soluble components to counteract or enhance solubility of linkers • Alter pH or reactant concentrations (dilute conditions)
Conjugating biomolecules without degradation or aggregation	Fundamental differences in properties or stability of reactants	• Adjust reaction environment (pH, ionic strength, or solvent composition), decrease temperature, decrease reaction time • Addition of stabilizing excipients • Use gentle agitation (end-over-end rotator rather than stirring)
Purification of biomolecules conjugated via CuAAC require additional considerations to remove trace Cu to allow for biocompatibility	Cu is difficult to remove via standard purification methods due to sequestering by biomolecules	• Use chelators such as EDTA to scavenge Cu at the end of the reaction • Use chromatographic separation methods or dialysis in conjunction with chelators • Use Cuprisorb with small molecules but not with biomolecules
Loss of activity of bioconjugates made via CuAAC	CuAAC reaction conditions may lead to side reactions such as oxidation of amino acids or small molecules, or reduction of cysteines	• Use Cu chelating ligands such as THPTA or TBTA to limit degradation • Use aminoguanidine to act as an arginine surrogate to limit degradation • Pre-incubate with iodoacetamide to inhibit cyclooctyne compounds from reacting with cysteine residues
Poor conjugation of biomolecules onto high MW polymers	Lower accessibility to reactive sites due to higher viscosity and potential folding of larger polymers	• Add sufficient denaturant such as DSMO to promote accessibility of reactive sites • Alter pH or ionic strength to disrupt intramolecular interactions • Use more dilute reaction conditions, or increase temperature or reaction time