Bioconjugate Chemistry

Review

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Site-Specific Antibody–Drug Conjugates: The Nexus of Bioorthogonal Chemistry, Protein Engineering, and Drug Development

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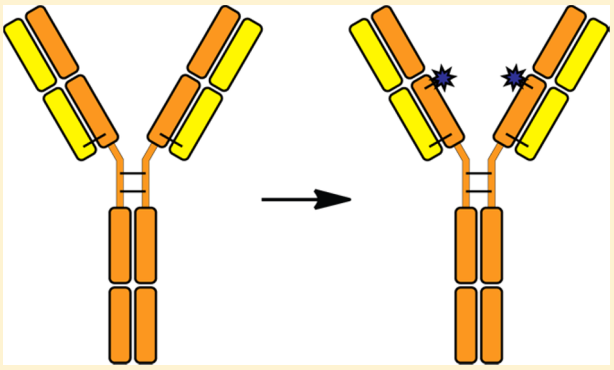
ABSTRACT: Antibody–drug conjugates (ADCs) combine the specificity of antibodies with the potency of small molecules to create targeted drugs. Despite the simplicity of this concept, generation of clinically successful ADCs has been very difficult. Over the past several decades, scientists have learned a great deal about the constraints on antibodies, linkers, and drugs as they relate to successful construction of ADCs. Once these components are in hand, most ADCs are prepared by nonspecific modification of antibody lysine or cysteine residues with drug-linker reagents, which results in heterogeneous product mixtures that cannot be further purified. With advances in the fields of bioorthogonal chemistry and protein engineering, there is growing interest in producing ADCs by site-specific conjugation to the antibody, yielding more homogeneous products that have demonstrated benefits over their heterogeneous counterparts *in vivo*. Here, we chronicle the development of a multitude of site-specific conjugation strategies for assembly of ADCs and provide a comprehensive account of key advances and their roots in the fields of bioorthogonal chemistry and protein engineering.



I. INTRODUCTION

Over the course of the twentieth century, the development of chemotherapeutic agents for cancer treatment generally followed the trend of enhancing drug cytotoxicity. Eventually, the strategy of searching for more toxic small molecules yielded diminishing returns: today's most potent classes of small molecules, such as tubulin polymerization inhibitors and DNA alkylators, are often too toxic to be of use as pharmaceuticals; in other words, their lack of tumor selectivity limits the doses at which they can be administered to levels below those that would show efficacy.[1] In order to improve the therapeutic index (the ratio of toxic dose to effective dose) of these toxins, much focus has been directed toward targeting drugs specifically to tumor cells.[2,3] Antibody–drug conjugates (ADCs) represent the recent pinnacle of such targeting efforts.

ADCs comprise an antibody specific for an antigen that is either uniquely expressed or overexpressed on cancer cells as well as a potent cytotoxin that, on its own, would not be tolerated systemically. The concept of using an antibody as a drug targeting moiety seems straightforward, but it has been difficult to execute. Research on ADCs began nearly 50 years ago,[4] but there have been few clinical successes: gemtuzumab ozogamicin (Mylotarg) was approved by the FDA for treatment of acute myeloid leukemia in 2000, but it was withdrawn from the US market 10 years later due to concerns about safety and lack of efficacy.[5,6] Since then, just two other ADCs, Seattle Genetics's brentuximab vedotin (Adcetris) and Genentech and Immunogen's trastuzumab emtansine (Kadcyla), have been approved, in 2011 and 2013, respectively.

An ADC has three basic components: an antibody, a cytotoxic drug, and a linker between the two. In developing ADCs, scientists have learned a great deal about the constraints on each of these components. The antibody must be highly specific and should be humanized to minimize immunogenicity. Additionally, the targeted antigen should be highly expressed on the surface of tumor cells in copy numbers sufficient to enable delivery of a cytotoxic dose, and it must internalize via a mechanism that permits drug access to its intracellular target. The drug must be extremely potent, and its physical properties must allow the attachment of several molecules to the antibody without inducing aggregation of the ADC. Finally, the linker must help to solubilize the often-hydrophobic drug (a molecule containing the drug and linker is typically prepared and then conjugated to an antibody under aqueous conditions), must be stable during circulation so that the drug is not prematurely released, and, depending on the application, may require intracellular cleavage to facilitate release of the free drug.[7]

Much work has been directed toward optimizing these details of ADC composition and manufacturing over the last 50 years. However, until the past decade, the fundamental conjugation

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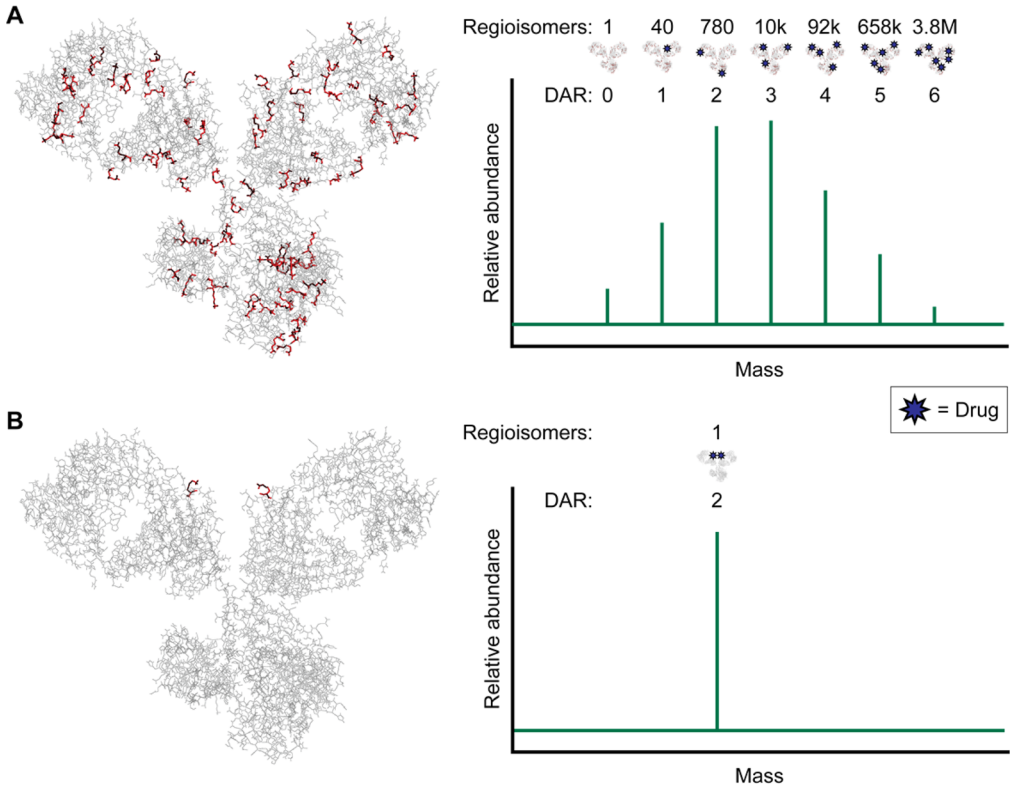

Figure 1. Potential sites of modification and theoretical product distributions for lysine-conjugated and site-specifically conjugated antibodies. (A) All lysine residues of a human IgG1 are highlighted in red, indicating potential sites of conjugation with activated esters. The number of regioisomers is calculated based on 40 reactive lysine residues. (B) A site-specifically modifiable antibody with one conjugation site on each heavy chain highlighted in red; a fully conjugated antibody has a DAR of 2. PDB ID: 1IGY.

chemistries used to connect the protein and drug-linker moiety remained essentially unchanged. Nonspecific acylation of lysine residues with activated esters and alkylation of cysteine thiols with maleimides are two classic bioconjugation methods that were used to make the first generation of ADCs. For example, both Mylotarg and Kadcyla are produced by nonspecific conjugation to lysine residues,[8,9] whereas Adcetris is constructed by alkylation of cysteine thiols that are exposed by prior reduction of the conserved hinge region disulfide bonds.[10] These conjugations are problematic, however, because they produce products that are heterogeneous in two regards illustrated here for lysine conjugation (Figure 1A):[11] first, the product population contains conjugates with a variable drug-to-antibody ratio (DAR). Second, any two conjugates with the same DAR are likely regioisomers, as many surface-accessible lysine residues (as well as the N termini of the light and heavy chains) are potential candidates for modification. For example, one study found that modification of an IgG with an *N*-hydroxysuccinimide ester reagent yielded a population of products with DARs ranging from 0 to 6 in which at least 40 of the 86 lysine residues in the antibody were modified to some degree; such a mixture potentially contains over 4.5 million unique molecules.[12] The eight hinge region cysteine residues offer less potential heterogeneity for maleimide conjugation methods, but, in practice, a distribution of multiple DARs is observed after the chemistry.[13]

Given the outcome of conventional bioconjugation methods, the clinically approved ADCs had to be developed and administered as heterogeneous mixtures. Even though the underlying protein scaffold remains constant in a heterogeneous population of ADCs, each conjugate has its own set of properties with respect to important parameters such as pharmacokinetics, toxicity, aggregation, antigen affinity, and drug release. This realization led several groups to contemplate the potential benefits of achieving site-specific conjugation of drug to antibody, wherein both the modification sites and DARs could be more precisely controlled. A drug that comprises one molecule (Figure 1B) instead of a mixture of thousands or millions could presumably be more easily optimized, manufactured, and developed through the clinical pipeline with fewer regulatory hurdles.

Although the rationale for site-specific conjugation in ADC production has long been apparent, most methods for achieving this goal have reached fruition only in recent years.[14,15] In general, site-specific modification first requires the use of a protein engineering method to endow the desired conjugation site with unique reactivity.[16] This has been achieved by engineering ultrareactive cysteine residues, introducing sugars or unnatural amino acids containing bioorthogonal functional groups, or by genetic encoding of peptide tags for further enzymatic or chemical modification. The appropriately tagged antibody is then reacted with a drug-linker molecule using a bioorthogonal transformation[17,18] or enzymatically conjugated to a drug-functionalized substrate. Many of these methods have now been commercialized and should be represented in clinical pipelines within the next decade.

Here, we review site-specific protein conjugation technologies that show promise for the production of next-generation ADCs (Table 1). For a broader view of the evolution of ADCs over the last few decades, we direct readers to an excellent recent review.[1]

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Table 1. Summary of Methods for Construction of Site-Specific ADCs

category	pros	cons	specific method	comments
cysteine conjugation	fast conjugation reactions; minimal structural perturbation	requires prereduction and may require reoxidation	global reduction/alkylation	DAR of 4 or 8 depending on linker chemistry
			cysteine to serine mutation	DAR of 2, 4, or 6
			THIOMAB	maleimide conjugates unstable, but newer chemistries exist
			N-terminal cysteine conjugation	oxazolidine conjugates intentionally unstable
glycoconjugation	no protein engineering required	site of modification is immutable; DARs tend to be lower due to glycan heterogeneity	periodate oxidation of fucose or sialic acid	methionine oxidation may be problematic
			enzymatic transfer of azidosugars	asymmetric cyclooctynes yield two regioisomers
			metabolic incorporation of thiofucose	DAR limited by metabolic incorporation efficiency
unnatural or noncanonical amino acid incorporation	minimal structural perturbation; potentially enables wide variety of bioorthogonal ligation reactions	technically complicated	amber codon suppression	aryl oxime ligation is slow
			cell-free amber codon suppression	can quickly screen variants; generates aglycosylated antibody
			selenocysteine incorporation	mostly conjugates with DAR of 1; C-terminal incorporation
peptide tags	minimal off-target reactivity; operationally simple	enzymatic conversion efficiency is site-dependent	deglycosylation followed by transglutaminase treatment	generates aglycosylated antibody
			transglutaminase tag	DAR of 2 or 4
			sortase tag	must be placed near C terminus
			aldehyde tag	conjugate stability requires Pictet–Spengler or alternative ligation

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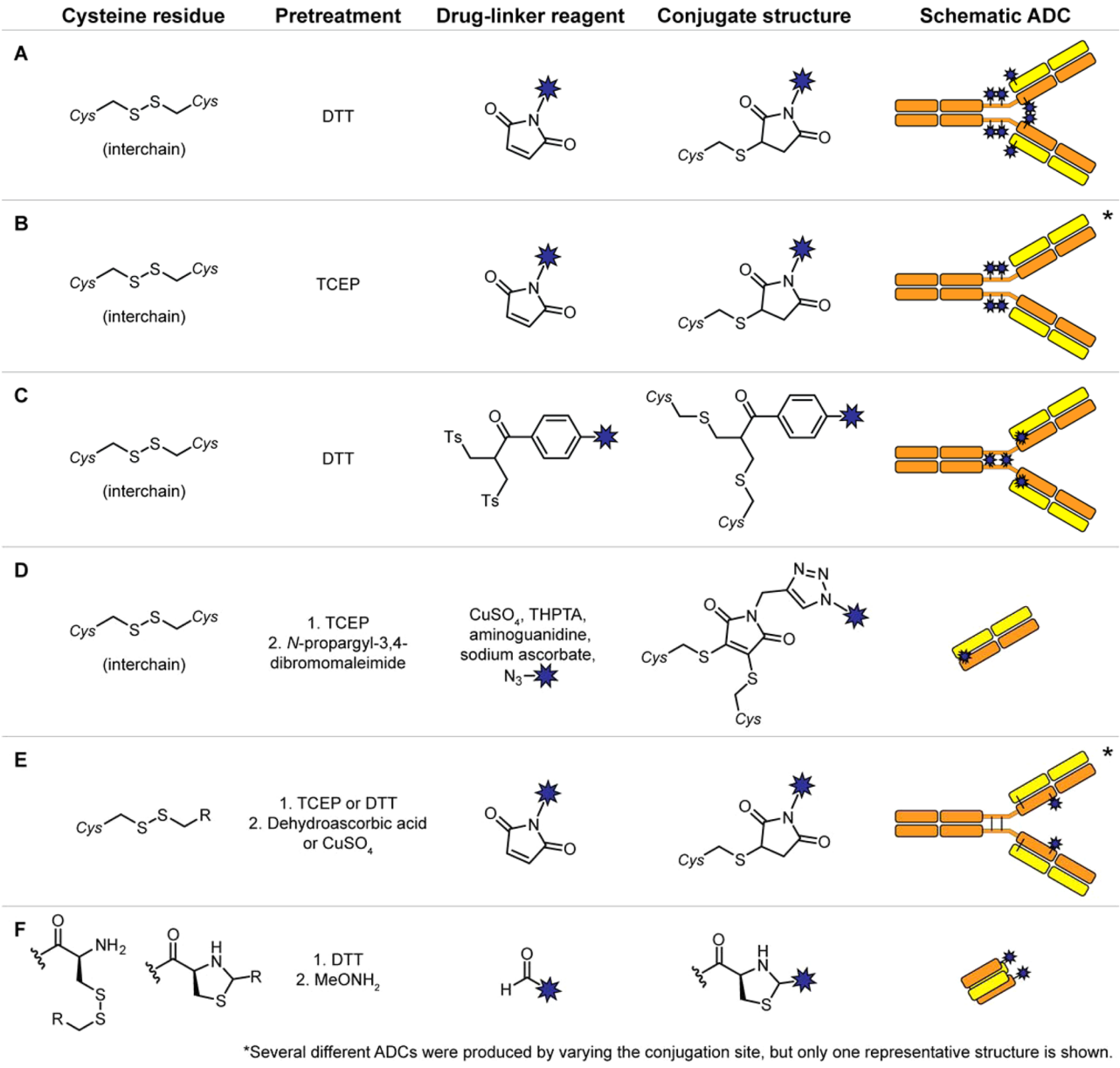

Figure 2. Methods for site-specific ADC production based on cysteine conjugation. (A) Reduction and alkylation of all interchain cysteine disulfides. (B) Reduction and alkylation of interchain cysteine residues on antibodies containing several cysteine-to-serine mutations. (C) Reduction and bridging alkylation of interchain cysteine residues with bis-sulfone linkers. (D) Reduction and bridging alkylation of interchain cysteine residues with propargyldibromomaleimide, followed by Cu-click ligation. (E) Reduction of cysteine residues followed by reoxidation of interchain disulfides and selective alkylation to produce THIOMABs. (F) Reductive and nucleophilic deblocking of N-terminal cysteine residues on a diabody followed by thiazolidine ligation. Abbreviations: DTT, dithiothreitol; TCEP, tris(carboxyethyl)phosphine; THPTA, tris(3-hydroxypropyltriazolylmethyl)amine.

II. CONJUGATION TO INTERCHAIN OR ENGINEERED CYSTEINE RESIDUES

A classic method for site-specific protein modification is to take advantage of a uniquely reactive cysteine residue on the protein's surface. While most proteins do not have reduced solvent-accessible cysteine residues, they can be engineered into the protein or generated by reduction of native disulfide bonds. The resulting cysteine residue selectively reacts with a variety of suitable electrophiles, with maleimide, haloacetamide, and disulfide reagents being the most common.

One way of obviating the heterogeneity that results from linker attachment at a variable subset of amino acid residues is to drive the reaction to completion, resulting in an ADC with all instances of a particular amino acid functionalized. Unlike many of the newer methods for production of site-specific ADCs, this method has the advantage that it does not require protein engineering and is, in principle, simple to execute. A typical IgG has far too many lysine residues for such an approach to be possible, but reduction of interchain disulfide bonds followed by conjugation to the resulting eight cysteine residues is more tractable (an IgG also has 12 intrachain disulfide bonds, but selective reduction of the four interchain disulfide bonds while keeping the IgG intact is straightforward). This approach was originally used by Trail, Firestone, and co-workers at Bristol-Myers Squibb to prepare a maleimide-linked doxorubicin conjugate of BR96, an antibody against the tumor antigen Lewis Y (Figure 2A).[19,20] The BR96–doxorubicin conjugate was tested in Phase II clinical trials, but gastro-

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intestinal toxicity that may have resulted from off-target antibody binding prevented further clinical use.[21]

Senter and co-workers from Seattle Genetics used this reduction−alkylation approach to prepare maleimide-linked auristatin conjugates of antibodies against Lewis Y and CD30 with a DAR of 8.[22] A follow-up study used a procedure of partial disulfide reduction followed by preparative chromatography to isolate antibody populations with DARs of exactly 2 or 4. Evaluation of the performance of these ADCs *in vivo* showed that antitumor activities of ADCs with DARs of 4 or 8 were equivalent at equal antibody doses but that maximum tolerated dose (MTD) and circulation half-life were inversely related to DAR.[23] The rapid *in vivo* clearance of the conjugate with DAR 8 may have been due to its hydrophobicity,[10] but whatever the cause, after a certain point a higher DAR was detrimental to the performance of the ADC. Additionally, ADCs with such high DARs are more prone to aggregation due to the hydrophobicity of many commonly used small molecule toxins (although the authors noted that it was not problematic with their auristatin E conjugates), potentially limiting the generality of the method of driving the interchain cysteine conjugation reaction to completion.

Given the notion that decreasing DAR might yield similarly potent ADCs with better therapeutic indices, Seattle Genetics next explored two strategies to simplify production of cysteine-alkylated ADCs with a lower DAR. The first method involved partial reduction of cysteine residues with either dithiothreitol (DTT) or tris(carboxyethyl)phosphine (TCEP) or full reduction and partial reoxidation with Ellman's reagent followed by conjugation to maleimido-auristatins.[13] Analysis of the product distributions from these reactions showed that each method yielded a distinct mixture of ADCs, with the reoxidation method affording more conjugates with a DAR of 4 at the expense of higher DARs. While this partial conjugation strategy could be used to skew the product distribution toward certain sites and/or DARs, it still produced mixed populations of ADCs with DARs of 0, 2, 4, 6, and 8. The second method to achieve lower DAR involved mutating some of the hinge cysteine residues to serine residues (Figure 2B).[24] Previous work had shown that $F(ab')_2$ variants lacking disulfides between their heavy and light chains could be assembled biosynthetically, remained intact in serum, and bound their epitopes with affinities as high as those of disulfide-bonded variants.[25] As expected, the cysteine-to-serine mutations in anti-CD30 did not negatively impact binding affinity, aggregation, or conjugate stability *in vitro*. However, empirically, there was no obvious advantage to the site-specific conjugates over a heterogeneous mixture with DARs ranging from 0 to 8: data comparing ADCs with 4 site-specifically linked drugs to those with an average of 4.4 heterogeneously linked drugs showed similar antitumor activities in mouse xenograft models and similar MTDs in rats. In the words of the authors, the main lesson from these studies was that for their conjugates, "the stoichiometry of drug attachment is a more critical determinant of antibody−drug conjugate potency and tolerability than is the site of drug attachment and conjugate homogeneity". This conclusion led Seattle Genetics to develop its anti-CD30 ADC functionalized with monomethyl auristatin E (MMAE), brentuximab vedotin (Adcetris), as a mixture of conjugates, albeit a relatively controlled one.

Given the desire to achieve low DARs from conjugation at interchain disulfides, Brocchini and co-workers devised a strategy to use each disulfide as a single point of attachment.[26] They found that treatment of interferon α2b, which contains two disulfide bonds, with a bis-sulfone reagent that generates a Michael acceptor *in situ* yielded tethered thioether products with a three carbon atom spacer. The technology has successfully been used for conjugations to Fab fragments as well.[27] Godwin and co-workers from PolyTherics subsequently attached MMAE to these bis-sulfone reagents to prepare trastuzumab−MMAE conjugates with DARs close to 3 (Figure 2C).[28] Importantly, these conjugates did not suffer from the instability inherent to the equivalent maleimide conjugates (see below), likely due to the dual sites of attachment. In an alternative strategy, Haddleton and co-workers used dibromomaleimides to covalently tether disulfides via a two carbon atom spacer.[29] Subsequently, Caddick, Boyle, and co-workers showed that treatment of a reduced Fab fragment with an *N*-propargyldibromomaleimide quickly yielded clickable conjugates with one point of attachment in high conversion (Figure 2D).[30] The authors of this study used Cu-click chemistry to assemble the requisite triazole linkage between the antibody and drug-linker, but the field has generally avoided Cu-click reactions on ADCs because of Cu-mediated generation of reactive oxygen species and oxidized reaction byproducts such as dehydroascorbate, both of which can react with proteins.[31,32] Adapting the dibromomaleimide to be compatible with Cu-free click chemistry or another bioorthogonal ligation would be straightforward. One potential advantage of the dibromomaleimide and bis-sulfone linkers is that they provide a stable covalent linkage between the heavy and light chains, unlike methods relying on alkylation of individual cysteine residues in which the heavy and light chains remain noncovalently associated; empirically, however, no important differences in stability have been observed.

Introduction of surface-accessible cysteine residues into an IgG was one of the first strategies used to prepare conjugates site-specifically. Owens and co-workers from Celltech (now part of Union Chimique Belge, UCB) prepared serine- or threonine-to-cysteine mutants at one of five sites on the CH1 domain of an antibody against the tumor-associated glycoprotein TAG72.[33] Assaying for cysteine sulfhydryl content revealed anywhere from 0.1−1.1 free thiols per mutant out of an expected 2. The blocked cysteine residues, which were not characterized in this study, likely existed as disulfides with cysteine or glutathione. Cysteine residues positioned at convex or flat parts of the antibody surface were more likely to be blocked than those in concave environments. While the authors could successfully conjugate the free cysteine residues, the resulting products were not homogeneous because the starting material existed as a mixture of antibodies with 0, 1, or 2 free sulfhydryl groups. Attempts to unblock the oxidized cysteine residues with small molecule reductants were hampered by inadvertent reduction of the hinge region disulfide bonds. A more recent study by Stimmel and co-workers employing a serine-to-cysteine mutant in the CH3 domain of several IgGs found that mercaptoethylamine could deblock the desired cysteine residue more selectively than stronger reductants such as DTT.[34] While this was a significant improvement, analysis of protease digests still showed that approximately 10−20% of a bromoacetamide conjugate was not associated with the desired peptide. Aside from incomplete or off-target conjugation, other complications can arise from introduction of cysteine residues depending on the insertion site. For example, a S444C mutation in the CH3 domain of an IgG yielded tail-to-tail linked IgG dimers,[35] and a S119C mutation in the CH1 domain

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yielded a compact, inflexible IgG as a result of an extra disulfide bond between the two heavy chains.[36]

In 2008, Junutula, Mallet, and co-workers at Genentech disclosed homogeneous ADCs prepared by conjugation to IgGs containing an unusually "hot" cysteine residue whose position they identified using phage display methods.[37,38] These ADCs, which they dubbed THIOMABs, were produced by global reduction of blocked cysteine residues and interchain disulfides, subsequent oxidation in the presence of $CuSO_4$ or dehydroascorbic acid to regenerate the interchain disulfide bonds, and then conjugation of the reactive cysteine thiol to maleimide reagents (Figure 2E). This method generated site-specifically modified ADCs with a DAR of 2.0 in high purity. The Genentech group went on to show that, in two mouse tumor xenograft models, an anti-MUC16-MMAE conjugate was at least as effective as a conventional thiol-conjugated ADC with approximately twice the DAR (produced with Seattle Genetics's reduction−alkylation approach, resulting in a range of DARs from 0−8). Importantly, the THIOMAB conjugate displayed a larger therapeutic index, as it was tolerated at much higher doses in animals. Further analysis showed that the THIOMAB was cleared from circulation in rats more slowly than the conventional ADC. The THIOMAB's cysteine−maleimide linkage also exhibited better serum stability than that of the conventional ADC. This 2008 study highlighted both the feasibility and benefits of site-specific antibody−drug conjugation and marked the beginning of a flurry of activity to produce site-specific ADCs.

One hypothesis for the improved *in vivo* stability of THIOMABs relative to nonspecific cysteine−maleimide conjugates was that the high solvent accessibility of the latter linkages may have facilitated their decomposition. In an effort to explore whether the conjugation site could modulate the stability of a cysteine−maleimide conjugate, a team from Genentech prepared three THIOMABs of trastuzumab in which the introduced cysteine residue was at a site with high solvent accessibility or a site with intermediate solvent accessibility and either positive or neutral charge.[39] Tracking the fate of the drug or fluorophore cargo after incubation in plasma showed stark differences in the stabilities of the conjugates: the solvent-accessible conjugate quickly underwent thiol exchange with serum albumin, the conjugate at a positively charged site underwent a succinimide hydrolysis reaction, resulting in improved stability, and the conjugate at a neutral site exhibited either behavior. Although it was known that succinimidyl thioethers could undergo ring-opening hydrolysis reactions,[40] such a thorough analysis on their stability had not previously been performed, particularly as it related to local protein environment and thiol exchange. With the growing realization that cysteine−maleimide conjugates are susceptible to decomposition, research on cysteine conjugation has yielded several new reaction partners in recent years,[41−43] including a self-hydrolyzing maleimide containing an amine that promotes an irreversible hydrolysis reaction to form a stable amide conjugate.[44]

The success of the THIOMAB reduction/reoxidation procedure has led others to use it as well. For example, Jeffrey and co-workers at Seattle Genetics recently reported an anti-CD70−pyrrolobenzodiazepine linked through engineered cysteine residues.[45] Initial attempts to prepare conjugates with reduced hinge cysteine residues resulted in significant ADC aggregation because of the combination of higher DARs and hydrophobicity of the pyrrolobenzodiazepines, but employing a method that limited the DAR to 2 solved the problem.

Cysteine conjugation chemistries have also been employed in "traceless" ADC-like molecules, wherein release of the drug yields a native protein with no chemically altered side chains that might provoke an immune response. Neri and co-workers have pursued this strategy using engineered antibody fragments that offer improved tissue penetrance while retaining the targeting ability of antibodies.[46] This group has used two less-conventional strategies for cysteine conjugation: thiazolidine ligation and disulfide modification. To install the 1,2-aminothiol necessary for a thiazolidine ligation, a cysteine residue was placed immediately downstream of the signal peptide cleavage site of a diabody (an scFv dimer), affording an N-terminal cysteine residue in the secreted protein (Figure 2F).[46] Alternatively, C-terminal cysteine residues could be treated with a maleimide reagent containing an aminothiol masked as its formaldehyde-derived thiazolidine. Following deblocking, conjugation of the aminothiol-containing proteins to an aldehyde-functionalized cematodin derivative provided site-specifically modified diabodies, although the reaction required up to 4 days at pH 4.5 to proceed. The thiazolidine linkages exhibited half-lives of roughly 2 days in PBS at 37 °C. While the field has generally moved away from linkers that provide slow nonspecific cleavage of the ADC (such as the hydrazone linker used to prepare Mylotarg) due to concerns about toxicity arising from premature drug release, the rationale for engineering a half-life into the linker here was to use it with antibodies targeting poorly internalized antigens. With the advent of a system to ribosomally incorporate an unnatural amino acid containing a masked 1,2-aminothiol that can be deprotected without disrupting native disulfide bonds,[47] the thiazolidine ligation could also be useful for modification at interior sites on an antibody; alternatively, the 1,2-aminothiol moiety could be treated with a cyanobenzothiazole reagent to form a more stable linkage.[48] In a subsequent study, the Neri group engineered cysteine residues at the C terminus of each CH4 domain of a small immune protein.[49] To conjugate the cysteine residues in a selective and traceless fashion, an umpolung strategy was employed: following selective reduction with TCEP, the C-terminal cysteine residues were oxidized with Ellman's reagent. Treatment of the resulting electrophilic disulfides with thiol-functionalized cematodin afforded the site-specifically modified proteins in high conversions and with short reaction times.

Cysteine conjugation is the testing ground on which much of our current knowledge about site-specific ADCs was built. In coming years, cysteine will continue to be a popular target for conjugation based on the new generation of cysteine-specific chemistries that promise to fix the problem of maleimide exchange. Cysteine alkylation reactions are typically quite fast, making these conjugation schemes an appealing choice.

III. CONJUGATION TO GLYCANS

Human IgG molecules have a conserved glycosylation site at each N297 residue in the CH2 domain. The inherent site-specificity of this post-translational modification and decades of research on glycoengineering make the pendant N-glycans a convenient target for site-specific conjugation. Additionally, the glycosylation site is sufficiently far from the variable region that conjugation to attached glycans is unlikely to impact antigen binding. However, glycosylation is a heterogeneous post-translational modification, rendering the generation of homo-

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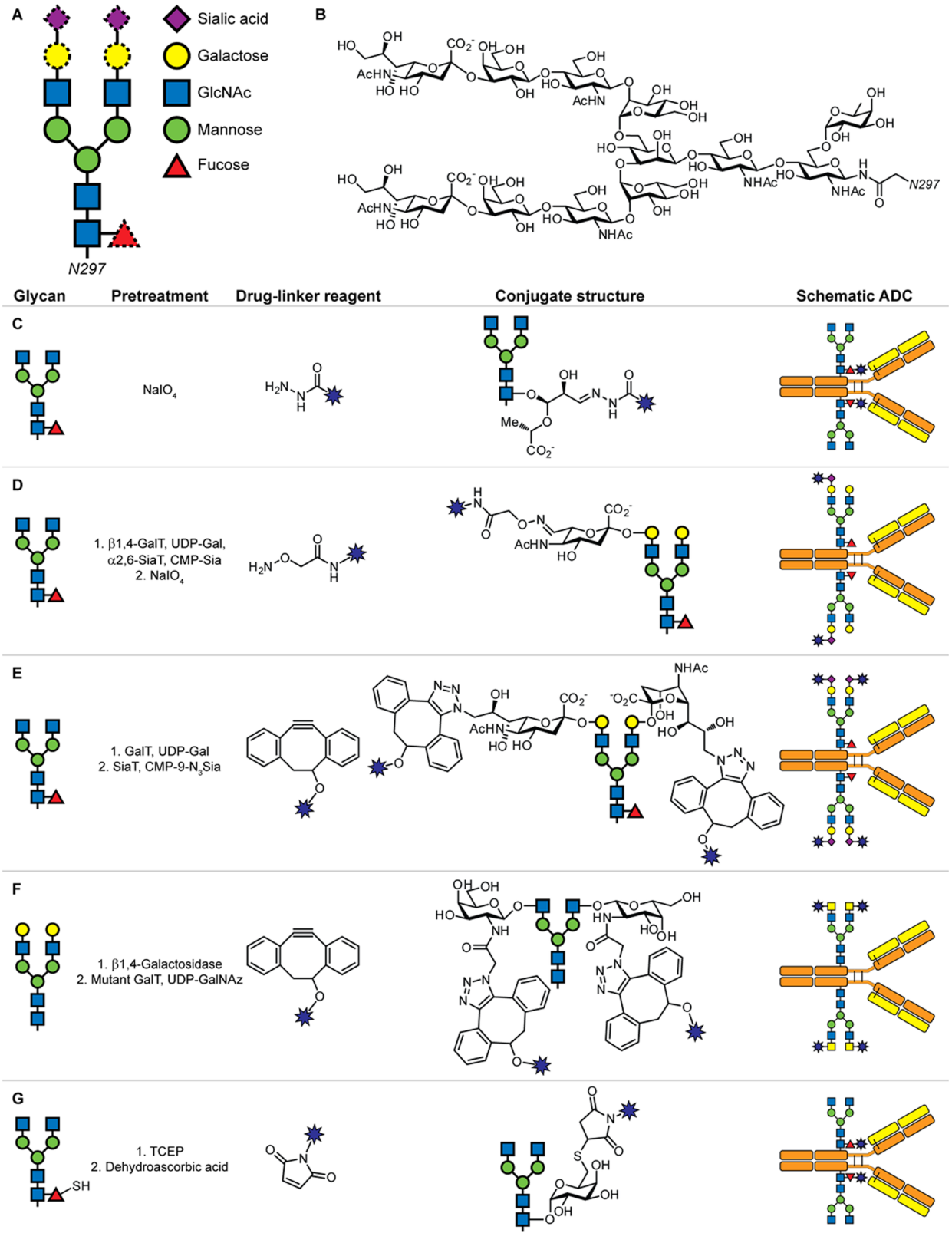

Figure 3. Methods for chemical conjugation on the N-glycan of IgG. (A) Schematic structure of glycans found at N297 of recombinantly expressed IgG. Dashed lines indicate partial occupancy. (B) Chemical structure of a fully elaborated complex-type N-glycan. (C) Periodate oxidation of fucose followed by hydrazone condensation. (D) Enzymatic transfer of galactose and sialic acid followed by periodate oxidation and oxime condensation. (E) Enzymatic transfer of galactose and 9-azidosialic acid followed by Cu-free click reaction. (F) Enzymatic removal of terminal galactose followed by enzymatic transfer of GalNAz and Cu-free click reaction. (G) Metabolic incorporation of 6-thiofucose followed by maleimide conjugation. Abbreviations: GalT, galactosyltransferase; SiaT, sialyltransferase; 9-N_3Sia, 9-azidosialic acid; GalNAz, *N*-azidoacetylgalactosamine.

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geneous glycans for chemical modification a formidable challenge. A variety of N-linked biantennary complex glycan structures can be found on serum-derived IgG; the core heptasaccharide containing *N*-acetylglucosamine (GlcNAc) and mannose can be modified with fucose and bisecting GlcNAc (although the latter is not observed on N-glycans expressed in cell lines such as CHO, NS0, and Sp2/0), as well as galactose and sialic acid on each of the antennae (Figure 3A,B).[50] Site-specific ADC production methods based on glycoconjugation often incorporate treatments to reduce glycan heterogeneity prior to the chemistry, but such efforts are generally imperfect, which can result in low DARs in the final products.

Glycans contain vicinal diol moieities that can be oxidatively cleaved with periodate to generate aldehydes. A large body of literature has examined the efficiency of periodate oxidation of antibodies and other glycoproteins,[51] as reductive amination with protein lysine residues has been a common method to produce protein−protein conjugates since its introduction in the 1970s.[52] Aldehydes produced via periodate oxidation constituted the first widely used bioorthogonal functional group, enabling reductive amination as well as conjugation to small molecule hydrazide and aminooxy compounds.

In 1984, in what was perhaps the first attempt to site-specifically modify an antibody with a small molecule, O'Shannessy and co-workers used the periodate oxidation method to prepare IgG and IgM conjugates with biotin hydrazide.[53] Several subsequent studies used similar methods to prepare site-specifically radiolabeled antibodies.[54,55] While the authors showed that their oxidized and conjugated antibodies did not suffer from diminished affinity compared to that of unconjugated antibodies, the high concentration of periodate employed (10−30 mM) may have resulted in a heterogeneous population of aldehydes on the glycans as well as oxidation of methionine residues.[56]

Recent efforts to produce glycan-linked ADCs via periodate oxidation have included more thorough characterization of the reaction products. Neri and co-workers expressed an IgG in CHO cells, which, under their culture conditions, resulted in a very high occupancy of fucosylation at the core GlcNAc residue.[57] By treating the resulting fucosylated IgG with 10 mM sodium periodate, they were able to oxidize the fucose residues to a new moiety containing a carboxylic acid (from over-oxidation) and an aldehyde. The aldehyde was used to prepare hydrazone conjugates with fluorophores and a dolastatin analogue (Figure 3C).[57]

Compared to other monosaccharides, sialic acid can be oxidized under relatively mild conditions. It is unique among the nine mammalian glycan building blocks in that it contains vicinal diols in the form of a glycerol moiety that is relatively unhindered and flexible compared to those found on the other monosaccharides. However, sialic acid is incorporated into antibodies at low levels: it is present in 25% of polyclonal IgG in human serum[58] and in less than 5% of antibody glycans in cell lines commonly used for antibody expression.[59] Thus, Zhou and co-workers from Sanofi-Genzyme used enzymatic means to install sialic acid, demonstrating that treatment of trastuzumab or two other IgGs with a mixture of β1,4-galactosyltransferase and α2,6-sialyltransferase as well as their respective donor substrates UDP-galactose and CMP-sialic acid yielded products with monosialylated glycans.[60] These glycans were then oxidized with 1 mM periodate and conjugated to aminooxy-drugs to form oxime-linked conjugates with DARs between 1.3 and 1.9 (Figure 3D). The oxidized trastuzumab bound the neonatal Fc receptor (FcRn) with diminished affinity compared to that of the unmodified antibody, suggesting that oxidative protein damage may have occurred. This hypothesis was confirmed by an analysis of tryptic peptides showing partial oxidation of two methionine residues involved in FcRn binding upon periodate treatment. ADCs with diminished FcRn binding could exhibit shorter half-lives *in vivo*.

Using a sialyltransferase to incorporate a modified sialic acid residue containing a bioorthogonal functional group would obviate the need for periodate treatment. Many sialyltransferases are known to exhibit relaxed substrate specificity for substitutions at the N-acetyl group and the C9 position, which is normally substituted with a primary hydroxyl group.[61] Boons and co-workers recently exploited the substrate tolerance of the commercially available sialyltransferase ST6Gal1 to incorporate a sialic acid derivative modified with an azide at the C9 position into an IgG.[62] After incubating the antibody with a galactosyltransferase and a sialyltransferase as well as their respective donor sugars, a cyclooctyne-conjugated fluorophore or drug was clicked on to the glycan (Figure 3E). Analysis of the azide-functionalized antibodies showed a ratio of 4−4.5 azides per antibody, with some glycosyltransferase-dependent conjugation unexpectedly occurring on the light chain. Given the permissive nature of the sialyltransferase, this approach could also be used in conjunction with sialic acids containing bioorthogonal functional groups other than the azide.

Work on using glycosyltransferases to introduce clickable monosaccharides into proteins dates to 2002, when Qasba and co-workers at NIH designed mutants of a β1,4-galactosyltransferase containing an enlarged sugar-binding pocket.[63] Whereas the wild-type enzyme showed poor catalytic efficiency with substrates larger than galactose, the best mutant efficiently appended the larger sugar *N*-acetylgalactosamine (GalNAc) to GlcNAc. Subsequently, the Hsieh-Wilson group showed that the mutant enzyme could transfer analogues of galactose containing a ketone or azide (*N*-azidoacetylgalactosamine, GalNAz) to GlcNAc-bearing substrates.[64,65] While these clickable galactose analogues were originally introduced to detect the presence of GlcNAc as a post-translational modification of serine and threonine residues, Qasba's group showed that the technology could also be used in conjunction with nonreducing GlcNAc residues for site-specific conjugations to antibodies.[66] This proof-of-concept experiment was followed by a more thorough study in which a cyclooctyne-functionalized radioisotope chelator was ligated to several GalNAz-functionalized antibodies.[67] These conjugates were prepared by galactosidase treatment of the antibody, addition of GalNAz in the presence of the mutant galactosyltransferase, and conjugation to the cyclooctyne-functionalized chelator (Figure 3F). Antibody conjugate preparations following this method yielded conjugates with an average of 3.3 triazole-linked products per antibody. The authors accounted for the lack of complete conjugation to tetrasubstituted products by making note of heterogeneity in the starting glycan structures (some native glycans may have been truncated or sialylated and thus not susceptible to the galactosidase treatment) and poor glycosyltransferase access to glycosylation sites due to steric hindrance. The biotechnology company Synaffix is also using a similar approach to ADC construction.[68]

An alternative to the above methods involving *in vitro* chemical or enzymatic modifications of native glycans is to glycoengineer the antibody as it is being expressed. This

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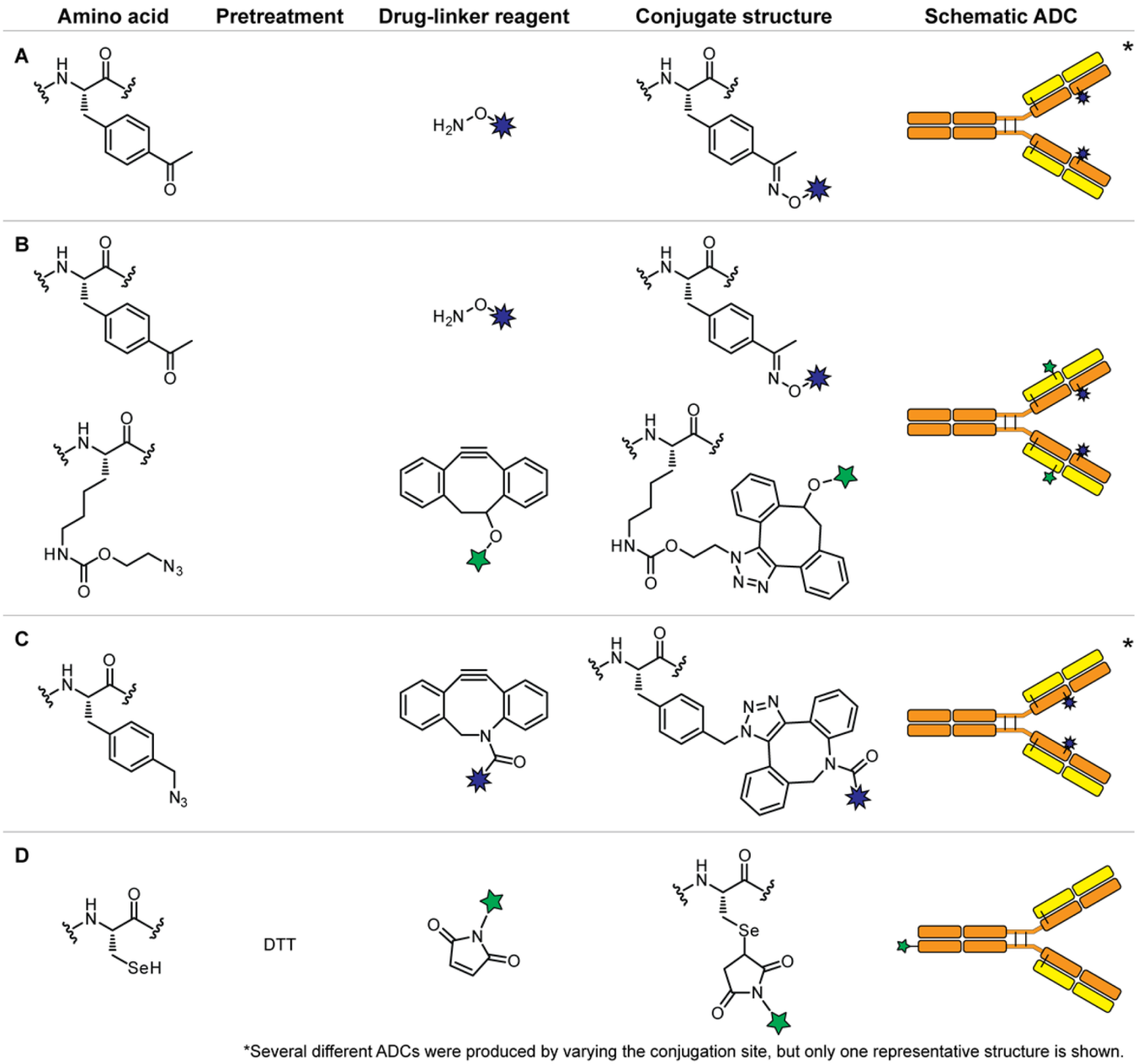

Figure 4. Conjugation methods based on UAA incorporation. (A) Incorporation of *p*-acetylphenylalanine followed by oxime condensation. (B) Simultaneous incorporation of *p*-acetylphenylalanine and an azido-lysine derivative followed by oxime condensation and Cu-free click chemistry to attach a fluorophore (green star). (C) Cell-free incorporation of *p*-azidomethylphenylalanine followed by Cu-free click chemistry. (D) Incorporation of selenocysteine followed by mild reduction and alkylation.

strategy is part of the larger field of metabolic oligosaccharide engineering, which has been used extensively to metabolically incorporate clickable or otherwise modified analogues of sugars into glycoproteins for applications in molecular imaging and proteomics or to modulate their functions.[61,69] Okeley and co-workers from Seattle Genetics used this approach to prepare antibodies with thiol-functionalized glycans for further modification.[70] During a screen of approximately 200 fucose analogues for inhibition of antibody fucosylation during expression,[71] some analogues were incorporated into antibody glycans at the usual fucosylation site. The observation that expression levels of fucose could be controlled metabolically led to the deliberate installation of 6-thiofucose, introduced in the cell culture medium in its peracetylated form for membrane permeability, as a clickable fucose analogue. At the employed concentration, 6-thiofucose was incorporated into 60−70% of glycans. Like surface-accessible thiols produced via insertion of cysteine residues, the thiols were present as a disulfide with free cysteine. Borrowing on the procedure developed to prepare THIOMAB conjugates, complete reduction of the antibody (including interchain disulfide bonds) followed by reoxidation and conjugation with a maleimide-linked MMAE yielded a conjugate with a DAR of 1.3 (Figure 3G). The thiofucose ADC displayed similar *in vitro* potency and superior plasma stability compared to that of a nonspecific ADC conjugated at interchain cysteine residues.

While these glycoconjugation methods have provided a straightforward means to achieve site specificity, one downside is the lack of control over site placement. So far, there are no data indicating that modification of the glycans at N297 is

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problematic, but other methods such as THIOMAB, unnatural amino acid incorporation, and peptide tag methods provide flexibility in the number of conjugation sites and their placement that glycoconjugation does not. In principle, one could introduce a new N-glycosylation site into the antibody, but, given the subtle factors governing whether a given NX(S/T) sequon is glycosylated and the large size of common N-glycans, such an approach would require careful engineering. Another complication associated with glycoengineering approaches is that the conjugates produced may be immunogenic. Antibodies against a variety of subtly modified glycans have been found in humans: for example, *N*-glycolylneuraminic acid, which differs from sialic acid by addition of a single hydroxyl group, acts as an antigen in humans,[72] and a variety of sialic acid glycoengineering studies have found that other unnatural sialic acids are immunogenic as well.[73]

IV. RIBOSOMAL INCORPORATION OF UNNATURAL AND NONCANONICAL AMINO ACIDS

Methods for ribosomal incorporation of unnatural amino acids (UAAs) into proteins provide an elegant solution to the problem of site-specifically engineering bioorthogonal functionality. In the most widely used method for UAA incorporation pioneered by Schultz, a mutant protein encoded by a gene with the amber stop codon (TAG) at the site of the desired UAA is expressed in cells, along with a corresponding orthogonal tRNA/aminoacyl-tRNA synthetase (aaRS) pair capable of installing the UAA at the amber stop codon site. This method for UAA incorporation has recently been reviewed.[74]

One of the first unnatural amino acids to be incorporated in *Escherichia coli* UAA expression systems was *p*-acetylphenylalanine, chosen for the bioorthogonal reactivity of its ketone.[75] Schultz, Smider, and collaborators at Ambrx have incorporated this amino acid into antibodies produced in mammalian cells.[76] Conjugation of an aminooxy-auristatin F to trastuzumab containing *p*-acetylphenylalanine provided oxime conjugates in high conversions (Figure 4A). The oxime conjugations in these studies proceeded for several days at pH 4−4.5, which is slow relative to other ligation strategies; however, many other small bioorthogonal functional groups have been introduced into proteins using amber suppression and could, in principle, be inserted into an IgG, so the slow reaction kinetics do not reflect an inherent limitation of the UAA technology. *In vitro* evaluation of a panel of three ADCs produced by oxime conjugation at various sites on the antibody showed no significant difference in activity in several HER2-positive cell lines. The site-specific ADCs exhibited a clearance rate in rats similar to that of unconjugated trastuzumab, in contrast to the faster rates of antibody clearance previously observed with nonspecifically conjugated ADCs when compared to those of their parent antibodies.[77] In collaboration with Pfizer, subsequent xenograft studies in mice using ADCs produced with this method showed that oxime-linked ADCs against HER2 and 5T4 were more effective against tumor growth than were nonspecifically cysteine-conjugated ADCs with twice the DAR. Additionally, conjugates containing a protease-cleavable linker at one of two sites exhibited different plasma stabilities, demonstrating that control over the site of modification is important in tuning the pharmacokinetic parameters of ADCs.[78]

Insertion of UAAs can be expanded to site-specifically incorporate more than one bioorthogonal functional group into the same protein, which would enable the preparation of well-defined theranostics or ADCs linked to toxins operating through different mechanisms. By simultaneously introducing two mutually orthogonal tRNA/aaRS pairs that insert unnatural amino acids in response to the amber and ochre stop codons in mammalian cells, the Schultz lab has demonstrated the ability to incorporate two different unnatural amino acids into one protein.[79] This system was used to prepare trastuzumab analogues containing *p*-acetylphenylalanine and an azide-functionalized carbamate derivative of lysine, which were then conjugated to an aminooxy auristatin and a cyclooctyne fluorophore reagent, respectively (Figure 4B).

One complication associated with expressing proteins containing UAAs in mammalian cells is that several exogenous components must work in concert to produce protein in high enough titers to be commercially viable. Additionally, amber suppression in mammalian cells such as CHO is complicated by a relatively high rate of amber stop codon usage, which likely leads to toxicity arising from unwanted amber readthrough products in the presence of charged tRNAs. Accordingly, antibody titers in CHO cells in the first ADC study from Ambrx were around 300 mg/L, which is relatively low compared to typical production scale titers of 2−5 g/L.[80] Since then, however, Ambrx has introduced a line of CHO cells named EuCODE that provides titers above 1 g/L.[78]

As an alternative to amber suppression in mammalian cells, Swartz and co-workers combined amber suppression with cell-free protein synthesis to optimize incorporation of several UAAs into model proteins.[81] In cell-free synthesis, a clarified *E. coli* lysate is used as the reaction medium in which UAA-containing proteins are expressed. Sato and co-workers from Sutro Biopharma recently described the optimization of this system for production of ADCs containing *p*-azidomethylphenylalanine (historically, other groups have incorporated *p*-azidophenylalanine, but the alkyl azide exhibits faster reaction kinetics and likely improves stability to reduction and photolysis).[82] An advantage of using cell-free synthesis relative to conventional cell culture expression is that a large number of conditions for protein expression can be quickly screened simultaneously in microtiter plates. This was demonstrated by expressing a 1760-member library of aaRS mutants to screen for optimal incorporation of *p*-azidomethylphenylalanine into green fluorescent protein, but, in principle, the workflow could be adapted to quickly screen many sites of UAA incorporation throughout an IgG. Expression of UAA-containing trastuzumab with the top 6 aaRS mutants from the screen followed by conjugation with a cyclooctyne-functionalized MMAF yielded conjugates with DARs of up to 1.9 that exhibited expected *in vitro* potencies (Figure 4C). In conjugates with lower DARs, the conjugation efficiency was likely lower due to the insertion of phenylalanine, tyrosine, or glutamine in place of the UAA by a lower-fidelity aaRS, although those mutants could not be detected by mass spectrometry. Importantly, proteins produced via cell-free synthesis are not glycosylated. Historically, aglycosylated antibodies have been avoided due to concerns about immunogenicity of the free N297 residue, increased susceptibility to aggregation, and poor pharmacokinetics; however, given the advancement of several aglycosylated antibodies into clinical trials, those concerns seem to have abated.[83]

Of all the methods used for site-specific ADC preparation to date, UAA incorporation offers the most flexibility. The ability to generate conjugates site-specifically based on a single amino acid mutation may prove to be valuable to researchers wishing

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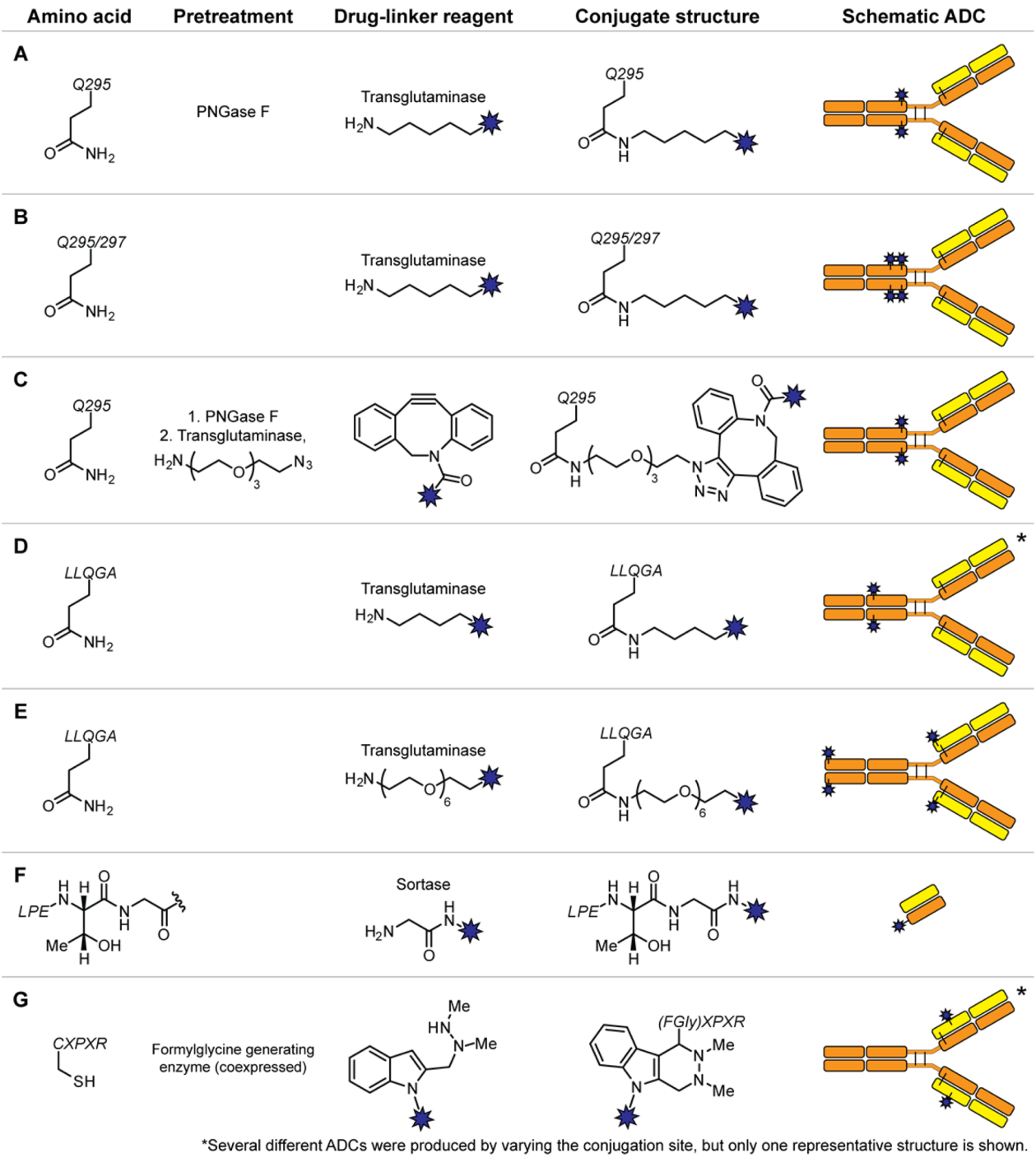

Figure 5. Conjugation methods based on enzymatic modification of peptide tags. (A) Glycosidase treatment for access to Q295 followed by transglutaminase-mediated conjugation of amine-functionalized small molecules. (B) Transglutaminase-mediated conjugation in an N297Q mutant at sites Q295 and Q297. (C) Glycosidase treatment followed by transglutaminase-mediated conjugation of an azido-PEG-amine and Cu-free click chemistry. (D) Transglutaminase-mediated conjugation of amine-functionalized drugs to an engineered LLQGA site or (E) several engineered LLQGA sites simultaneously. (F) Sortase-mediated conjugation of a glycine-functionalized chelator near the C-terminus of an scFv. (G) Formylglycine generating enzyme mediated conversion of cysteine to formylglycine followed by HIPS ligation. Abbreviations: PNGase, peptide N-glycosidase; FGly, formylglycine; HIPS, hydrazino-Pictet−Spengler.

to investigate structure−activity relationships (SAR) on ADCs. Additionally, the ketone and the azide are only two of many possible functional groups that could be used with these systems; Schultz, Chin, and others have shown that many functionalities can be incorporated into proteins, allowing the use of other bioorthogonal reactions.[84] The main obstacle to using UAAs to generate ADCs seems to be the initial investment required to express the requisite UAA-containing antibodies; in contrast, other methods can be used with native antibodies or those with just a few mutations.

An alternative to incorporating a UAA is to incorporate selenocysteine, a noncanonical amino acid that is found in 25 human proteins.[85,86] Selenocysteine is encoded by the opal stop codon (UGA) when in proximity to a selenocysteine insertion

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sequence in the 3′ untranslated region of an mRNA transcript.[87] Selenocysteine's nucleophilicity and low pK_a of 5.5 make it useful for protein conjugation chemistry under slightly acidic conditions, where competing nucleophiles such as cysteine and lysine are unreactive. Arnér and co-workers initially demonstrated the ribosomal incorporation of selenocysteine into nonselenoproteins.[88] Subsequently, Rader and co-workers at NIH used selenocysteine to prepare immunoconjugates by reacting an Fc fragment containing selenocysteine near its C terminus with a maleimide under mildly acidic conditions.[89] An important property of this platform is the moderate incorporation efficiency (20%) of selenocysteine into the protein. The majority of assembled Fc proteins containing selenocysteine are thus heterodimers of a selenocysteine-containing chain with an opal termination chain, resulting in the ability to attach one equivalent of small molecule per Fc (the presence of an affinity tag downstream of the opal codon allows selenocysteine-containing proteins to be purified away from homodimeric opal termination products). In a subsequent study, rituximab was used as a model for incorporation of selenocysteine near the C terminus of a full-length antibody (Figure 4D).[90] Expression of the full-length antibody yielded increased incorporation of selenocysteine (30−50%), resulting in a mixture of products containing one or two selenocysteine residues. Maleimide conjugation to selenocysteine did not negatively impact antigen affinity or significantly affect FcRn or Fcγ binding. Selenocysteine thus provides a chemical handle for reactions with electrophilic small molecules; so far, its incorporation into nonselenoproteins has been demonstrated only near the C terminus, but, in human proteins, it can be found upstream of α helices as well.[86]

V. MODIFICATION OF PEPTIDE TAGS

Following the success of green fluorescent protein fusion tags for fluorescence microscopy in the late 1990s, several efforts were pursued to minimize the size of fluorescent tags. These efforts resulted in fusions to progressively smaller proteins that were capable of selectively reacting with or binding small molecules, such as the SNAP tag and the TMP tag.[91] Proteins possessing highly selective reactivity or binding affinity cannot be miniaturized indefinitely, so the field next turned to peptide tags that allowed their carrier proteins to be enzymatically modified with small molecules. These types of enzymatic tagging methods were popularized in the mid 2000s by the Ting lab, which showed that biotin ligase,[92] transglutaminase,[93] and lipoic acid ligase[94] were capable of site-specifically ligating small molecules to their peptide substrates within a variety of proteins.

Transglutaminase catalyzes amide bond formation between glutamine side chains and small molecules containing a primary amine, and it is notable for its relaxed small molecule substrate specificity. Work on using microbial transglutaminase to modify antibodies was first published by Schibli, whose group showed that Q295, the natural amino acid residue that is situated near the N297 glycosylation site, could be a substrate for transglutaminase, but only if the N-glycan was first removed.[95] Such treatment of an IgG makes Q295 more sterically accessible and increases the flexibility of the loop in which the residue is situated. Treatment of rituximab or an anti-L1-CAM antibody with the glycosidase PNGase F followed by transglutaminase in the presence of various amine-containing small molecules led to isopeptide bond formation at Q295 (Figure 5A). Alternatively, transglutaminase treatment of the N297Q mutant yielded products modified at Q295 and Q297, resulting in up to four small molecule additions per antibody (Figure 5B).

The Schibli group next collaborated with Innate Pharma to use microbial transglutaminase for production of ADCs.[96] In the Schibli group's initial study, amine-functionalized small molecules were directly coupled to antibodies in the presence of transglutaminase.[95] When this approach was applied to PNGase-treated IgG1 using amine-functionalized auristatin derivatives, only 50−80% of heavy chains were modified despite the presence of 40 equiv of drug-linker. Hypothesizing that transglutaminase would more readily accept a smaller substrate with a clickable functional group, the authors instead modified several antibodies with an azido-PEG-amine to yield fully modified conjugates (Figure 5C). Subsequent reaction with a cyclooctyne-functionalized MMAE yielded a uniform product with a DAR of 2 by MS analysis of the heavy chain, suggesting >95% coupling efficiency.

Strop, Rajpal, and co-workers from Rinat-Pfizer have used a transglutaminase from *Streptoverticillium mobaraense* to prepare ADCs.[97] Rather than removing the glycans from N297 for accessibility to Q295, this group introduced the amino acid tag LLQGA into various sites on the heavy or light chains to direct the attachment of fluorophores or auristatins directly to the introduced glutamine residue. The best LLQGA sites, one on the light chain and one on the heavy chain, exhibited DARs of 1.8−1.9 when coupled directly with a monomethyl auristatin D (MMAD)-functionalized amine (Figure 5D). A comparison of the pharmacokinetics of these two anti-M1S1 conjugates *in vivo* showed a striking dependence on the conjugation site: in mice, the two conjugates as well as the parent antibody exhibited similar clearance rates, whereas in rats, the heavy chain conjugate was cleared very quickly. Additionally, in rats, the stability of a protease-cleavable linker containing a valine-citrulline dipeptide was dependent on the conjugation site: the heavy chain ADC accumulated DAR 1 species, whereas the light chain ADC remained almost completely intact. Finally, a comparison of the site-specific ADCs to a conventional cysteine conjugate with DAR 3.6 showed that the former were better tolerated at high doses in rats, consistent with the findings from an initial study on THIOMABs.[37]

In a subsequent study on transglutaminase-mediated ADC production, the Rinat-Pfizer group examined tryptic digests of their ADCs by LC-MS/MS and found that 1.3% of the heavy chains were inadvertently modified with MMAD at Q295.[98] This impurity was introduced on the small portion of the antibody that was aglycosylated. When a Q295N mutant was used as the substrate, no sites of off-target conjugation could be detected by analysis of tryptic peptides, suggesting that the reaction proceeded with >99.8% site specificity. Finally, the group installed the LLQGA tag at both a heavy and a light chain site simultaneously to prepare ADCs with a DAR of 3.8, a rare example of using a site-specific conjugation method to prepare an ADC functionalized at several sites (Figure 5E). This study highlighted the importance of thorough conjugate characterization with respect to site specificity and DAR: the presence of a 1−2% impurity would be difficult to reproducibly detect by intact protein MS, but analysis of tryptic digests in this study was capable of detecting femtomole quantities of peptides with a sensitivity of approximately 0.2%.

Bacterial sortase can also be used to direct amide bond formation with a primary amine at a specific site on a protein of interest. Sortase is a transpeptidase that covalently attaches

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proteins to the peptidoglycan layer in the cell wall of Gram-positive bacteria. Mao and co-workers originally showed that the LPXTG consensus sequence for sortase modification could be installed recombinantly into proteins to direct the addition of the primary amine in glycine-functionalized cargo molecules.[99] Sortase cleaves the LPXTG peptide between the threonine and glycine residues, replacing the original glycine residue and subsequent C-terminal amino acid residues with the glycine-functionalized cargo molecule. Hagemeyer, Donnelly, and co-workers have used sortase-mediated conjugation to modify an scFv with a radioisotope chelator (Figure 5F).[100] Importantly, the product of a sortase-mediated conjugation also contains an LPXTG motif, and the reaction is therefore reversible. The authors found that glycine-functionalized small molecules incorporating PEG or sterically bulky tryptophan residues led to higher conversions, perhaps because they minimized the propensity for the reverse reaction and/or hydrolysis of the thioester acyl-enzyme intermediate. The optimized conjugations proceeded with around 90% conversion, and following installation of the radioisotope, the conjugates were used for PET/CT imaging. The sortase-tagging method has also been used by Harrenga and co-workers at Bayer to modify an Fab[101] as well as by the Ploegh group to ligate peptides and proteins to the heavy chain of a full-length antibody.[102]

While the transglutaminase and sortase methods use peptide tags to direct an amide conjugation to a desired site on the protein, a peptide tag could instead be used to direct an enzymatic transformation that yields a bioorthogonal functional group. This is the strategy behind the aldehyde tag technology, which was developed in our lab as a general method for site-specific protein modification. A protein containing the peptide sequence CXPXR at the desired site of modification is coexpressed with a formylglycine generating enzyme, which catalyzes oxidation of the cysteine residue within the sequence to formylglycine.[103] During studies in which we used aldehyde-tagged proteins to prepare antibody−protein conjugates[104] and Fc fragments with defined glycan structures,[105] our lab observed that formylglycine-derived oximes are unstable to hydrolysis over the course of several days at ambient temperature. The need for a stable conjugation chemistry for formylglycine led us to develop the Pictet−Spengler ligation, a reaction in which a stable C−C bond is formed between the aldehyde and an aminooxy-functionalized tryptamine analogue.[106] Rabuka and co-workers at Redwood Bioscience (now part of Catalent Pharma Solutions) disclosed ADCs made from aldehyde-tagged proteins using the hydrazino-Pictet−Spengler (HIPS) ligation,[107] a variant of the Pictet−Spengler ligation that proceeds more quickly near neutral pH. The CXPXR tag sequence was introduced into trastuzumab at three sites with cysteine-to-formylglycine conversions of 86−98%, and the resulting antibodies were conjugated to HIPS-maytansinoids with over 90% conversion (Figure 5G).[108] Comparisons of tagged and untagged protein showed no changes in thermal stability or FcRn binding at pH 6, while the ADCs showed a slight decrease in thermal stability and slightly slower dissociation from FcRn at neutral pH. The group next evaluated how several important parameters varied depending on the conjugation site. Evaluation of the plasma stability of three maytansine conjugates showed that stability was site-dependent, likely resulting from accelerated hydrolysis of the maytansine ester in certain microenvironments. *In vivo* half-life and efficacy in a mouse xenograft model were also site-dependent, with a nonspecifically lysine-functionalized trastuzumab−maytasine ADC exhibiting a shorter antibody half-life than any of the site-specific conjugates. Finally, in results consistent with previous studies comparing site-specific and nonspecific ADCs, a toxicity study showed that a C-terminally tagged ADC was much better tolerated than the nonspecific ADC. The Redwood Bioscience team has also used the aldehyde tag platform to conduct SAR studies on a variety of linkers.[109] Because all of the early linker development for ADCs was done on mixtures of conjugates, decisions about linker structure were often made empirically after screening a variety of linkers. The ability to interrogate SAR on homogeneous conjugates will likely lead to an improved understanding of the factors relevant to linker degradation and drug release.

Work with transglutaminase, sortase, and formylglycine generating enzyme has shown that chemoenzymatic strategies can be a convenient way to produce site-specific ADCs. A variety of other chemoenzymatic protein modification techniques exist that could also be used for site-specific ADC production.[110] Additionally, some methods involving peptide or protein tags do not require enzymatic modification. For example, peptide tags can be used to direct chemical modification to the N terminus of an Fc via transamination and oxime ligation;[111] alternatively, expressed protein ligation has been used to modify an IgG at the C terminus,[112] and the SNAP tag has been used to site-specifically modify an scFv.[113] Also of note is the Meditope technology, in which a domain with affinity for a corresponding Meditope peptide can be grafted onto an antibody.[114]

One potential downside to using peptide tags to direct site specificity is that the inserted tag sequence may be immunogenic in humans. In such a case, the likely recourse would be to find an alternate peptide sequence that acts as a substrate, which could also require engineering the enzyme, depending on its promiscuity. Preliminary work can also help rule out problematic sites of modification; for example, in the Redwood Bioscience study, computational analysis as well as an *ex vivo* assay for T cell activation suggested that antibodies tagged at the chosen sites and the resulting ADCs would not be immunogenic.[108]

VI. CONCLUSIONS

Thanks to advances in protein engineering, bioorthogonal chemistry, and analytical methods, the field of site-specific ADC development is advancing rapidly. We can now covalently append a drug molecule to many sites on an antibody, but the chemistries for doing so are still few in number. Many of the site-specifically modified ADCs in coming years will likely be linked via maleimides, oximes, and triazoles, functional groups that bear the signatures of classic bioorthogonal reactions. As these functional groups see widespread use, we will also come to understand their strengths and limitations. Cys−maleimide conjugations, once considered the gold standard of protein bioconjugation reactions for their speed and selectivity, are now understood to yield products that undergo thiol exchange and succinimide ring-opening reactions. Hydrazone and oxime conjugations, widely appreciated for their reliability and simplicity, suffer from slow reaction kinetics and potential hydrolytic instability. Additionally, many commonly used bioorthogonal reagents do not react stereospecifically. For example, reactions of maleimides, bis-sulfone cross-linkers, dibenzocyclooctynes, hydrazides and alkoxyamines, and tryptamine analogues with their respective protein-bound reaction

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partners all yield at least two diastereomeric products, with complications sometimes arising from formation of several regioisomers as well. As currently performed, these conjugation methods, even when applied to homogeneous starting materials with site-specifically installed bioorthogonal functionality, cannot generate homogeneous products. As the field progresses, the continued development and refinement of protein engineering techniques in conjunction with new bioorthogonal chemistries will enable the production of homogeneous conjugates, and will allow parameters such as stability, potency, pharmacokinetics, and immunocompatibility to be tuned.

Protein engineering tools have been used extensively by biopharmaceutical companies in the development of biologic therapies over the last 3 decades. In contrast, the primary drivers of bioorthogonal chemistry have been academic laboratories, and their intended purpose typically has been to develop research tools for studying biological systems. The brimming pipeline of site-specific ADC technologies shows that biopharmaceutical companies are now thoroughly vetting the collection of bioorthogonal reactions, and the scope of applications of bioorthogonal chemistry is poised to expand into clinical use.

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Notes

The authors declare the following competing financial interest(s): C.R.B. is a co-founder of Redwood Bioscience.

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