*Antibody Therapeutics, 2020, Vol. 3, No. 4* **271–284**
*doi:10.1093/abt/tbaa027*
*Advance Access Publication on 18 December 2020*

Review Article

# Site-specific conjugation of native antibody

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Received: September 18, 2020; Revised: November 20, 2020; Accepted: December 15, 2020

## Abstract

**Traditionally, non-specific chemical conjugations, such as acylation of amines on lysine or alkylation of thiols on cysteines, are widely used; however, they have several shortcomings. First, the lack of site-specificity results in heterogeneous products and irreproducible processes. Second, potential modifications near the complementarity-determining region may reduce binding affinity and specificity. Conversely, site-specific methods produce well-defined and more homogenous antibody conjugates, ensuring developability and clinical applications. Moreover, several recent side-by-side comparisons of site-specific and stochastic methods have demonstrated that site-specific approaches are more likely to achieve their desired properties and functions, such as increased plasma stability, less variability in dose-dependent studies (particularly at low concentrations), enhanced binding efficiency, as well as increased tumor uptake. Herein, we review several standard and practical site-specific bioconjugation methods for native antibodies, i.e., those without recombinant engineering. First, chemo-enzymatic techniques, namely transglutaminase (TGase)-mediated transamidation of a conserved glutamine residue and glycan remodeling of a conserved asparagine N-glycan (GlyCLICK), both in the Fc region. Second, chemical approaches such as selective reduction of disulfides (ThioBridge) and N-terminal amine modifications. Furthermore, we list site-specific antibody–drug conjugates in clinical trials along with the future perspectives of these site-specific methods.**

**Statement of Significance: Compared with their non-specific counterpart, site-specific bioconjugation produces more homogenous, well-defined, and developable antibody conjugates that are likely to achieve their desired properties and functions. In this review, we present practical site-specific methods to conjugate native (non-engineered) antibodies, as well as highlight their clinical applications and discuss future directions.**

**KEYWORDS: hybrid modality; bioconjugation; transglutaminase; glycan remodeling; antibody–drug conjugate (ADC)**

## INTRODUCTION

Hybrid Modality Engineering of Protein is a platform that introduces non-canonical chemical moieties or scaffolds, i.e., effectors—and thus functions—into peptides and proteins, e.g., native antibodies, to confer novel and improved functions otherwise unavailable via recombinant technology. Common protein engineering approaches include mutagenesis, insertion or deletions of peptides or incorporation of unnatural amino acids. These approaches have several shortcomings, such as providing only a linear architecture, and in some cases, it requires laborious optimization and low yield results. As reviewed herein,

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a powerful alternative is the site-specific conjugation of native antibodies, i.e., those without recombinant engineering.

### Site-specific vs. non-specific (stochastic or random)

Until recently, most antibody conjugates were constructed through traditional chemical methods, such as acylation of amines on lysine residues and N-termini, as well as alkylation of thiols on cysteine residues. These processes have been used to construct antibody–drug conjugates (ADC) approved by the food and drug administration (FDA) [2, 3]. Unfortunately, these strategies are typically not site-specific, which result in heterogeneous mixtures, produce positional isomers, and suffer poor reproducibility. For example, Lou *et al.* [4] have shown that all amines in an IgG (76 lysine residues in both light and heavy chains [HCs]) were modified via N-hydroxysuccinimide (NHS) chemistry to observable degrees. These reactions yield heterogeneous constructs with a widely distributed drug-to-antibody ratio (DAR) of 0–8. The multiple sites of modifications may cause structural changes to the antibody and have been shown to perturb their underlying biological function [1, 5–7]; for instance, modifications at or near the complementarity-determining region (CDR) in antibodies may reduce antigen binding and specificity.

Further complications arise from the challenges in the characterization of conjugates. To start, antibodies themselves consist of various post-translational modifications (e.g., charge variants) such as deamidation, oxidation, glycosylation, or other reactive metabolites [8–17]. Therefore, the analysis of antibody conjugates constructed via the non-specific approaches is rather complicated, and the detection of all modifications can be difficult, thereby underestimating the sites of modifications [18]. For example, the modification sites are missed due to false negatives' prevalence during analysis [18]. Furthermore, non-specific methodologies can produce side reactions that are often underappreciated [18]. Overall, these species—both unmodified and modified antibody conjugates—exponentially increase the complexity of the analysis after non-specific chemical modifications and further increase the resulting product's heterogeneity.

To overcome non-specific conjugation hurdles, site-specific methods to modify antibodies produce homogenous and well-defined conjugate and do not compromise the conjugate's biological activity (Fig. 1). For instance, these chemistries avoid modifications to the antigen-binding region (Fab) and have been shown to increase the binding efficiency compared with their non-specific counterpart [1]. With the advent of click and other bioorthogonal chemistries [19–21]—which are fast, high yielding, and with commercially available reagents—various antibody conjugates have been constructed via site-specific conjugation with a wide range of applications in analysis, imaging, diagnosis, and therapy.

Some of the previously reported site-specific conjugation technologies include linchpin directed modification, which conjugates drugs and fluorescent tags to the Fab and monoclonal antibodies, and regioselective and chemoselective lysine modification with sulfonyl acrylate reagents [22–24]. In 2019, a novel proprietary technology—affinity peptide-mediated regiodivergent functionalization (AJICAP™) was reported, which utilizes Fc affinity reagents to target selective lysine residues, onto which the payloads are attached [25].

This review will focus on both chemical and enzymatic site-specific methods for the conjugation of native antibodies without the need for genetic engineering. In this context, native antibodies refer to antibodies produced or naturally existing in various species (e.g., human, mouse or goat) and antibodies that are recombinantly produced, where the conserved sequences are preserved and not engineered.

### Improved properties and functions

Site-specific methods offer advantages over their non-specific counterparts in the conjugate's properties and functions, such as enhanced plasma stability, increased tumor uptake, enhanced binding efficiency, less variability in dose studies, and a higher level of binding (Fig. 2). The work conducted by Kristensen *et al.* [6], measuring the percentage of intact tracer present in plasma, demonstrated increased stability for site-specifically labeled conjugates compared with their stochastically labeled counterparts. After 168 h, the site specifically labeled conjugates, ß- [1–4] galactosidase and endoglycosidase S2, were stable in plasma with >99% and 91% intact tracer, respectively. On the other hand, random labeling showed a decrease to 80% intact tracer in plasma (Fig. 2a) [6]. Moreover, site-specific conjugates exhibited a higher tumor uptake than non-specific conjugates (Fig. 2a) [6]. The tumor uptake was "twice as high for the site-specific conjugates, 14.2 ± 1.7 %ID/g ($\beta$-Gal) and 16.7 ± 1.5 %ID/g (endoS2), compared with 6.5 ± 0.5 %ID/g for the randomly labeled trastuzumab 120-h post-injection" (Fig. 2a) [6]. Sadiki *et al.* [1] reported a two-fold higher binding efficiency for the site-specific compared with the non-specific constructs (Fig. 2b). Less variability of site-specific conjugates (center and right panels) at low concentrations (between 10 nM and 10 pM) compared with the non-specific conjugates (left panel) was observed by Pellegrino *et al.* [5] (Fig. 2c). Randomly labeled conjugates (left panel) showed an impaired level of binding compared with the site specifically labeled conjugates (center and right panels) (Fig. 2d).

### Site-specific conjugation chemistries

In the following sections, chemo-enzymatic methods, namely transglutaminase (TGase) and glycan-mediated conjugation, and chemical methods such as selective reduction of disulfides (ThioBridge) and N-terminal modification are presented.

## CHEMO-ENZYMATIC

Chemo-enzymatic methods are typically site-specific and generally proceed under mild reaction conditions. Furthermore, for a given family of enzymes, their activity and specificity can be tailored by tuning the reaction conditions, choosing multiple isoforms that confer different

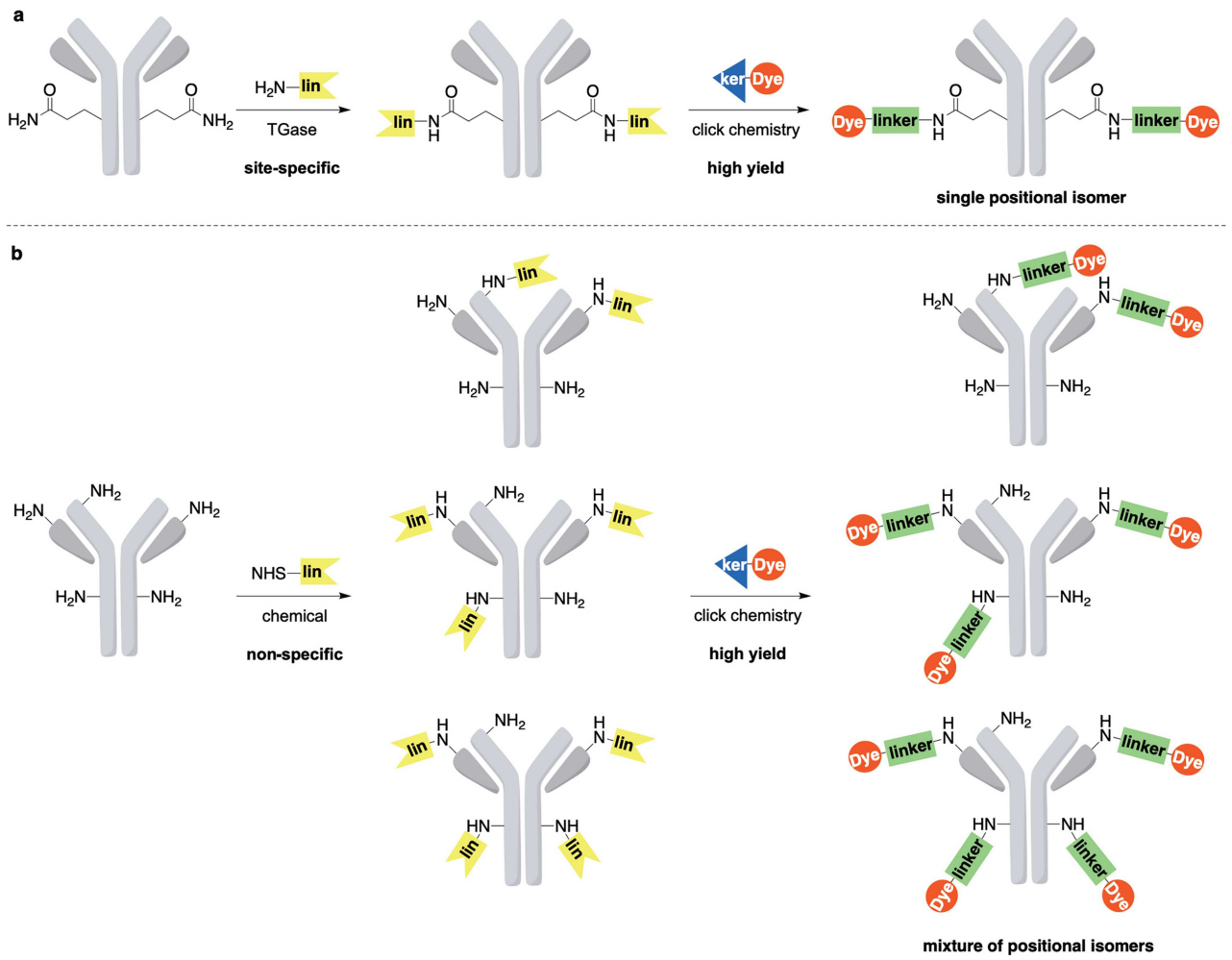


**Figure 1.** Antibody conjugates constructed via a convergent assembly [1]. (a) Site-specific, transglutaminase (TGase)-catalyzed conjugation leads to homogenous constructs that preserve binding affinity. (b) Non-specific chemical modification, such as acylation of amines, yields a heterogenous mixture that ultimately reduces binding affinity. Adapted with permission from Sadiki *et al.* [1]. Copyright © 2020, John Wiley, and Sons.

specificities, as well as protein engineering [27, 28]. In the next section, we introduce two of these approaches: transglutaminase-mediated modification of glutamine and glycan remodeling, which occur in the conserved Fc region of an antibody.

## Transglutaminase

As early as 2000, antibodies were reported to be modified by transglutaminase (TGase, EC 2.3.2.13) [29]. Although over 60 glutamine (Gln) residues exist in an antibody, only a single conserved residue in the HC is typically modified, as reported by Schibli *et al.* [30, 31] [EEQ$^{295}$YN*S; of note, the Asn (N*) residue is typically glycosylated]. This residue resides in the Fc region of deglycosylated antibodies and is efficiently modified by the corresponding amine substrate under the catalysis of TGase. The deglycosylation of the conserved asparagine (two residues C-terminal to the glutamine), ensure that the adjacent glutamine residue is accessible for enzymatic transformation. Interestingly, Marculescu *et al.* [32] recently reported that microbial transglutaminase (mTGase) also modifies another glutamine residue (Gln3) in the HC region, albeit to a lesser extent. We and others [1, 31, 33–35] have shown that desired, and commercially available amine reagents can be conjugated into antibodies via one-step (Fig. 3) or two-step (Fig. 1) convergent processes mediated by click and other bio-orthogonal chemistries. Various reports of one-pot dual conjugation [36], branched [37], heterobifunctional [34] and proteolytically cleavable [36, 38] linkers, as well as non-canonical substrates [35] such as hydrazines, hydrazides, and alkoxyamines, have been conjugated to antibodies and thus illustrates the versatility of this family of enzymes as a tool for bioconjugation. Moreover, recombinant peptide tags, as well as reactive glutamine and lysine residues, have been introduced into antibodies through genetic engineering; however, these systems are outside the scope of this review and have been summarized by others [39, 40]. Since only a conserved Gln295 is the primary modification site, in principle, IgG1, IgG2, IgG3, and IgG4 can also be modified without the need for genetic engineering. These methodologies have produced

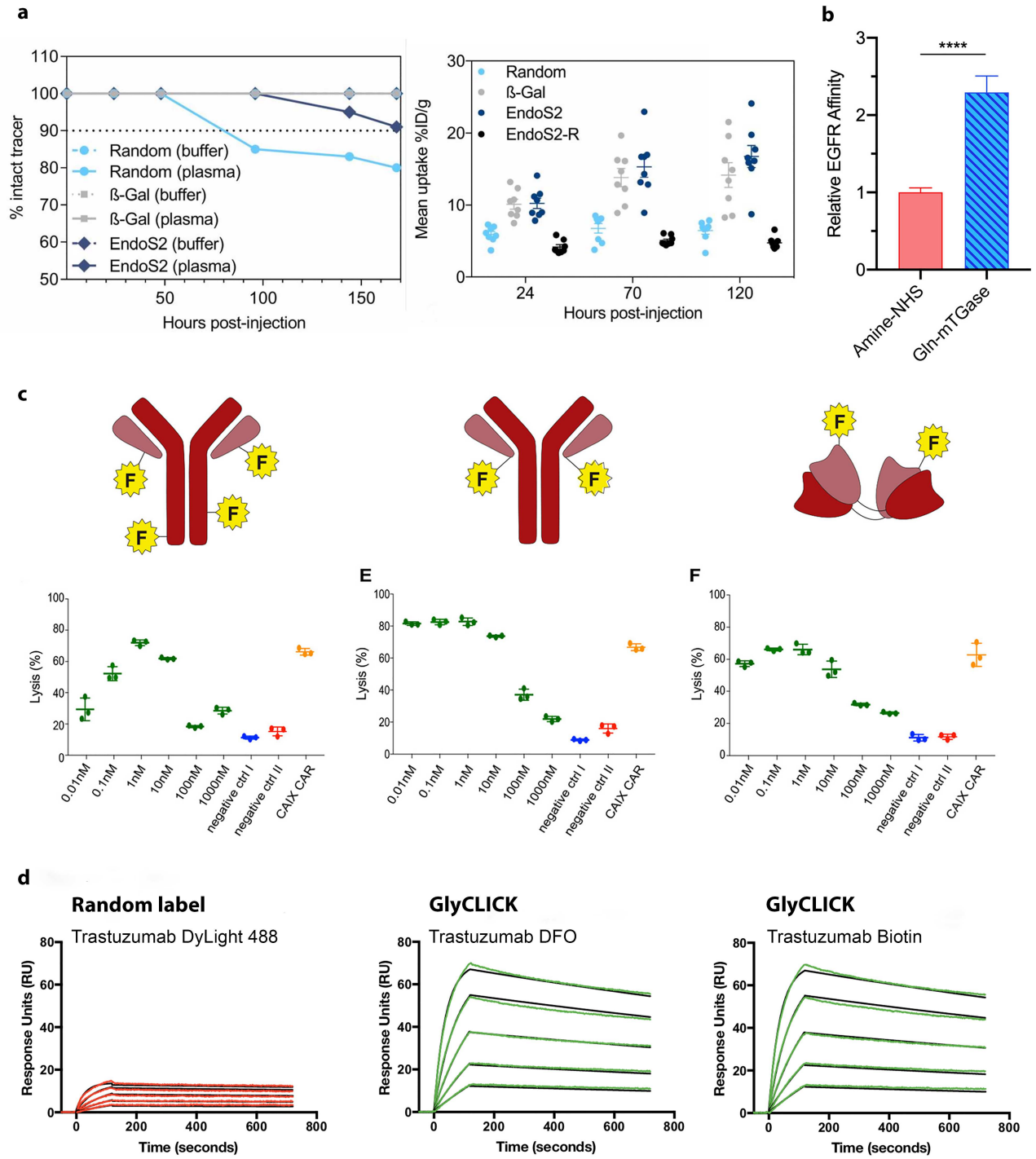


**Figure 2.** (a) Site-specific conjugates (modified via $\beta$-Gal and EndoS2) showed higher tracer stability in plasma compared with non-specific (random) labeled conjugates in plasma. Mean tumor uptake is more elevated for site-specific conjugates ($\beta$-Gal and EndoS2) compared to non-specific (random) conjugates post-injection in mice (right) [6]. (b) Site-specific conjugation (mediated by transglutaminase) demonstrated a two-fold increase in binding efficiency compared with its non-specific counterpart (i.e., acylation of amines) [1]. (c) Site-specific conjugates (center and right panels) show less variability at a low concentrations between 10 nM and 10 pM compared with non-specific conjugates (left panel) [5]. (d) SPR analysis of trastuzumab conjugates formed by random conjugation (left panel) and site-specific conjugation to introduce DFO (Deferoxamine) and Biotin (center and right panels) [26]. The randomly labeled conjugate showed impaired binding compared with the site-specifically conjugated antibody. Modified and adapted with permission from Kristensen *et al.* [6], Sadiki *et al.* [1], Pellegrino *et al.* [5], and Genovis. Copyright © 2020, John Wiley and Sons; Copyright © 2014, American Chemical Society.

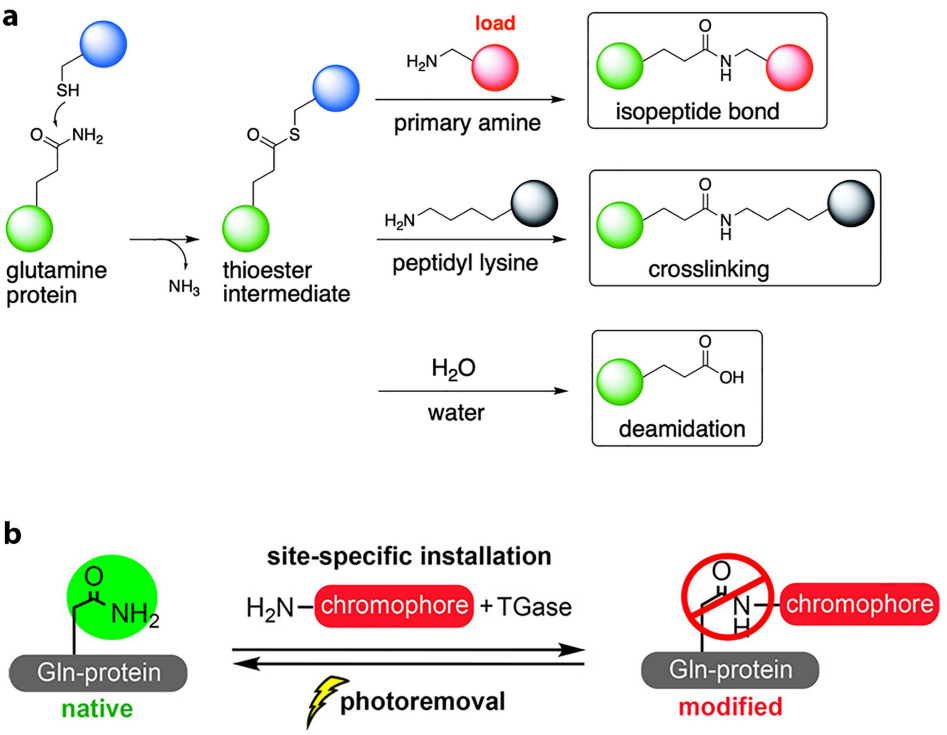


**Figure 3.** (a) Transglutaminase (TGase)-mediated transamidation of glutamine residues. First, a nucleophilic attack of the cysteine thiol in the active site of TGase to generate a thioester intermediate. Second, an amine-containing molecule nucleophilic attacks the thioester forging a new iso-peptide amide bond. Lastly, competing reactions include cross-linking of glutamines with lysine residues in peptides/proteins and deamidation of glutamine to generate glutamic acid via hydrolysis. These competing reactions can be entirely suppressed by a sub- or low-millimolar concentrations of exogenous amines. (b) TGase-mediated conjugation of protein to generate photoremovable conjugates. First, amine-containing chromophores are installed into glutamines by TGase. Second, light-mediated removal of the chromophores to regenerate the native peptide or proteins [51]. Modified and adapted with permission from Moulton *et al.* [51]. Copyright © 2019, American Chemical Society.

homogenous antibody conjugates for various applications, including imaging via radioimmunoconjugates [41], cytotoxicity using ADCs [38], PEGylation [40], and functional virus-based nanoparticles [42]. Lastly, Pfizer's Trop-2 ADC constructed via transglutaminase-mediated conjugation showed promise in preclinical studies [43] and was further translated to a phase 1 (NCT02122146; Table 1) clinical trial in solid tumors [44]. The Phase 1 results show that the iso-peptide amide bond is stable in both *in vitro* and *in vivo* settings.

Transglutaminases (TGases, EC 2.3.2.13) are a family of enzymes that catalyze an iso-peptide amide bond formation between an unsubstituted side-chain amide group of glutamine residues (as acyl donor) and a nucleophilic amine substrate (as acyl acceptor), e.g., a primary amine (Fig. 3), under mild reaction conditions [45, 46]. The resulting amide bond on the glutamine side chain is as chemically stable as other amide bonds in proteins and appears to be stable in biological systems (i.e., no enzymatic hydrolysis has been reported). Numerous isoforms of TGases have been identified, produced, and engineered with tailored broad/narrow specificities, [47, 48]. The substrate specificity of TGases is governed primarily by solvent accessibility of the glutamine or lysine residues, dynamic and flexibility of the modified region, and flanking residues proximal to the modification site [49, 50]. Therefore, typically only a few glutamine or lysine residues are modified in any given protein. Competing reactions include cross-linking of glutamines with lysine residues in peptides/proteins and deamidation of glutamine to glutamic acid (Fig. 3); however, in practice, these reactions can be entirely suppressed by a sub- or low-millimolar concentrations of exogenous amines [1, 38, 46, 51].

Due to its site-specific nature, requiring no co-factor, robustness, commercial availability, and low cost, mTGase from *Streptomyces mobaraense* [52] is widely used to construct protein–DNA, protein–glycosylation, protein–polymer, protein–protein conjugates, surface immobilization, as well as cross-linking in the food industry, academic research, and pharmaceutical development [40]. mTGase displays broad specificity toward primary amine substrates [53, 54]; for example, various commercially available primary amines containing azides [1], strained alkenes and alkynes [1, 55], tetrazines [55], and bulkier substrates such as polyethylene glycol (PEG) [56], have been incorporated into proteins using mTGase mediated conjugation. Besides, experimental conditions can be tuned to enhance selectivity, e.g., addition of organic co-solvents [57]. Lastly, this enzyme has been immobilized on solid-matrices such as glass [58] and agarose [59] beads, facilitating easy removal from the reaction mixtures via simple filtration.

Native human antibodies are poor substrates for TGase and require deglycosylation for efficient modification. To

**Table 1.** Representative native ADCs using site-specific conjugation methods that are currently in phase 1 clinical trials for cancer therapy [44, 95, 111, 112]

| Chemistry | Name | Developer | Drug to antibody ratio | Description | Clinical trial identifier |
|---|---|---|---|---|---|
| TGase-glutamine | PF-06664178 | Pfizer | 2 | • Anti-TROP2 IgG1<br>• Valine-citrulline cleavable linker<br>• Auristatin-based cytotoxic payload | NCT02122146 |
| Glycan remodeling | ADCT-601 | ADC Therapeutics | 2 | • Anti-Human AXL IgG1<br>• Valine-citrulline cleavable linker<br>• PBD (pyrrolobenzodiazepine)—cytotoxic dimer payload | NCT03700294 |
| | XMT-1592 | Mersana Therapeutics | 6 | • Anti-NaPi2b IgG1<br>• Proprietary platform (Dolasynthen)<br>• Auristatin-based cytotoxic payload | NCT04396340 |
| ThioBridge | OBI-999 | OBI-Pharmaceuticals | 4 | • Anti-Globo H IgG<br>• Proteolytically cleavable linker<br>• Auristatin-based cytotoxic payload | NCT04084366 |

ameliorate such restrictions, ongoing efforts on engineering TGases and other transferases [60] with altered specificity—e.g., modification of antibodies without the need to deglycosylate the conserved asparagine [61]—will undoubtedly facilitate clinical translation.

Our laboratory devised a novel photo-reversible conjugation chemistry based on TGase, which adds versatility to the field (as depicted in Fig. 3). The technology site-specifically installs photo-switches into peptides and proteins and generates photoremovable conjugates that allow the native proteins to be regenerated by light (i.e., photo-caging) [51]. This methodology is simple, robust, and easily adaptable and thus can be utilized to spatially and temporally control the biological functions of antibodies by light—e.g., binding to their respective antigen or effector functions such as antibody-dependent cellular cytotoxicity. Transglutaminase-mediated reactions are orthogonal to other site-specific approaches—i.e., genetic engineering such as unnatural amino acids, chemical methods, such as, thiol bridge or N-terminal modification, and enzymatic methods, such as, sortase. Therefore, combining these tools enables generations of novel conjugates that were previously unavailable, e.g., introducing two orthogonal proteolytically cleavable linkers like matrix metalloproteinase-2 and cathepsin-B using mTGase and recombinantly produced a tag for lipoate-acid ligase A [36].

## Glycan-mediated, site-specific conjugation

Glycan-mediated conjugation offers a generally applicable and exclusively site-specific approach for antibodies' bioconjugation due to several factors [62]. First, almost all IgG-type antibodies have one—and only one—glycan at a conserved site in the Fc region [63]. For this review, "glycan-mediated conjugation" refers to the chemo-enzymatic installation of payloads onto the conserved N-glycosylation sites of IgG-type antibodies. This installation is achieved via glycan remodeling, which entails the addition or removal of saccharides from these conserved sites, typically through galactose- or sialic acid-based chemistry.

The most common approach to glycan-mediated conjugation involves enzymatic transglycosylation and is composed of two steps: first, trimming of the antibody's native glycan via $\beta$-galactosidase, which cleaves the terminal galactose moieties; or via endoglycosidase, which cleaves after the innermost N-acetylglucosamine or GlcNAc moiety; and second, rebuilding of the glycan by installing an unnatural sugar moiety containing a handle for further bioconjugation [64–74]. This glycan-mediated conjugation strategy is further discussed in the following sections. Other techniques also exist. For example, Zhou *et al.* [71] chemoenzymatically introduced terminal sialic acid moieties onto the antibody's native glycan and functionalized them for conjugation.

*GlyCLICK.* GlyCLICK is a glycan-mediated conjugation technology marketed as a kit by Genovis AB (Sweden and Cambridge, MA, USA).

As illustrated in Fig. 4, the first step (typically performed at room temperature), involves a proprietary immobilized endoglycosidase that cleaves the native glycan after the innermost GlcNAc moiety; the trimmed antibody is readily

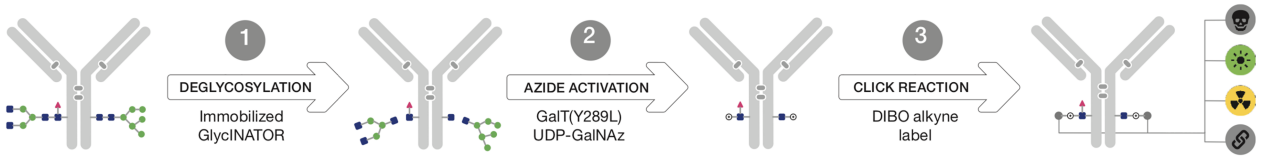


**Figure 4.** An overview of the steps for glycan-mediated conjugation using GlyCLICK: first, trimming the native antibody glycan using endoglycosidase; second, installation of the azide via $\beta$-1,4-galactosyltransferase; and third, click reaction using a payload with suitable chemistry [64]. Adapted with permission from Toftevall *et al.* [64]. Copyright © 2020, Springer Science Business Media, LLC, part of Springer Nature.

separated from the immobilized enzyme. In the second step, $\beta$-1,4-galactosyltransferase-mediated installation of the azide-functionalized unnatural sugar occurs overnight at 30°C. Lastly, strained alkyne-functionalized payload reacts with the azide handle at room temperature without catalysts to afford conjugates containing fluorophores, affinity tags, chelators, or custom chemistries [64, 67].

GlyCLICK produces homogeneous conjugates with well-controlled DARs. It is worth noting that the two attachment points per Fc domain can afford DARs greater than two if branched or dendrimeric linkers are used. For example, Thompson *et al.* [75] employed strategies similar to elements of GlyCLICK to produce glycan-remodeled conjugates with DARs of 2 and 4.

Because the Fc glycan is distant from the CDRs, glycan-mediated conjugation typically preserves the antibody's antigen-binding capacity. Indeed, surface plasmon resonance (SPR) studies of trastuzumab conjugates produced via GlyCLICK show overlapping curves for native trastuzumab, whereas the product of stochastic conjugation exhibits impaired binding levels [64].

*Scalability, scope, and limitations.* Wide and robust applications have been demonstrated for glycan-mediated conjugation, including human IgG1–IgG4s and IgGs from goats, mice, rabbits, rats, monkeys, sheep, cows, and horses [64]. van Geel *et al.* [76] successfully produced conjugates of several human or humanized IgG1s, IgG2s, and IgG4s, including a rituximab-BCN-PEG$_8$-doxorubicin conjugate up to the 5 g scale. All conjugates demonstrated site-specific incorporation of the linker and payload, negligible aggregation, hydrolytic stability, and homogeneous DARs. Also, trastuzumab-maytansine conjugate developed via glycan remodeling showed more favorable *in vitro* and *in vivo* efficacy data than Kadcyla, a commercial ADC composed of the same components but generated through stochastic conjugation methods [76].

*Applications: preclinical stage.* Preclinical use of glycan-mediated conjugates is growing [6, 77–79]. Recently, Kristensen *et al.* [6] demonstrated that conjugates of trastuzumab to $^{89}$Zr-SCN-Bn-DFO exhibited increased positron emission tomography (PET) tumor uptake and substantially higher *in vitro* stability and immunoreactivity compared with conjugates generated via traditional lysine acylation, as seen in Fig. 2. Similarly, Christensen *et al.* [77] showed an anti-PD-L1 antibody-$^{89}$Zr-DFO-6E11 conjugate for PET imaging increased relative tumor uptake of the imaging agent and enabled quantification of PD-L1 expression differences in tumors and spleens of irradiated tumor-bearing mice.

*Applications: clinical stage.* At least two ADCs developed using GlycoConnect from SynAffix (a glycan-mediated conjugation approach similar to GlyCLICK) have advanced to early-stage clinical trials: ADCT-601 from ADC Therapeutics and XMT-1592 from Mersana Therapeutics. ADCT-601, an anti-human AXL-pyrrolobenzodiazepine-dimer toxin conjugate, is undergoing Phase I evaluation in patients with solid tumors (NCT03700294; Table 1), whereas XMT-1592, a NaPi2b-targeting antibody conjugated to an auristatin payload, recently initiated Phase I dose-escalation studies in patients with non-small cell lung cancer adenocarcinoma or ovarian cancer (NCT04396340; Table 1).

## CHEMICAL CONJUGATION

Chemical conjugations, such as the acylation of amines and alkylation of thiols, are typically not site-specific (i.e., random or stochastic), and the solvent-accessible residues are typically modified. This method generates a mixture of ADC species with a variable DAR and mixed tethering sites. However, as detailed in the following section, two site-specific chemical methods are available: first, selective reduction of disulfide followed by alkylation (ThioBridge); and second, N-terminal transamination and imidazolidinone formation. Although there are several other approaches for selective reduction of disulfide bridging bonds such as bis-sulfone reagents, dibromo pyridazinediones (PDs), and next-generation maleimides acting as cysteine cross-linking reagents [80–83], this paper will focus specifically on ThioBridge.

### Selective reduction of disulfide (ThioBridge)

Together with the acylation of amines (N-termini and lysine residues), alkylation of thiol (cysteine residues) is the most common and conventional bioconjugation chemistry. In human native antibodies, all cysteine residues exist as disulfides. There are four isoforms of IgG (IgG1, IgG2, IgG3, and IgG4), each of which contains 12 intra-chain disulfide bonds that are conserved. In addition, an IgG1 antibody has four interchain disulfide bonds typically used as potential sites for conjugation. To some degree, these interchain disulfides bonds can be selectively reduced by judiciously combining some reductants under certain conditions, such as tris (2-carboxyethyl) phosphine (TCEP) or dithiothreitol (DTT) [84–88]. Selective reduction of the interchain

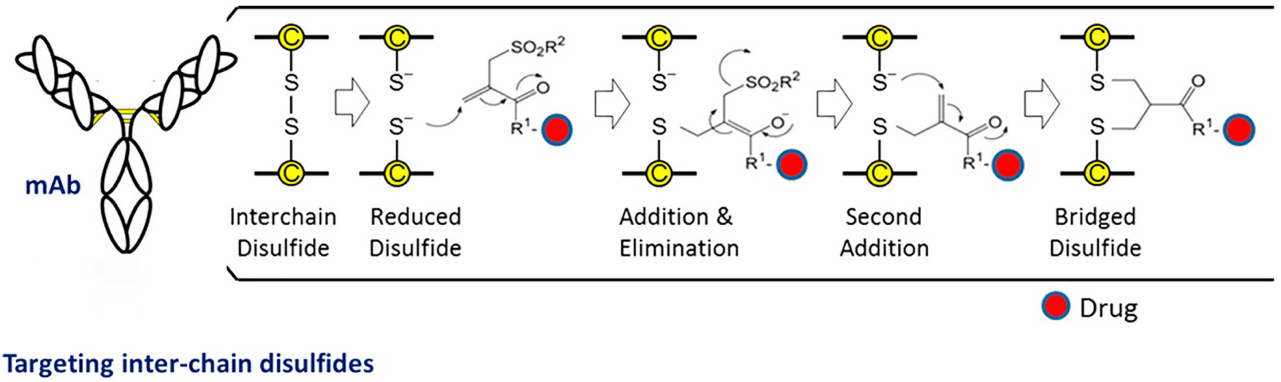


**Figure 5.** Conjugation of a drug to the site-selective reduced interchain disulfide bonds of an antibody, via bis-thiol reactive reagent that cross-links the reduced disulfide bonds, involving a sequence of Michael addition and elimination reactions [85]. Modified and adapted with permission from Badescu *et al.* [85]. Copyright © 2014, American Chemical Society.

disulfide bonds is a vital step as it yields different DARs (e.g., 2, 4, 6, or 8) [85, 88].

Disulfide bonds, which are often referred as "disulfide bridges," are crucial in holding different antibody domains together. Modification of disulfide bonds/cysteines can be achieved using salts such as vinyl sulfonium [89–91]. Reduction followed by a single alkylation of each resulting cysteinyl thiol like the one obtained via the venerable maleimide chemistry often leads to significant destabilization of antibody structures. Modification of the four inter-chain disulfide bonds in the antibody often lead to increased conjugation heterogeneity and reduced site-specificity. Furthermore, alkylation by maleimide is reversible (i.e., deconjugation), which has been observed *in vivo* [92]. These limitations are rectified by the rebridging approach detailed next.

In 2014, Badescu, Godwin, and co-authors reported the first disulfide re-bridging strategies with bis-alkylating reagents—namely ThioBridge. This technology was developed by PolyTherics (a subsidiary of Abzena) as a novel way to prepare more homogeneous and stable ADCs without protein engineering [85]. As depicted in Fig. 5, the two-step method uses bis-sulfone reagents that are selective for a pair of cysteine thiols in close proximity, which is the case for the thiols generated from the reduction of disulfides in antibodies.

*Step 1: site-specific disulfide reduction to liberate free cysteine thiols for conjugation.* Antibodies generally have minimum to no free thiols. The four accessible interchain disulfide bonds can be selectively reduced fully or partially, using DTT or TCEP, respectively. Moreover, different degrees and sites of reductions can be achieved under controlled conditions, e.g., tuning the equivalence of the reductants to the antibody, reaction time, and temperature. For example, DTT reduced all four interchain disulfide bonds generating eight free thiols that can be re-bridged to make up to four conjugation sites. Whereas, at 1–2 equivalent of TCEP per disulfide, varying loading ratios can be achieved (e.g., DAR of 1–4) [88].

*Step 2: bridging of reduced antibody with drug-containing reagent.* The pair of thiols (reduced disulfide) is then followed by bis-alkylation, proceeding through Michael addition and elimination, to conjugate both thiols derived from the original disulfide bond. This reaction results in covalent re-bridging of the disulfide bond via a three-carbon bridge leaving the antibody's structure less perturbed compared with single alkylation [85]. Naturally, once the ThioBridge linkage is introduced, various conjugates (drugs or payloads) can be incorporated, such as PEG or clickable handles [85, 93].

*Stability*. In the ThioBridge technology, the disulfides are reannealed, resulting in greater stability and homogeneity. In contrast, ADCs produced using maleimide are unstable due to deconjugation and cross-conjunction to free thiols of albumin serum [94]. Shen *et al.* [85, 94] demonstrated that in rat serum, the conjugated fluorophore (i.e., Alexa Fluor 488) was lost, based on maleimide, yet, the ThioBridge counterpart was stable and showed no detectable cross conjunction to serum albumin. Due to its *in vivo* stability, ThioBridge technology is being developed by OBI Pharma to construct a glycolipid antigen targeting ADC, globohexaosylceramide (Globo H, OBI-999). This molecule is currently under clinical evaluation in Phase I/II study (NCT04084366; Table 1) [95].

## N-terminal conjugation

The N-terminal transamination of antibodies and imidazolidinone formation are two site-specific methods that are not reactive toward the amine on the lysine side chain for two main reasons. First, only two N-termini exist in antibodies, one on the light and one of the HC. Second, the origin of chemical selectivity of the N-terminal amine stems from the adjacent carbonyl group of the amide [96, 97]; in comparison, the epsilon amine on the side-chain of lysine is adjacent to methylene (alkyl) groups. [98, 99].

*N-terminal modification with pH control offers limited selectivity:* Contrary to common assumption, even at low pHs, exclusive selectivity cannot be achieved for the N-terminal amines via acylation by NHS ester and reductive

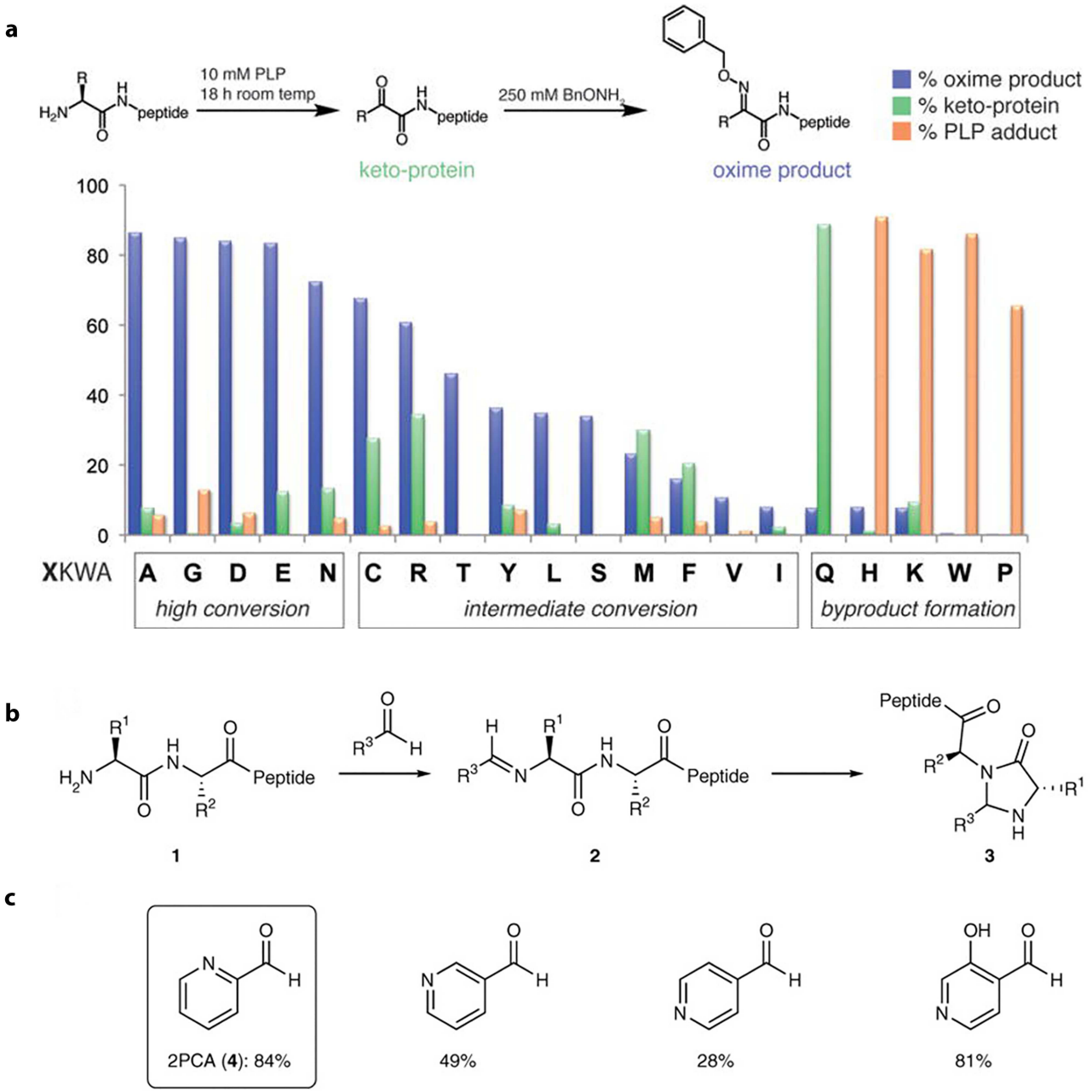


**Figure 6.** (a) Transamination of the N-terminal amine by pyridoxal phosphate (PLP) and reactivities of different amino acids [101]. (b, c) Modification of protein N-termini by pyridine aldehyde and the formation of imidazolidinone [102]. Adapted with permission from Witus *et al.* [101]; MacDonald *et al.* [102]. Copyright © 2014, John Wiley and Sons; Copyright © 2014, Springer Nature.

amination; in other words, under these conditions, amines on the lysine side chains can nonetheless be modified, albeit to a reduced degree. A contributing factor to this misconception is the incomplete characterization of bioconjugate products and side-products. [18, 100].

*Transamination.* N-Terminal modification via transamination reaction using carbonyl agents directly converts the N-terminal amine to a carbonyl group, which can be further conjugated via established chemistries, such as with hydrazines, hydrazides, or hydroxylamines. In 2006, Francis and co-authors reported a mild method based on biomimetic transamination reaction using various aldehydes, such as pyridoxal phosphate (PLP) (vitamin B6) [101, 103–105].

*Conditions and by-products.* Transamination reaction can occur in mild conditions, near physiological pH and temperature, and can thrive in various buffers, and does not require any particular catalysts. Of note, PLP-mediated transamination reaction often forms pyridoxal phosphate (PLP) aldol adducts as by-products, especially at high pyridoxal phosphate (PLP) concentrations and long reaction

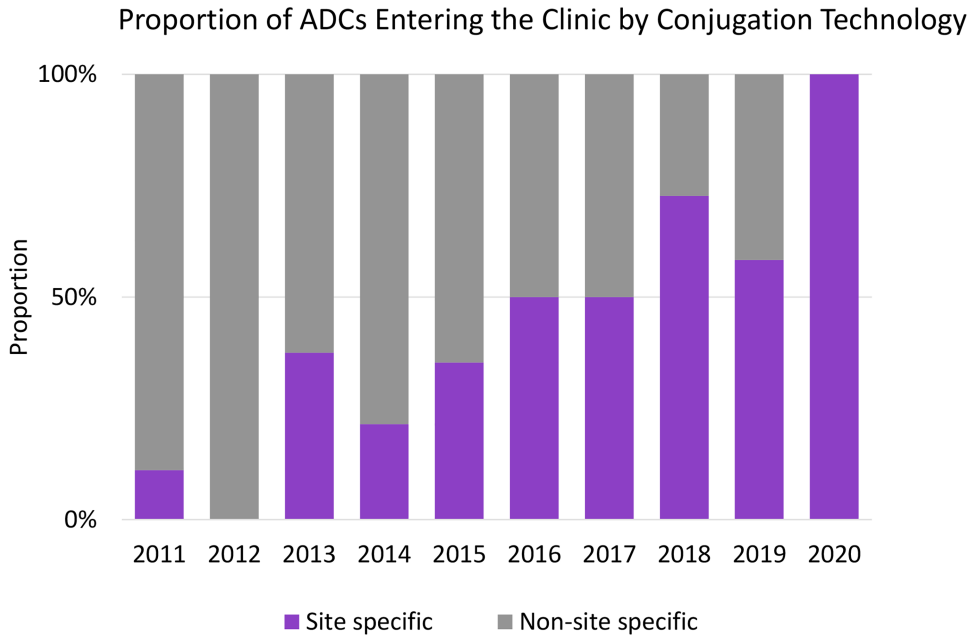


**Figure 7.** Site-specific ADCs entering clinical trials via conjugation technology over the years from 2011 to 2020. The percentage of site-specific antibody–drug conjugates (denoted in purple) entering clinical trials has steadily increased over the last decade. The upward trend of site-specific ADCs demonstrates a transition of prospective clinical ADCs to those constructed through site-specific conjugation. ADCs with undisclosed conjugation approaches are excluded. Cut-off date 3 September 2020. Insights provided by Beacon Targeted Therapies, Beacon-intelligence.com [110].

times (Fig. 6a); however, the aldol adducts can nevertheless be further conjugated as well [101].

*Reactivities of different amino acids*. In 2010, Witus and Francis reported that high yields, up to 90% conversion, can be achieved for some amino acid residues such as alanine, glycine, asparagine aspartic acid, and glutamic acid, as shown in Fig. 6a, whereas others have much lower conversion due to the formation of various by-products (i.e., PLP adducts) [101]. Of note: aspartic acid undergoes decarboxylation before transamination [103, 106].

*Cyclization: N-terminal modification of antibodies via one-step cyclization using pyridine carboxaldehyde (2PCA)*. Another practical one-step site-specific N-terminal conjugation was first reported in 2015 by Francis, Macdonald, and co-workers, which included the reaction of 2-pyridinecarboxyaldehydes (2PCA) to form imidazolidinone by cyclization of the adjacent amide. It proceeds through an imine to afford stable imidazolidinone as the final product [102]. Thus, the reaction is selective against the amine on the lysine side chain, which does not have an amide, resulting in imidazolidinone formation with aldehydes, Fig. 6(b,c).

*Potential issues and limitations: pyroglutamic acid formation*. One potential limitation is the formation of pyroglutamic acid (PyroGlu or PyroE) at the N-termini of proteins, including antibodies. If the N-terminal residue is glutamine (Gln) or glutamic acid (Glu), pyroglutamic acid can be formed readily; the resulting product is an amide, which resists further reactions for amines. Moreover, both glutamine and glutamic acid are common at the N-termini of antibodies, hence Pyro-Glu formation. For instance, in 2006, Chelius *et al.* [107] reported pyroglutamic acid formation from N-terminal glutamic acid residues in both light and HCs of recombinant monoclonal antibodies. In 2011, Flynn *et al.* [108] showed the conversion of glutamate residues at the N-terminal of human IgG2 antibody into pyroglutamate *in vivo*. Since antibodies contain two N-termini, the formation of Pyro-Glu at one N-terminus may enhance the remaining N-terminus' selectivity.

*Clinical trials*. To our best knowledge, at present, no ADCs are using N-terminal conjugation chemistry entering therapeutic studies. One reason may be the concern that N-termini's close proximity to the antigen-binding sites could potentially perturb binding affinity or specificity. On the other hand, such circumstances can be readily addressed by standard tests.

## ADCs IN CLINICAL TRIALS

Site-specific conjugation of native antibodies has several advantages that lend to their clinical translation (Table 1). First, these antibody conjugates are homogenous and well-defined constructs [1]. Second, these conjugates are more stable in plasma [6, 109], have enhanced binding efficiency [1], as well as increased cellular uptake [6] compared with non-specific methods. From 2011 to 2020, several site-specific ADCs have entered clinical trials, as illustrated in Fig. 7. As a general trend, the percentages of site-specific ADCs have been steadily increasing over the years, and is 100% in 2020.

## FUTURE PERSPECTIVE

Genetic engineering methods [1, 2] have been used to construct antibody conjugates. For instance, reactive

cysteine (i.e., THIOMAB) was introduced into antibodies and evaluated in preclinical settings [94, 113, 114]. Other approaches include the incorporation of unnatural amino acids [115], which introduce a chemical handle for further conjugation; for example, an ADC based on para-acetyl phenylalanine (pAF) is being developed by Ambrx [116] and is currently in the clinical trial (NCT03255070). In addition, tags incorporated genetically to antibodies have been chemo-enzymatically conjugated using formyl glycine-generating enzymes (FGE; NCT03682796) and sortase [117–120]. These genetic approaches are orthogonal to the site-specific methods presented herein. Therefore, antibodies can be conjugated by both methods in a combinatorial fashion to produce multi-purpose conjugates.

Coupling site-specific conjugation and biorthogonal click chemistries, a diverse array of antibody conjugates can be envisioned. Various linkers can be coupled to native antibodies site-selectively, including exogenous cleavable linkers using chemical or light [51], endogenous cleavable linkers mediated by proteases, pH, or redox [121], as well as non-cleavable linkers. The design of these linkers can be branched (e.g., dendrimer) or polymers with higher payloads (e.g., branched polymers that are currently in the clinic) [122]. These advances will undoubtedly enable more efficacious and multi-functionalized antibody conjugates for many applications. Indeed, the future applications will combine different aspects and advantages of multiple approaches, as we outline in our Hybrid Modality Engineering of Protein concept.

## ACKNOWLEDGMENTS

We would like to thank Tyler Dost and Olaf Tobias Bjornstal for providing their critical review of the manuscript and the reviewers for helpful discussion.

## FUNDING

A.S thanks the support of Amgen Wolfgang Goetzinger Scholar Awards in Life Science Analysis and John Hatsopoulos Scholar Award. This work was supported by Alpha Fund (Center for Entrepreneurship Education) by Northeastern University, the National Science Foundation's Innovation Corps (I-Corps) program (NSF Award 1735260 to Marc H. Meyer at Northeastern University), and the National Institute of Health (National Institute of General Medical Sciences 1R01GM101396 to Z.S.Z.).

## CONFLICTS OF INTEREST STATEMENT

M.E.D. is an employee of Millennium Pharmaceuticals, Inc., a wholly owned subsidiary of Takeda Pharmaceutical Company Limited.

Z.S.Z. holds the position of Editorial Board Member for Antibody Therapeutics and is blinded from reviewing or making decisions for the manuscript.

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