The AAPS Journal, Vol. 17, No. 2, March 2015 (© 2015)
DOI: 10.1208/s12248-014-9710-8

Review Article

Theme: Critical Considerations for Design and Development of Antibody Drug Conjugates
Guest Editors: M. Tabrizi, I. Figueroa, and S. Sadekar

Antibody Drug Conjugates: Design and Selection of Linker, Payload and Conjugation Chemistry

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Received 18 September 2014; accepted 19 December 2014; published online 22 January 2015

Abstract. Antibody drug conjugates (ADCs) have emerged as an important pharmaceutical class of drugs designed to harness the specificity of antibodies with the potency of small molecule therapeutics. The three main components of ADCs are the antibody, the linker, and the payload; the majority of early work focused intensely on improving the functionality of these pieces. Recently, considerable attention has been focused on developing methods to control the site and number of linker/drug conjugated to the antibody, with the aim of producing more homogenous ADCs. In this article, we review popular conjugation methods and highlight recent approaches including "click" conjugation and enzymatic ligation. We discuss current linker technology, contrasting the characteristics of cleavable and non-cleavable linkers, and summarize the essential properties of ADC payload, centering on chemotherapeutics. In addition, we report on the progress in characterizing to determine physicochemical properties and on advances in purifying to obtain homogenous products. Establishing a set of selection and analytical criteria will facilitate the translation of novel ADCs and ensure the production of effective biosimilars.

KEY WORDS: ADC; antibody drug conjugate; biopharmaceutics; enzymatic ligation; therapeutics.

Antibody drug conjugates (ADCs) couple the highly desirable pharmacokinetic (PK) profile and targetability of monoclonal antibodies (mAbs) with the potent cytotoxicity of small molecule drugs. Such a combination can potentially minimize dose-limiting toxicities while maximizing desired therapeutic effects. Yet, initial ADCs pairing standard anti-cancer agents, such as doxorubicin, were ineffective in clinical trials (1). These failures were linked to (1) the limited number of drug molecules that can be conjugated to one antibody without affecting antigen binding and (2) the limited number of antigens on target cell surfaces, preventing therapeutic levels of drug accumulation in cells. To date, the most successful approaches to overcome these challenges are improved linker technology and the selection of extremely potent drugs to the pair with the antibody (e.g., ado-trastuzumab emtansine and brentuximab vedotin) (2,3). Innovations in linker design are focused on multiple issues ranging from serum stability to mechanism of release to drug to antibody ratio (DAR). As linkers become increasingly sophisticated, more emphasis is being placed on the methods of bioconjugation between linker and antibody, with the goal of producing homogeneous ADC populations. Several methods of characterization are now employed to assess the composition of such conjugates and to increase our understanding of correlations between ADC structure and efficacy. These many facets of ADC synthesis will be addressed in this review.

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CONJUGATION

The majority of ADCs are built on IgG1 scaffolds. These complex, ~150-kDa biomolecules contain multiple native sites for conjugation and can be modified to include additional reactive sites. Most conjugation methods involve nucleophilic residues, while others use special genetic engineering techniques to introduce electrophilic handles such as aldehydes or ketones. In any approach, chemical reactive sites at the scaffold surface must be utilized and conjugation must not affect biophysical integrity.

Non-specific Conjugation Through Native Residues

Reactive side chains of naturally occurring amino acids such as lysine and cysteine are attractive sites of conjugation. The main advantage of linkage through native residues is facile reactivity that does not require preliminary processing/modification of the antibody. The main disadvantages of these methods are the variability and heterogeneity of the resulting products (4,5).

The IgG scaffold has over 80 lysines. With over 20 residues found at highly solvent-accessible sites, conjugation to lysines leads to a wide range of possible drug to DARs at varying conjugation sites (6). For example, amidation of

lysines to produce trastuzumab emtansine (T-DM1) results in an average of 3.5 drugs attached per antibody molecule (2). Separately, Acchione *et al.* reported average DARs between 6 and 14 depending on the equivalents of a model linker used (4). The heterogeneous mixtures produced contain several species that are difficult to purify and characterize (7).

Non-specific conjugation often alters the electrostatic properties (isolectric points) and hydrophobicity of the parent antibody, which influence ADC stability and PK (5). An in-depth analysis of antibody stability by Wankakar and co-workers found that most solvent-accessible lysines are located in the CH_2 domain and that conjugation in this region destabilizes the antibody and leads to aggregation (8).

Cysteines are less prevalent in IgGs than free amines and are more uniformly distributed. There are 16 cysteine pairs in a full IgG scaffold: 12 intra-chain and 4 inter-chain disulfide bonds. Due to greater solvent accessibility, the four inter-chain disulfide bonds are the main targets for conjugation (9). Disulfide bonds are integral structural components of the IgG scaffold but must be reduced prior to modification. Therefore, reduction–oxidation conditions must be carefully controlled to allow for conjugation while ensuring overall structure integrity. Despite improved dispersity, cysteine conjugation still results in a mixture of products with varying sites and number of drugs attached (Fig. 1) (9,10). Due to the limited number of potential sites (DAR≤8), this method produces ADCs that are easier to characterize than the lysine coupling method with lower DARs, a feature that has been correlated with increased efficacy (11,12).

To further direct the site of attachment, McDonagh *et al.* systematically replaced cysteine residues with serine to limit the available disulfide bridges for conjugation (13). They reported high conversion rates (89–96%), creating mixtures of conjugates with DARs of 0, 2, 4, and 8. The products were purified by hydrophobic interaction chromatography (HIC), affording nearly homogeneous mixtures (80% DAR=4).

Overall, utilizing cysteines from inter-chain disulfide bridges is an effective strategy that does not require expensive and time-consuming reengineering of the antibody structure. The major limitation is the inability to produce truly homogenous populations without extensive purification. Disrupting inter-chain disulfide bonds may also compromise the physical stability of antibodies (14).

An innovative approach by Godwin and co-workers addressed the instability associated with reducing disulfide bonds by utilizing a bis-thiol linker to maintain inter-chain bridging while incorporating a drug payload. In this method, the linker is inserted between two reduced bridging cysteines (Fig. 2) (15). Thus, the DAR can be controlled by the concentration of the reducing agent and the stoichiometry of reactants. For example, a fully reduced trastuzumab antibody was treated with six equivalents of bridging linker, resulting in a mixture containing 78% of a conjugate with a DAR of 4. Milder reducing conditions and fewer equivalents of linker yielded a DAR of 2.8. Although this method does not necessarily lead to more homogeneous ADCs, it does provide the ability to reliably prepare ADCs with lower DAR while maintaining the intact antibody structure.

Although the majority of reported work is focused on lysine and cysteine nucleophiles, any reactive side chain of native amino acids, such as the hydroxyl group of tyrosine, could serve as a target for conjugation (16). The advantages of using native residues are facile expression and minimal purification prior to conjugation; however, product heterogeneity can lead to inconsistent PK profiles (17). Despite the inherent dispersity, all three FDA-approved ADCs to date utilize native lysines or cysteines.

Site-specific conjugation through genetically engineered sites

To increase the site specificity of ADC conjugation, reactive handles can be introduced by altering amino acid sequences. A simple approach pioneered by Lyons *et al.* and later built upon by Kull and co-workers introduces a single solvent-accessible cysteine into an IgG4 scaffold, providing a site-specific handle for conjugation (18,19). After expression and reduction to remove cysteine or glutathione adducts, the mutant antibodies were functionalized using a bromoacetyl-linked payload. Junutula and co-workers improved this method by screening for more suitable modification sites, naming the resulting ADCs "THIOMABs" (20). Using carefully optimized reduction conditions, conjugates with as high as 92% DAR=2 and conversions of over 98% were achieved. This efficiency makes this technology more amenable to industrial-scale production; however, the required reduction prior to conjugation remains a liability. In addition, cysteine incorporation often leads to aggregation caused by disulfide bridging between antibodies.

Several limitations of this method may be avoided by incorporating more discriminate residues, especially unnatural amino acids. Alternatively, ligating enzymes can be used to catalyze bond formation between specific sequences or chemical groups.

Unnatural Amino Acids

The standard genetic code has been allocated to include unnatural amino acids in proteins (21). Axup et al. used this approach in successfully incorporating a *p*-acetylphenylalanine (pAcPhe) group into the heavy-chain Fab region of an anti-Her2 antibody (22). A drug molecule bearing a terminal alkoxyamine or hydrazide can be coupled to the keto group by analine-catalyzed condensation to form a stable oxime or hydrazone bond. They generated ADCs linking alkoxy-amine functional auristatin molecules to pAcPhe (Fig. 3). This site-specific and highly efficient conjugation did not interfere with antigen binding, and the resulting ADCs were shown to be homogeneous (DAR=2) by SDS page and ESI/MS with >95% coupling. This method is limited, however, by the requirement for extensive genetic engineering and the notorious inefficiency of unnatural amino acid incorporation (23). In addition, oxime formation requires long reaction times of 1–4 days.

Selenocysteine (Sec) is a rare but naturally occurring amino acid that features a highly nucleophilic selenol group. Insertion of selenocysteine residues into proteins does not require synthetic tRNA, but only that the 3′ end of cDNA is modified to include a selenocysteine insertion sequence (24). Once a Sec-containing antibody is isolated, maleimide or iodoacetamide can be used to create selenoether conjugates (25). One group sought to increase the DAR of Sec antibodies by inserting multiple Sec residues into the C termini of full IgGs and fragments (26). Similar to cysteine

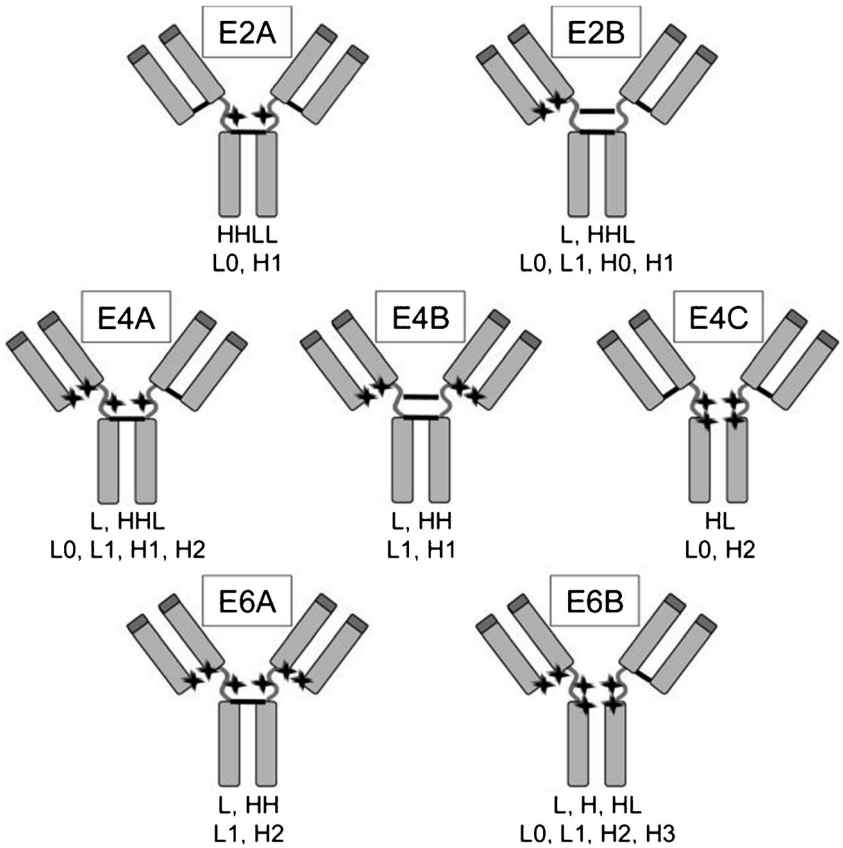

Fig. 1. Potential isomers from native cysteine conjugation. The locations of conjugation are indicated by *stars* and intact disulfide bonds are shown as *bars*. Below the isomer are the chain compositions under denaturing conditions (*first line*, nonreducing; *second line*, reducing). For denaturing and nonreducing conditions, the possible species formed are L, H, HL, HH, HHL, and LHHL. For denaturing and reducing, the possible species formed are L0, L1, H0, H1, H2, and H3, in which the *numbers* indicate how many drug molecules are attached to the light or heavy chain (adapted with permission from *Bioconjug. Chem. 16*, 1282–90. Copyright 2005 American Chemical Society)

conjugation, selenocysteines require reduction for nucleophilic activity, raising concerns about concomitant reduction of disulfide bridges within the antibody.

In the field of bioconjugation, there are a multitude of unnatural amino acid strategies to explore, each with distinctive reactivity and properties. For ADC development, systematic evaluation is imperative to establish criteria to pair the location and functionality of a given unnatural amino acid with a desired outcome (e.g., DAR, release, etc.).

Enzymatic Ligation

Recently, Sortase A (SortA)-mediated peptide coupling has seen increasing utility in various bioconjugation

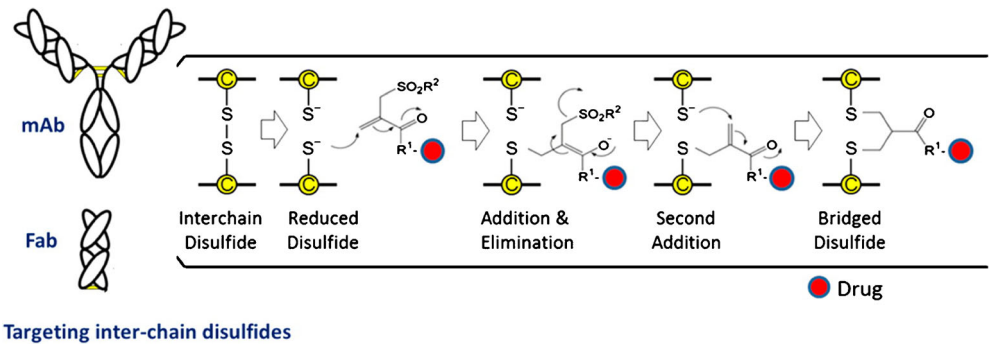

Fig. 2. A bis-thiol reactive linker was used to cross-link reduced disulfide bonds and simultaneously incorporate a drug. The method required a PEG_{25} chain due to low solubility of the linker and payload (shown as R^1) (adapted with permission from *Bioconjug. Chem. 25*, 1124–36. Copyright 2014 American Chemical Society)

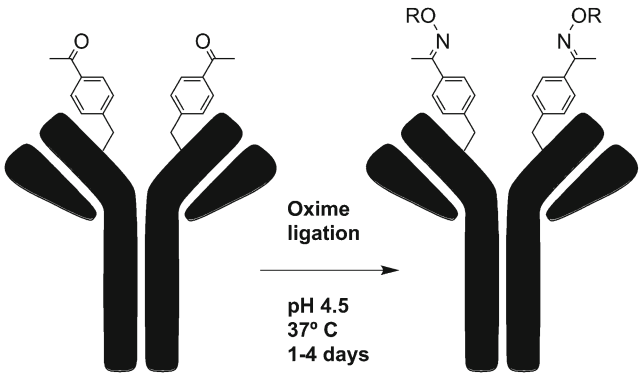

Fig. 3. Site-specific conjugation of alkoxy-amine-derivatized auristatin to anti-Her2 Fab and IgG with pAcPhe. The IgG is coupled by oxime ligation to drug derivitized with a terminal alkoxy-amine through pAcPhe residues (adapted from *Proc. Natl. Acad. Sci. U. S. A. 109*, 16101–6)

applications. SortA recognizes a C-terminal pentapeptide sequence (LPXTG) and creates an amide bond between threonine within the sequence and glycine in the N-terminus of the conjugation partner (Fig. 4) (27).

There are several examples of SortA ligations in the development of next-generation ADCs (28–30). NBE-Therapeutics, for example, recently announced a patent-pending Sortase-mediated antibody conjugation ($SMAC_{TM}$)-Technology. This technique has been used to conjugate biotin functional handles to the C-terminus of a single-chain Fv fragment derived from an anti-EGFR antibody (27). A more recent account demonstrates the fusion between the heavy-chain C terminus of an anti-Her2 Fab and the 30-kDA plant toxin gelonin (29). Levary and co-workers verified the flexibility of SortA by ligating a variety of oligo-glycine-tagged biomolecules to IgGs without disrupting antigen binding (28).

One advantage of using SortA is that the recognition sequence can be incorporated to either conjugation partner.

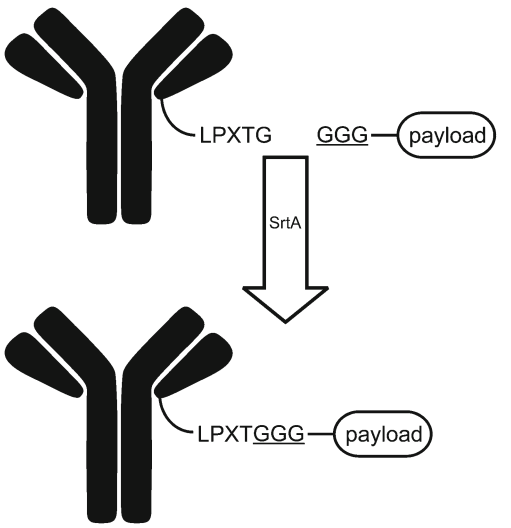

Fig. 4. Structures of N and C-terminal fusion partners denoting site and sequence of sortase A recognition motifs of each domain (adapted from Levary DA, Parthasarathy R, Boder ET, Ackerman ME (2011) Protein–Protein Fusion Catalyzed by Sortase A. PLoS ONE 6(4): e18342. Copyright 2011 Levary et al.)

Further, the LPXTG sequence does not require any unnatural amino acids, allowing expression to be carried out under a wide variety of conditions. A potential disadvantage is that SortA ligation is currently limited to C and N termini.

Another example of enzymatic ligation employs bacterial transglutaminases (mTGs) that catalyze the coupling of glutamine side chains to alkyl primary amines, such as lysine. Conveniently, bacterial mTGs are unable to modify glutamine residues in native IgG1s. Schibli and co-workers reported, however, that deglycosylating IgGs at N297 exposed a glutamine residue at the 295 position to enzymatic ligation to create ADCs with a DAR of 2 (31). Further, by producing a N297 to Q297 mutant IgG1, they were able to introduce two viable sites for enzymatic labeling to create ADCs with a DAR of 4 (31,32).

This method has also been used to modify native IgGs through the development of a unique "glutamine tag" (LLQG) that can be incorporated at variable locations in the antibody scaffold (Fig. 5) (33). Strop *et al* identified as many as 12 sites within an anti-EGFR antibody amenable to efficient conjugation (as high as 99% with a DAR=2). Several of these sites were verified for compatibility and broad applicability using an anti-Her2 or anti-M1S1 antibody.

Rabuka and co-workers offer an interesting example that combines enzymatic ligation with unnatural amino acids (34). By encoding a short consensus sequence, CXPXR (where X is serine, threonine, alanine, or glycine), a formylglycine residue, can be introduced by co-expressing the gene with formylglycine-generating enzyme (FGE). FGE oxidizes cysteine residues within the consensus sequence to formylglycine residues (fGly) with conversion efficiencies of up to 98%. Drugs can be attached through the aldehyde functionality of fGly by means of a Hydrazino-iso-Pictet-Spengler (HIPS) ligation. HIPS proceeds with efficiencies above 90% and creates a carbon-bonded heterocyclic linkage that is stable under physiological conditions.

Guided by the X-ray crystal structure of a human IgG1, these researchers used in silico analysis to identify potential locations for aldehyde tag incorporation based on maximum solvent accessibility and minimal immunogenicity. Using a library approach, eight model antibodies were assessed for their propensity for aggregation. Based on these results, three trastuzumab mutants were expressed and conjugated to a maytansine derivative and characterized *in vitro* and *in vivo*. Interestingly, each ADC variant exhibited a unique PK profile, although minimal changes to FcRn binding were observed.

The varying properties that result from each type and site of enzymatic ligation portend the questions of how to correlate ligation location with desired PK. The versatility in location of consensus sequence incorporation could increase the potential to include advanced linker features.

Oligosaccharides

All native mAbs exhibit some level of post-translation glycosylation, bestowing an additional site-specific target for conjugation (35). Glyco-conjugation is advantageous if limited conjugation sites for drugs are desired as a DAR of around 2 is easily achievable. Importantly, the antibody scaffolds often require minimal derivitization to achieve

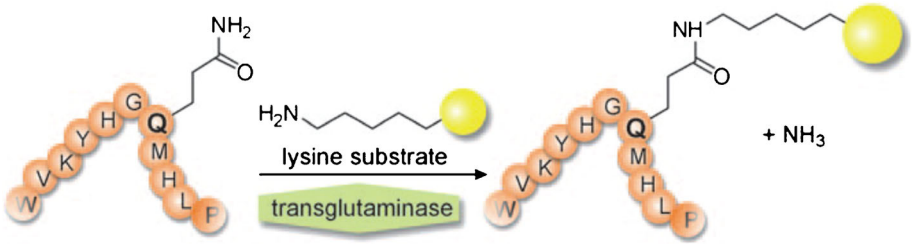

Fig. 5. A glutamic acid side chain found in a conserved amino acid sequence is ligated to a lysine side chain by transglutaminase (adapted with permission from Jeger, S., Zimmermann, K., Blanc, A., Grünberg, J., Honer, M., Hunziker, P., Struthers, H. and Schibli, R. (2010) Angew. Chem. Int. Ed., 49: 9995–9997. Copyright 2010 Wiley Periodicals Inc.)

efficient conjugation. Initial accounts of carbohydrate-based conjugation involved the oxidation of terminal sugar residues using periodate to yield aldehyde functionalities. Strong oxidizing conditions can lead to undesired oxidation of protein residues that can potentially disrupt antibody binding and stability (36). Zhao and co-workers were able to use milder conditions alongside glycoremodeling to produce ADCs with DAR of 1.5–1.7 (37). Using a two-step process, native sugars are modified with terminal sialic acid residues that are oxidized to generate aldehydes, which can then be coupled with primary amines by reductive amination or with hydrazides in aniline-catalyzed condensation to create acid-labeled hydrazones or with aminooxy groups to form oximes (Fig. 6) (37–39). Nevertheless, even under mild conditions, off-site oxidation may interfere with native antibody structure and function as observed during the development of gemtuzumab ozogamicin (Mylotarg) (36).

Qasba and colleagues circumvented detrimental oxidizing conditions by introducing bioorthogonal functional sites. They first homogenize mAb glycoforms via galactosidase digestion and subsequently integrate keto- or azide-modified galactose residues using mutant galactosyltransferases (40,41). Similarly, a recent report by Senter and co-workers exploits the promiscuity of native fucosyltransferases to incorporate thiol-functional fucose analogues for thiol-maleimide conjugation to MMAE (42). This method, however, requires reduction/oxidation steps to selectively liberate active thiol nucleophiles from cysteine adducts and suffers from limited integration efficiency (60–70%). Regardless, fucosyl engineering is of interest due to evidence that afucosylated IgGs exhibit significant enhancements in antibody-dependent cell-mediated cytotoxicity (ADCC) (43,44).

Current examples of glyco-conjugated ADCs often fail to discuss the effects of conjugation on the physical stability of the IgG structure. It has been suggested that this method prevents aggregation by producing a more homogeneous ADC population with lower DARs (37). In theory, structural integrity may be unaffected by drug conjugation provided that stabilizing interactions between the inner core sugars and the CH_2 domain are not disrupted. These weak interactions serve to promote an optimal "open" conformation within the Fc. Conversely, complete deglycosylation closes the CH_2–CH_2 gap, leading to IgG destabilization, aggregation, and therefore diminished effector functions (45,46). Unfortunately, it is currently difficult to predict how glyco-modification will affect Fc-receptor binding and related activity, although studies are reported to be forthcoming.

UV Cross-Linking

Bilgicer *et al.* reported a unique example of site-specific functionalization that does not require genetic engineering or pre-activated scaffolds (47,48). Payloads featuring a UV-reactive indole-3-butyric acid (IBA) moiety can be covalently linked to an IgG at a known "nucleotide binding site" (NBS)

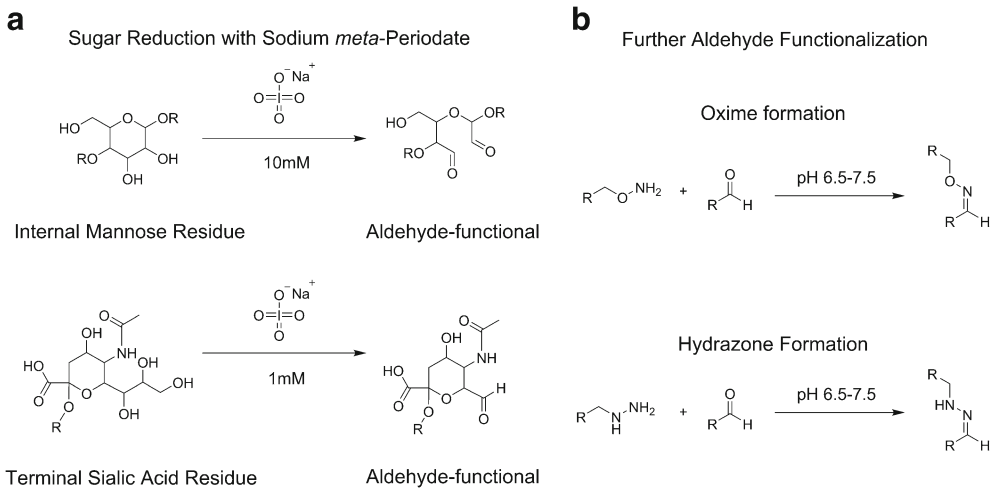

Fig. 6. Native sugars in the glycosylated sites of antibodies can be conjugated to functional linkers in a two-step process: **a** internal mannose or terminal sialic acid residues are reduced to aldehydes and **b** aldehydes react with linkers containing hydroxylamine or hydrazines to produce oxime and hydrazones, respectively

upon exposure to 254-nm light (Fig. 7). A computational analysis suggests that a phenylalanine residue at position 42 within the aromatic NBS region is the site of cross-linking. Conjugates of rituximab with an average DAR of 1.5 were synthesized using this method, confirming that each antibody is able to cross-link one or two IBA molecules in a concentration-dependent manner. The optimal UV intensity was found to be between 0.5 and 5 J/cm^2, above which damage to the CDR can occur.

LINKER

Although many drugs are amenable to direct conjugation to an antibody scaffold, heterobifunctional linkers often facilitate ADC bioconjugation. At the most basic level, linkers provide a functional handle for efficient conjugation to antibodies through methods described in the previous section. More sophisticated linkers increase effector solubility, improve stability throughout the production process, prevent premature drug release, and facilitate the liberation of active drug at the target. Critical aspects of linker chemistry include the functionality that allows conjugation to antibody, the mechanism for drug release, and the physical properties of the linker itself.

Reactive handles often featured in linker chemistry are grouped based on the site of conjugation. *N*-hydroxysuccinimide esters are the most common choice for functionalizing amines, especially when coupling to ε-lysine residues. For conjugation to cysteines, thiol-reactive maleimide is the most applied handle, although it is also possible to create a disulfide bridge by oxidation with a linker bearing a sulfhydryl group. Aldehyde or keto functional groups such as oxidized sugar groups or pAcPhe unnatural amino acids can be reacted with hydrazides and alkoxyamines to yield acid-labile hydrazones or oxime bonds. In addition, a hydrazine can be coupled with an aldehyde via HIPS ligation to generate a stable C–C linkage. As additional forms of copper-free click chemistry and unnatural amino acid incorporation become more efficient, it is possible that related methods will see more utility in the future.

The mechanism of drug release is an important consideration in linker selection. Non-cleavable linkers rely on degradation of the scaffold within the lysosome after internalization. Alternatively, cleavable linkers respond to physiological stimuli such as low pH, high glutathione concentrations, and proteolytic cleavage. Each strategy has inherent advantages and disadvantages, but ultimately the optimal combination of linker and conjugation chemistry must be uniquely tailored to correlate each unique facet: the antibody, the drug molecule, and the profile of the disease to be treated.

Non-cleavable Linkers

Several non-cleavable alkyl and polymeric linkers have been explored in ADC development. A notable example is the MCC amine-to-sulfhydryl bifunctional cross-linker featured in T-DM1 (Fig. 8a) (49). This linker is especially useful as the cyclohexane ring provides steric hindrance that decreases the rate of hydrolysis of the resulting thioether.

The greatest advantage of using non-cleavable linkers is their increased plasma stability when compared to many cleavable linkers. Despite the limited "bystander" effect, the resistance to cleavage outside of target cells may actually increase the specificity of drug release. Several *in vivo* studies and clinical data, for example, have shown that non-cleavable linked ADCs outperform their cleavable counterparts *in vivo* (2).

Non-cleavable linkers require mAb degradation within the lysosome after ADC internalization to release active drug. With this mechanism, differences between parent drug and potential ADC metabolites must be taken into consideration. For example, MMAE, a protein-based anti-mitotic drug, is most potent in its native form and is therefore poorly suited for derivitization with non-cleavable linkers. Conversely, MMAF retained its potency even when linked with a simple alkyl chain *in vitro* and *in vivo* (50). One proposed mechanism for the decreased efficacy of non-cleavable linked ADCs is that drugs bearing charged amino acids suffer from decreased membrane permeability, limiting their ability to kill nearby cells. A major motivation for employing cleavable linkers is to improve this "bystander" effect (51).

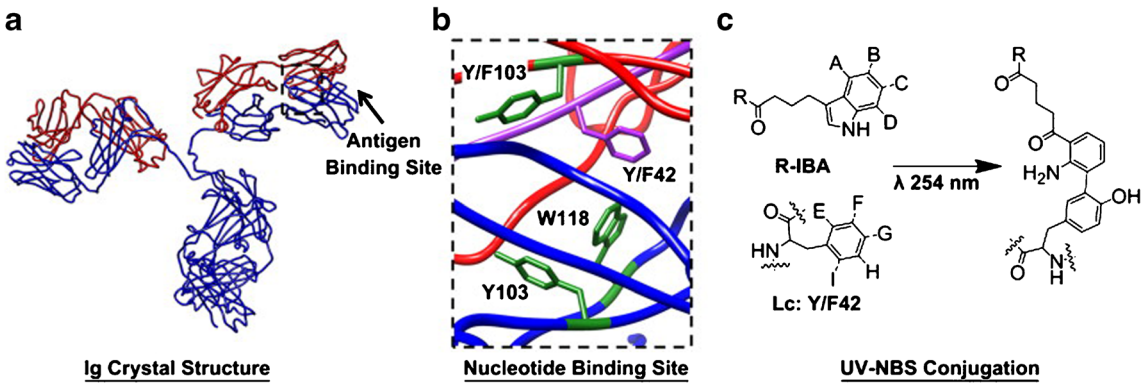

Fig. 7. **a** IgG antibody crystal structure: light chains (*red*), heavy chains (*blue*), nucleotide binding site (*NBS*, *boxed*). **b** Rituximab (PDB: 2OSL) with the four NBS residue side chains depicted, two on the light chain and two on the heavy chain; site of conjugation highlighted in *purple*. **c** Proposed UV-NBS cross-linking mechanism between the IBA-ligand (R-IBA) and NBS light chain residue Y/F42 (reprinted from Alves, N. J.; Champion, M. M.; Stefanick, J. F.; Handlogten, M. W.; Moustakas, D. T.; Shi, Y.; Shaw, B. F.; Navari, R. M.; Kiziltepe, T.; Bilgicer, B. *Biomaterials* 2013, *34*, 5700–5710, with permission from Elsevier)

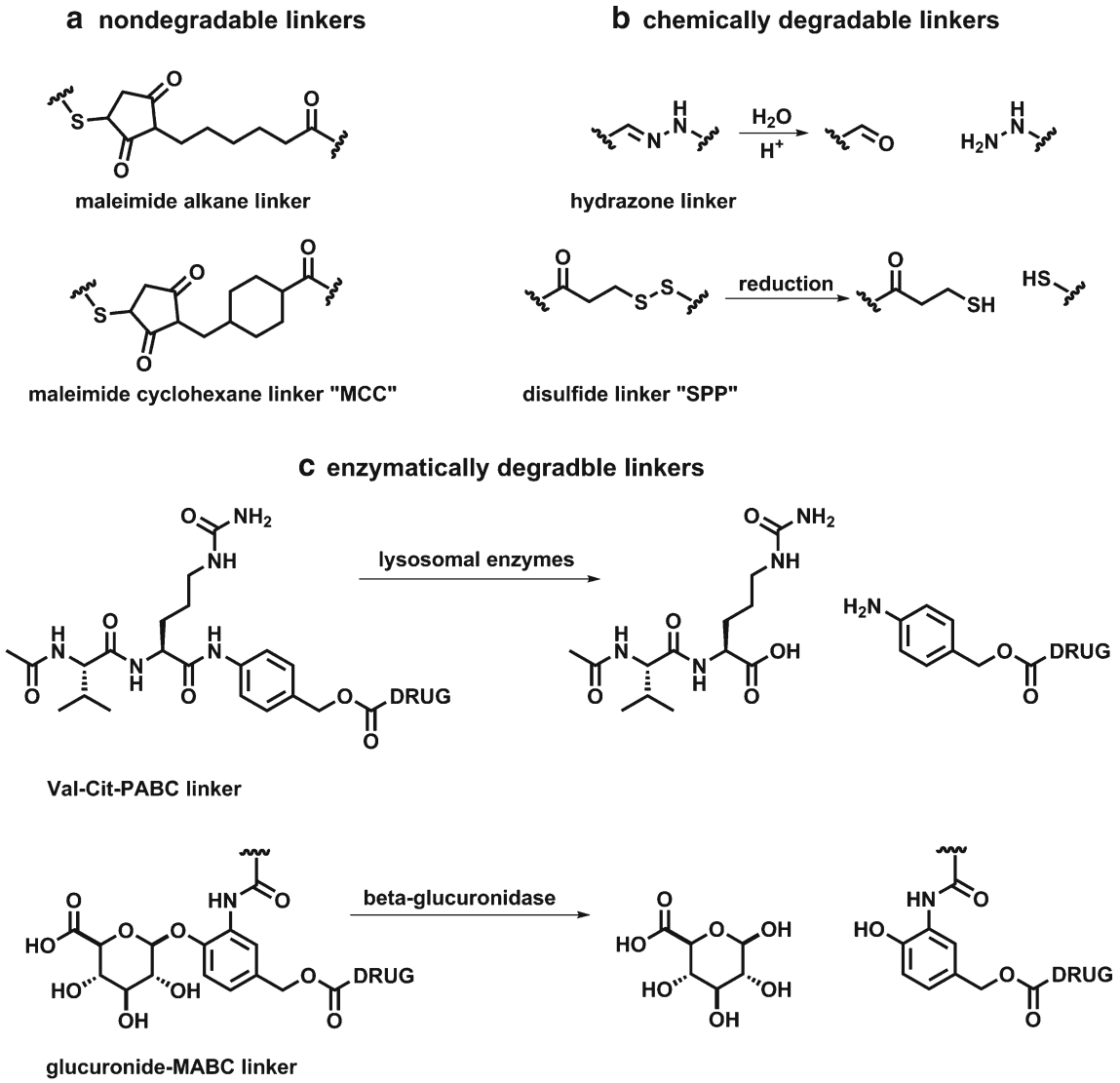

Fig. 8. Linkers provide a functional handle to conjugate drug payloads to the antibody scaffold. One important aspect of linker chemistry is the mechanism of drug release. Representative examples of each type are shown: **a** nondegradable linkers, **b** chemically degradable linkers can be cleaved by hydrolysis or reduction, and **c** enzymatically degradable linkers are first cleaved and may further degrade by inclusion of self-immolative benzyl-alcohol spacers

Chemically Labile Linkers

Cleavable linkers are popular in the ADC clinical pipeline with acid-sensitive linkers such as hydrazones and silyl ethers at the forefront (52). Hydrazones are easily synthesized and have a plasma half-life of 183 h at pH 7 and 4.4 h at pH 5, suggesting that they are selectively cleavable under acidic conditions such as those found in the lysosome (Fig. 8b) (10). The first-generation ADC gemtuzumab ozogamicin (Mylotarg) contains a hydrazone linker that was deemed necessary for drug potency (36). Acidic conditions, however, are often found in various places in the body, increasing the potential for nonspecific drug release. Mylotarg was recently withdrawn from the US market due to toxicities that were attributed in part to poor plasma stability of hydrazone, spawning the need to further tailor acid-labile linkers (53).

Disulfide bridges are envisioned to take advantage of the cellular reducing environment (54). After internalization and degradation, disulfide bridges can release drugs in the lysosome. Erickson *et al.* established that lysosomal processing is necessary for drug activation and found a significant population of lysine-bound, disulfide-linked drug among the metabolites of ADC degradation (55). These results suggest that the majority of disulfide-linked drugs are first liberated intact by proteolytic degradation of the antibody and only then released as active metabolites through disulfide exchange or by reducing agents such as glutathione. The methylated drug metabolite is then able to diffuse through the lipid membranes to the relevant site of action (56).

Although several side-by-side studies have shown that steric hindrance enhances the plasma stability of disulfide-linked conjugates, the factors governing disulfide-linked metabolite processing are poorly understood (57). This fact is exemplified by an account where a trastuzumab-MCC-DM1 conjugate outperforms several disulfide-linked conjugates, including trastuzumab-SPP-DM1 in *in vivo* models, a clear reversal of the proposed trend (2). Interestingly, the SPP

linker caused significant weight loss compared to the MCC analogue, hinting at potential toxicity of such conjugates. As such, the exact mechanism of disulfide-linked drug release remains elusive and should be elucidated to improve the efficacy of these linkers.

Enzymatically Cleavable Linkers

Enzymatically cleavable linkers are gaining significant attention in ADC development due to superior plasma stability and release mechanism. The most popular enzymatic cleavage sequence is the dipeptide valine-citrulline, combined with a self-immolative linker *p*-aminobenzyl alcohol (PAB). Cleavage of an amide-linked PAB triggers a 1,6-elimination of carbon dioxide and concomitant release of the free drug in parent amine form (Fig. 8c) (58).

A library of dipeptide linkers was screened by Debowchik and co-workers to measure the rate of doxorubicin release by enzymatic hydrolysis (59,60). They found that Phe-Lys was cleaved most rapidly with a half-life of 8 min, followed closely by Val-Lys with a half-life of 9 min. In stark contrast, Val-Cit showed a half-life of 240 min. They also found that removal of the PAB group reduced the cleavage rate, presumably through steric interference with enzyme binding. Substituting the PAB group for a glycine residue provides adequate spacing for cleavage but does not allow the release of free drug.

Another study compared the potency of auristatin derivative MMAE linked by dipeptide linkers Phe-Lys and Val-Cit and an analogous hydrazone linker. The Val-Cit linker proved to be over 100 times as stable as the hydrazone linker in human plasma. Most significantly, the Phe-Lys linker was substantially less stable than Val-Cit in human plasma, which accounts for its current popularity (10).

Non-peptide cleavable linkers are also being investigated. A glucuronide linker incorporates a hydrophilic sugar group that is cleaved by the lysosomal enzyme beta glucuronidase. Once the sugar is cleaved from the phenolic backbone, self-immolation of the PAB group releases the free drug. Initially, this linker was used to conjugate MMAE, MMAF, and doxorubicin propyloxazoline to various antibodies to create ADCs (61). In a subsequent study, glucuronide- and Val-Cit-PAB-linked ADCs were evaluated side by side for aggregation and efficacy. The glucuronide-linked conjugates show minimal aggregation (<5%) compared to dipeptide-linked conjugates, which show up to 80% aggregation. Though *in vitro* efficacy results were similar for the two ADCs, the glucuronide linker exhibited greater efficacy *in vivo*; however, the glucuronide-linked ADC was not well tolerated *in vivo* compared to Val-Cit-PAB (58).

Overall, enzymatically cleavable linkers provide antibody drug conjugates with plasma stabilities comparable to that of non-cleavable linkers while boasting a more defined method of drug release compared to disulfide-linked or acid-labile linkers. The ability to pair these linkers with self-immolative chemical groups bestows the release of free drugs with minimal derivation; the main constraint being the requirement for the drug to bear an amine or hydroxyl group to conjugate with PAB.

In concert with the numerous methods for bioconjugation to antibodies, the linker is an integral aspect of ADC development. The selection of linker should depend on the application and conditions a given antibody is likely to encounter.

PAYLOAD

The basic criteria for selecting the ADC payload are solubility, amenability to conjugation, and stability (62). In addition, the poor clinical efficacy of first-generation ADCs is attributed to sub-therapeutic levels of drug reaching the target. As such, drug potency is also a vital criterion for current ADC delivery mechanisms.

Lipophilic drugs readily pass cell membranes and therefore have a greater potential to escape the lysosome after release. Conversely, a potential payload must be sufficiently soluble to allow for conjugation to the antibody in aqueous buffers as high concentrations of organic solvent lead to antibody scaffold denaturing. The low solubility of many candidate payloads may be balanced by hydrophilic linkers, such as those containing sulfonates or poly(ethylene glycol), allowing for higher DAR than hydrophobic linkers such as SMCC (63).

Many potent drugs lack chemically functional handles that are necessary for conjugation. Modification to incorporate such handles can have deleterious effects on drug action. Likewise, conjugated drugs that are not released as the free, parent form may suffer decreased efficacy as is often seen when paired with non-cleavable linkers. Self-immolative linkers such as PAB facilitate the release of appended drugs back to the original unconjugated form; however, the PAB moiety itself is hydrophobic and may limit the use of certain payloads. As with solubility, a balance must be found between amending candidate drugs to allow conjugation and maintain efficacy.

Following conjugation, the payload must remain stable in circulation, through cellular processing and release, to reach the cytosolic target. Acid-sensitive drugs may degrade in the lysosome prior to reaching the site of action; disulfide-, alkene-, and epoxide-containing drugs may be reduced or transformed by cellular enzymes. Such drugs must be protected or modified.

Presently, the vast majority of ADC payloads in clinical trials fall into two categories: anti-mitotic or DNA damaging. The two most recently approved ADCs both contain anti-mitotic payloads (64,65). The effector in trastuzumab emtansine is a maytansinoid, and brentuximab vedotin employs an auristatin. The IC50s of each of these families of drugs are in the sub-nanomolar range. Additional anti-mitotic drugs investigated as ADC payloads include the taxanes and vinca alkaloids (66,67). DNA-damaging drugs including duocarmycin, pyrrolobenzodiazepine, calicheamicins, and doxorubicin have also been explored as ADC payloads. The first FDA-approved ADC, gemtuzumab ozogamicin, carries a derivative of calicheamicin with IC50 values in the low nanomolar range (36). Several calicheamicin-based ADCs, including inotuzumab ozogamicin, are currently being tested in clinical trials.

The physicochemical properties of ADCs limit the choice in payloads. Typically, a DAR >4 can diminish ADC solubility, impair binding, and influence PK. In addition, cellular trafficking of ADCs is restricted by the target antigen. Until these challenges can be circumvented, ADC payloads will be confined to extremely potent drugs with narrow therapeutic indices.

CHARACTERIZATION

Each of the components discussed can influence the stability, PK, and pharmacodynamics of an ADC. Significant efforts have been made in monitoring the physicochemical characteristics of ADCs (68); unfortunately, there are no standardized guidelines that correlate a specific characteristic with a given outcome. The major analytical techniques currently used to evaluate ADCs are mainly focused on DAR and dispersity. Several less-implemented techniques are also being explored for the routine evaluation of ADC properties, including dynamic light scattering (DLS) to measure aggregation (69), differential scanning calorimetry to measure changes in higher-order structure (4,8), IR-MALDI (70) to determine DAR, and immunoassays to detect disrupted binding of the Fc region.

UV/Vis

Possibly the most reported characteristic of ADCs is the DAR. UV/vis spectroscopy is the most commonly implemented technique to determine DAR. This molar ratio can be derived from the Beer–Lambert law by comparing the IgG absorbance maxima at 280 nm to that of a drug molecule in simultaneous equations (34,67,71–74). ADCs with a variety of payloads have been evaluated using this method, including DM1, methotrexate, calicheamicin analogues, and auristatins (11,75,76). Consideration should be given for any other contributions to the 280-nm absorbance and for the variance in extinction coefficients that result from different buffers (70,77). Moreover, UV/vis can only be used to determine the average DAR for the whole sample, unless orthogonal methods are employed to delineate the sample composition.

Chromatography

ADC synthesis often yields mixed products that differ in the number and site of conjugated payload. An assortment of chromatographic techniques can be applied to resolve ADCs based on characteristics specific to conjugation, linker, and payload. Capillary electrophoresis (CE) and HIC are especially useful in resolving native ADCs (9,78,79). Although size exclusion chromatography is most useful as a means of ADC purification, it can be used to characterize variants in ADC samples, particularly when aggregates are suspected; however, the hydrophobicity of the payload may lead to non-specific interactions with the stationary phase of columns. Importantly, conjugation of small drugs may not provide a large enough shift in hydrodynamic radius to be resolved by SEC (34,37,63,80,81).

Conjugating drugs onto the mAb structure can greatly alter their electrostatic characteristics, making charge-based separations such as CE useful in the characterization of ADCs (8,9,78). For example, conjugating through surface lysine residues can result in decreased positive surface charge when compared to the unconjugated mAb (82). Ion exchange chromatography and isoelectric focusing can offer information about such changes in ADC isoelectric point (5,83). These methods are not applicable to heterogeneous products with only small differences in isoelectric point.

Unnatural amino acid-linked conjugates and other site-specifically linked ADCs are well suited for characterization by standard reverse-phase high performance liquid chromatography (HPLC) using C18 columns (20). Direct characterization of intact inter-chain cysteine-linked ADCs is difficult, however. Disruption of the inter-chain disulfide bridges leads to instability under denaturing HPLC conditions due to incomplete covalent linkage between subunits; nevertheless, the heavy and light chains can be characterized separately (13).

HIC in particular is advantageous for characterization of intact ADCs because of the pH-neutral and non-denaturing salt gradients used for separation (79). This method is most amenable for relatively homogeneous conjugations such as conjugates formed by inter-chain disulfide linkage and those formed by other site-specific conjugation methods such as glycoengineering (9,11,20,37).

The information regarding ADC composition from chromatography is substantially enhanced by coupling sepa-

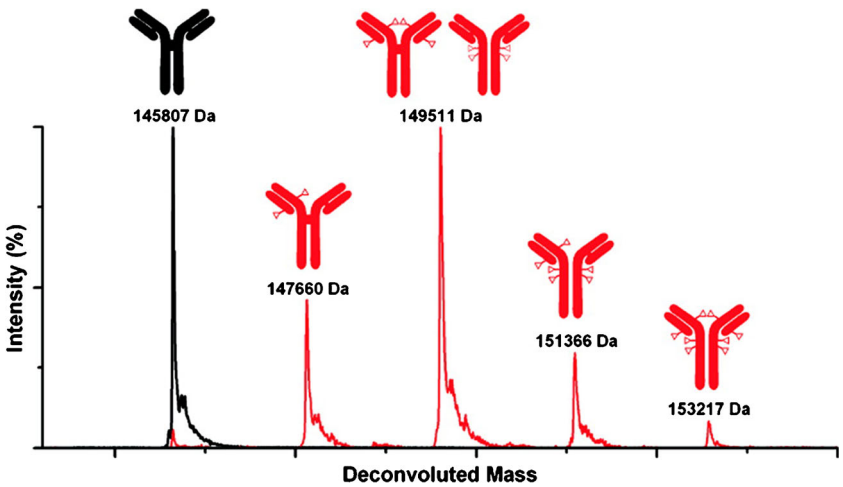

Fig. 9. ESI-MS is used to produce these deconvoluted mass spectra of a deglycosylated ADC in *red*. The ADC mixture of six different species in *red* is compared to that of the unconjugated mAb in *black* (reprinted with permission from *Anal. Chem. 84*, 2843–2849. Copyright 2012 American Chemical Society)

ration with other analyses such as UV/vis and mass spectrometry. For example, using HIC-UV/Vis, Hamblett and coworkers were able to separate and identify individual peaks corresponding to mAb species with zero to eight molecules of conjugated vc-MMAE (11).

Mass Spectrometry

Innovations in ionization techniques such as MALDI and ESI have expanded the utility of mass spectrometry (MS) to analyze larger macromolecules, including antibodies (68). These systems allow access to mass ranges over 4000 mass-to-charge ratio when paired with TOF or quadrupole detectors (84,85). The acquisition of mass spectra is relatively straightforward; however, it is important to remove all salts and other excipients that may interfere with protein ionization. Tandem liquid chromatography-mass spectrometry is often employed to simplify desalting and to improve resolution (7,86,87). In addition, the deglycosylation of mAb samples using enzymes such as PNGase is common practice and beneficial to simplify MS analysis.

MALDI-TOF MS can be used to detect mass shifts between parent mAb and ADC that correlate with payload conjugation. Although MALDI-TOF lacks mass accuracy to resolve individual species with different numbers of conjugated drugs, the obtained peak spectra can be analyzed to confirm conjugation and to determine the average DAR of the ADC. For example, Safavy and co-workers used MALDI-TOF to assess the DAR of paclitaxel-conjugated anti-EGFR antibodies (88).

ESI-MS produces "charge envelopes" of multiple-charged ionized molecules. This increases the accuracy and resolution of different DAR variants. Lazar and coworkers were able to analyze intact lysine-linked huC242-DM4 conjugates using ESI-MS. The complex chromatograms can be deconvoluted to reveal peaks of individual species. ESI-MS is also amenable to analyze intact disulfide-linked ADC, as demonstrated by Vallerie-Douglass *et al.* Using mild buffer conditions and by deconvoluting the resulting spectra, they were able to resolve individual forms of MMAF-conjugated antibodies (Fig. 9) (89). The observed DAR results were consistent with results from an orthogonal HIC analysis.

The combination of multiple analytical techniques can elucidate detailed information on ADC structure. Wang and colleagues paired enzymatic digestion with protein mapping to identify individual sites of conjugation within a huN901-DM1 conjugate (78). In addition to deglycosylation, ADCs were reduced by DTT, after which heavy and light chains were purified by SEC and subsequently digested and analyzed by ESI-MS. Through this method, they were able to identify seven light chain and 13 heavy chain modifications. Molecular modeling verified that the identified lysine sites are generally located at solvent-accessible regions with structural flexibility.

Many bioanalytical techniques are well established for the characterization of antibody drug conjugates, but these methods require precise selection and optimization for a specific mAb, drug, and linker chemistry. Well-defined and standardized analysis will become increasingly important as ADCs and many other biologics come under growing quality control scrutiny and standardization (90).

CONCLUSIONS AND PROSPECTUS

The versatility of antibodies and the increasing sophistication of bioconjugation methods have moved ADCs into the forefront of next-generation therapies for a widening pool of diseases. The content of this review demonstrates the vast diversity of strategies in ADC development, many of which are featuring a heavy focus on linker and conjugation chemistry. A systematic evaluation of the site and type of modification is essential to better understand the relationships between ADC structure, mechanism of action, and efficacy. Questions probing conjugation's effects on stability, the rate and specificity of payload release, and the resulting effects on PK must be thoroughly addressed. As site-specific conjugation becomes increasingly reliable, so will the opportunities for elegant linking systems be. Finally, advanced methods of characterization will be extremely important as this class of biologics is translated to industrial-scale production.

Future work in this area must address the limitations posed by the narrow therapeutic indices of highly potent drugs such as ematansine and vedotin. Control of cellular internalization, the targeting of multiple pathways, and the attachment of multiple orthogonal payloads may be promising areas of interest in the development of next-generation ADCs. As our understanding of antigen biochemistry increases, ADCs may see more utility in disease areas outside of cancer.

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