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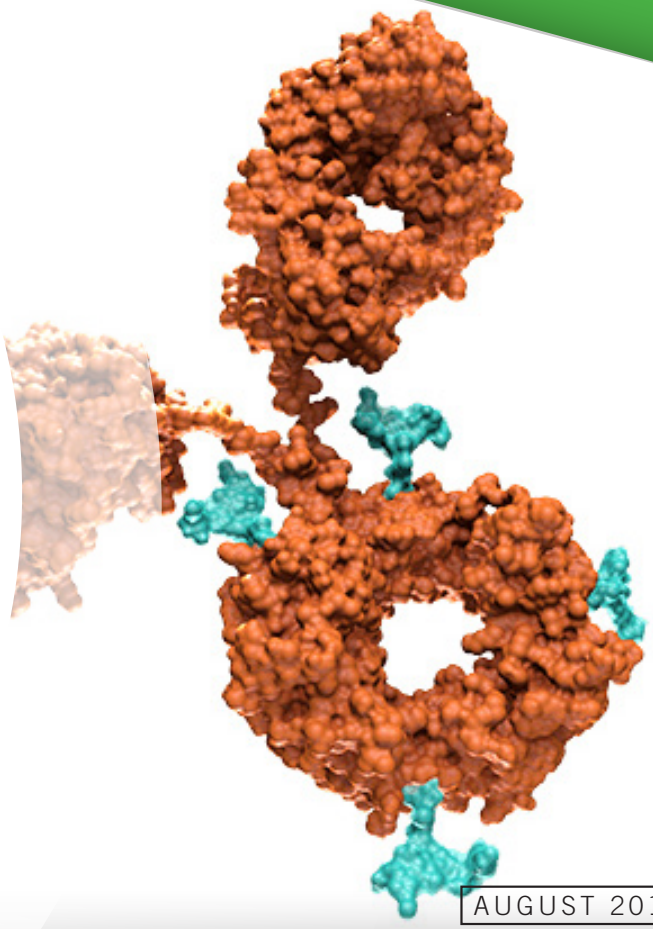
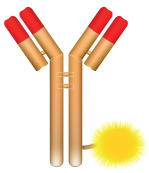


Advances in Biopharmaceutical Characterization: Antibody–Drug Conjugates

AUGUST 2018



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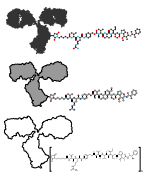

Solving the Analytical Challenges of ADC Characterization
An interview with Krisztina Radi

Tackling Analytical Method Development for ADCs
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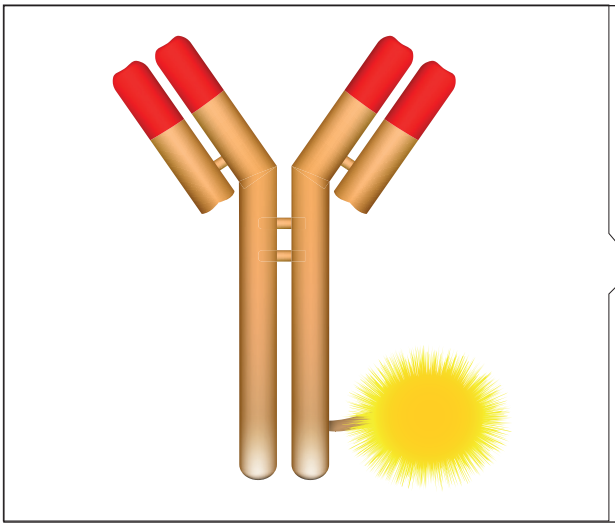


Antibody–Drug Conjugates: Perspectives and Characterization

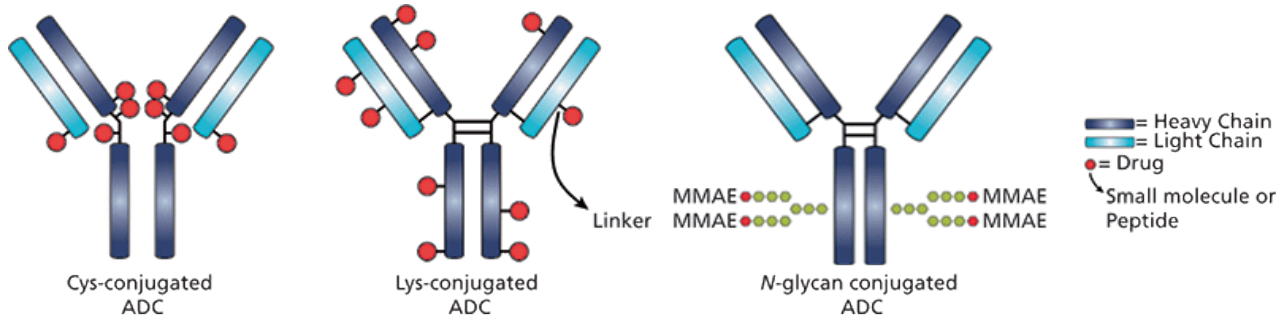
Rowan E. Moore, Kelly Broster, Ken Cook, Kyle D'Silva, Eric Niederkofler, Aaron O. Bailey, Jonathan Bones, and Michael W. Dong

This installment of "Perspectives in Modern HPLC" provides an overview of antibody–drug conjugates (ADCs) as a new class of biotherapeutics and describes their analytical characterization for quality assessment with examples from extensive applications libraries.

Chemotherapeutic agents have been the mainstay of anticancer therapy since the early 1940s. Chemotherapy, or the use of cytotoxic agents in medical oncology to inhibit the process of mitotic cell division, is routinely administered with curative intent, to prolong life or as part of palliative care. Although the use of chemotherapy can result in a significant response—for example, in the treatment of testicular cancer—its use is associated with a range of adverse effects. Many of the adverse effects of chemotherapy are the result of damage to healthy cells that divide rapidly and are thus sensitive to antimitotic drugs.

Antibody–drug conjugates (ADCs) are an increasingly important class of biotherapeutics that utilize the specificity of monoclonal antibodies (mAbs) and the cytotoxicity of a potent anticancer payload (1–3). The two molecules are connected via chemical linkers, and the result is a therapy that is able to provide sensitive discrimination between healthy and diseased tissues. The antibody targets and binds to a selected antigenic cell-surface receptor that is, ideally, only expressed on the target cancer cell. After an ADC binds to its target cell, the cell internalizes the ADC through receptor-mediated endocytosis, and the cytotoxic payload is then released inside the lysosomal cellular compartment to provide precise, selective delivery to the cancerous cells. Payload conjugation typically takes place on the amino groups of lysine residues or the sulfhydryl groups of interchain cysteine residues as is the case in ado-trastuzumab emtansine (Kadcyla, Genentech/Roche) and brentuximab vedotin (Adcetris, Seattle Genetics/Millennium Pharmaceuticals),

Figure 1: ADC structures showing different sites of attachment to mAb of the drug with the linker. MMAE = monomethyl auristatin E, an extremely potent synthetic antineoplastic agent.



respectively. With 80–100 lysine residues and only eight interchain cysteine residues available in each mAb molecule, lysine conjugation yields a more heterogeneous mixture of species compared to cysteine-conjugated ADCs. **Figure 1** depicts examples of common payload conjugation types, namely lysine, cysteine, and glycoconjugates (4).

In addition to the described primary amino acid structure, mAbs and ADCs also have distinct higher order structures that dictate their function and immunogenicity. They may be influenced by the above-described modifications and can appear as dimers or aggregates that also have the potential to induce immune responses and affect clearance rates.

For an ADC to demonstrate efficacy, it must incorporate a mAb that recognizes a specific tumor-associated antigen, a linker that has systemic stability but is specifically released at the target cell, and a cytotoxic agent that exhibits toxicity to the tumor cell as a stand-alone modality.

ADC Regulations

Whether submitting to the United States Food and Drug Administration (U.S. FDA), European Medicines Agency (EMA), or other regulatory bodies, ADC developers are covering new territory. Since ADCs incorporate both biologics and small-molecule moieties, these complex therapeutics are difficult to characterize, and multiple health authority experts are required to evaluate different aspects of the end product.

An ADC may be based on a previously approved mAb. For example, trastuzumab (Herceptin) is the mAb portion of the ADC Kadcyla. In such instances, new analytical technologies that have emerged since the development of the original mAb drug product should be evaluated for use in characterizing the related ADC. Consistent with the principles of quality by design (QbD), regulators expect sponsors to use the most current and effective technologies available to build product and process knowledge into controlling product quality.

With the approvals of Kadcyla, Adcetris, and more recently inotuzumab ozogamicin (Besponsa, Pfizer), gemtuzumab ozogamicin

(Mylotarg, Pfizer), and more than 50 ADCs in clinical trial pipelines, the clinical application of ADCs is accelerating rapidly (5).

It is important to have a clear understanding of the relationship between the conjugation and manufacturing process, and the resulting product quality and heterogeneity of the ADC. The potency of an ADC is due, in part, to the extent of drug-linker incorporation on the mAb. Methods that can structurally characterize the drug load and distribution have been developed and proven to be critically important for understanding ADC product quality. Wakankar and colleagues have summarized several considerations for the development of analytical methods that measure quality attributes unique to ADCs, such as drug load and drug distribution (6). In addition, several articles documenting the analytical strategies (7) as well as chromatographic and electrophoretic techniques for the characterization of ADCs have been published (8–10).

Characterization and Quality Control Requirements

Quality control (QC) testing of an ADC needs to account for its identity, purity, concentration, and activity (potency or strength)—the same as for any other biopharmaceutical product. Because of the inherent structural complexity of mAbs along with the covalently linked cytotoxic agents, several QC tests are required (8–10). A full understanding of the manufacturing process and its effect on the physicochemical and biological attributes of an ADC must be ascertained. However, in the case of ADCs, even the well-established QC terminology is not straightforward—for instance, the terms potency and strength have different meanings depending on whether the molecule being developed is large or small. The International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use (ICH) Q6A for small molecules lists strength (or assay) as a measure of the amount of an active pharmaceutical ingredient (API) (11). ICH Q6B for large molecules uses the term potency as a quantitative measure of biological activity (12). For an ADC that includes both of these components, total function (or potency) would need to be measured with a cell-based assay that assesses overall structure, antigen binding, drug loading, and drug delivery.

Unlike their pharmaceutical predecessors and more straightforward protein-based therapeutics, there is limited availability of certified standards for ADC test method development or comparison. Recently, Merck launched SigmaMAb Antibody-Drug Conjugate Mimic for use as a standard for mass spectrometry (MS) and high performance liquid chromatography (HPLC). SigmaMAb is an "ADC mimic" that conjugates SigmaMab (MSQC4), an IgG1 mAb, to dansylcadaverine fluorophores via a succinimidyl-4-(N-maleimidomethyl)cyclohexane-1-carboxylate (SMCC) crosslinker (13). At this time, the onus is completely on the developers to devise and implement a set of critical tests for identity and purity, involving the most appropriate analytical technologies. Each intermediate (mAb, linker, and drug) should have a reference standard in addition to an ADC reference standard, to be used in designated release and stability tests. These standards are critical reagents used for analytical

method system suitability and in characterization, stability, and bridging studies, as is currently expected for all pharmaceutical and biopharmaceutical products. The cohort of tests would be performed as part of chemistry, manufacturing, and control (CMC) efforts during drug development. Many of these tests would become assays for critical quality attributes (CQA) or analytical methods for specification testing in lot release.

Small-molecule conjugation to mAbs, using any type of strategy, has enormous potential to produce several variant isoforms. Appropriate tests are needed to measure heterogeneity and ensure product consistency. Routine QC testing and characterization may measure the following characteristics:

- Aggregates and fragments
- Charge variants
- Free drug
- Average drug-to-antibody ratio (DAR)
- Drug load distribution, including unconjugated mAb
- Endotoxins or bioburden

Because of the heterogeneity of ADCs, isoforms derived from mAb glycosylation and other post-translational modifications (PTMs) are often controlled at the point of mAb release. The inclusion in the certificate of analysis (CoA) for routine testing of other product-related impurities—such as aggregates, fragments, charge variants, and unconjugated antibodies—discussed above should be assessed product by product. For example, data could be generated to show that an unconjugated antibody is adequately monitored and controlled as part of DAR testing.

Chemical impurities other than free drug or drug-related substances may be evaluated with both ICH Q3B (R2) limits and pharmacology or toxicology input for the specific product (14). Some process-related impurities might be omitted from release testing with sufficient data and process experience over multiple ADC lots or multiple ADC products using the same conjugation platform.

Regulators consider compendial monographs, which exist for small-molecule intermediates, to be the minimum standard for chemical components when used in ADCs.

Drug and Linker: Approaches and Chemistries

The conjugation of anticancer payloads to lysine or cysteine residues found in mAbs results in the generation of ADCs that exhibit significant heterogeneity, with some of the ADC potentially having altered antigen-binding properties leading to suboptimal potency, solubility, stability, and pharmacokinetics. To reduce heterogeneity, expand payload options, and prolong circulating stability, novel site-specific conjugation approaches are actively being pursued within the field (15).

The hydrophobic nature of the payloads used in current ADCs leads to the creation of conjugates of increasing hydrophobicity versus their starting mAb scaffolds. The hydrophobicity of ADCs can promote aggregation, which in turn can lead to hepatotoxicity (16) or increased immunogenicity (17). The hydrophobicity of ADCs can also promote drug resistance via increased affinity for multidrug resistance transports, with the incorporation of hydrophilic linker chemistries shown to bypass multidrug resistance (18).

ADCs use three main tumor-specific microenvironmental factors to selectively release their cytotoxic payloads: cleavable linkers ex-

hibiting protease-sensitivity, pH-sensitivity, and glutathione-sensitivity. Within each of these main linker release mechanisms, significant linker technology advancements are ongoing.

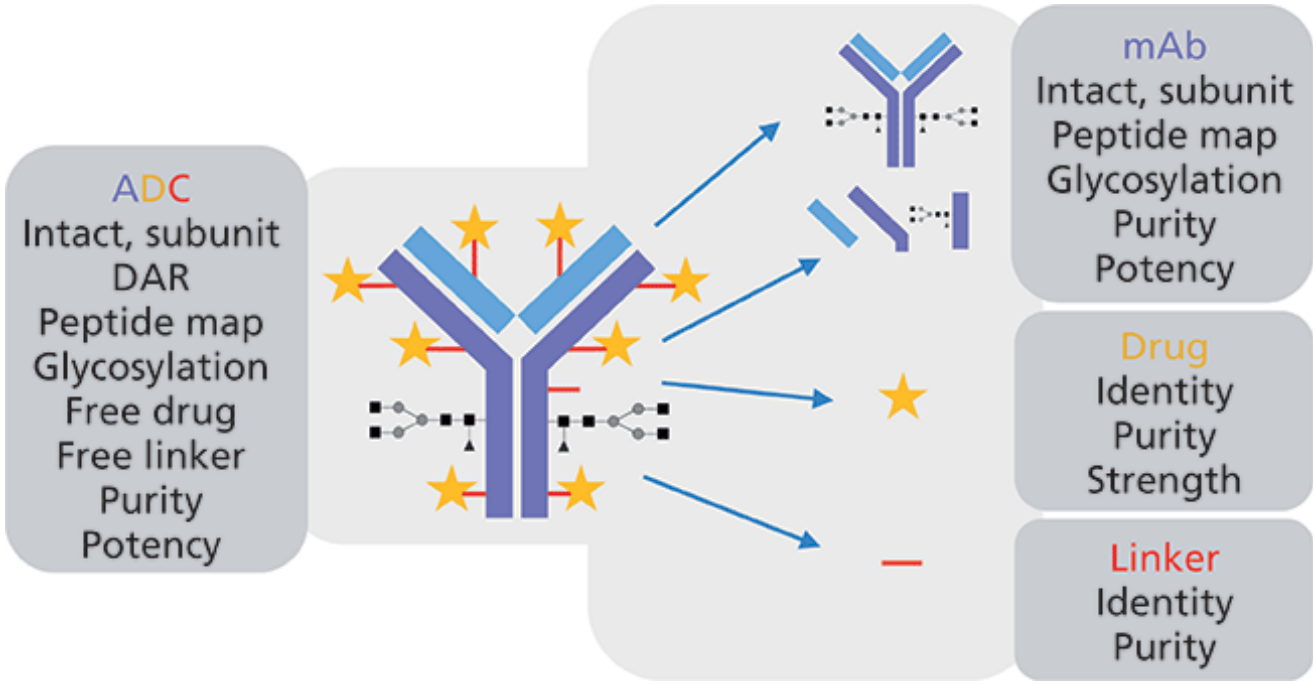
Among the types of conjugation chemistries, enzyme-based site-specific modification shows great potential by eliminating the potential interruption of an antibody–antigen interaction and providing a highly reproducible and modular conjugation system when compared to standard lysine and cysteine conjugation.

Developments in linker chemistries also provide a greater opportunity to incorporate increasingly potent cytotoxic payloads. Quaternary ammonium linkers now enable stable conjugation of payloads with tertiary amine residues (19); the extremely potent synthetic antineoplastic agent monomethyl auristatin E (MMAE) has been linked to mAbs via a linker that is selectively cleaved by cathepsin (for example, in Adcetris) upon entrance into the tumor cell (20). A conjugate with the potent maytansinoid DM1 has been approved (for example, Kadcyla), and Seattle Genetics recently published work on a novel methylene alkoxy carbamate (MAC) self-immolative unit for hydroxyl-containing payloads within ADCs (21). The latter compound enables direct conjugation of drugs through alcohol functional groups that are present on a diverse range of synthetic drugs as well as natural cytotoxic products. Most recently, Spirogen (now part of the AstraZeneca Group) developed a potent and flexible class of ADC payload based on a proprietary pyrrolobenzodiazepine (PBD) technology. PBDs are a family of sequence-selective DNA minor-groove binding agents and are among the most cytotoxic agents known. They are ideally suited for antibody–drug conjugation because of their unique mechanism of action that retains activity against cancer stem cells and is compatible with multiple linker and conjugation technologies. There are two ADCs currently undergoing clinical trial from the collaborative efforts of Spirogen and Seattle Genetics (22), and many more are in the pipeline. As previously mentioned, most of the payload and linker technologies used or studied today impart increasing levels of hydrophobicity on the mAb scaffold (10); for example, DM1 has an estimated LogP value of 3.95 per molecule incorporated. PBDs are even more hydrophobic, with an estimated LogP value of 5.08 per incorporated molecule. To address this issue, hydrophilic spacers (for example, para-aminobenzyl alcohol [PAB]) and linkers (such as polyethylene glycol [PEG]) are often incorporated as part of the bioconjugation chemistry to balance out the increased hydrophobicity introduced by the conjugation of the payload.

Chromatography for mAb, Drug, Linker, and ADC

Various ultrahigh-pressure liquid chromatography (UHPLC) techniques have proved to be useful for analyzing ADC heterogeneity at the intact level, including hydrophobic-interaction chromatography (HIC), ion-exchange chromatography (IEC), size-exclusion chromatography (SEC), and reversed-phase chromatography. Where appropriate, the coupling of these separation techniques with high-resolution accurate mass spectrometry (HRAM MS) presents a powerful characterization tool. Further structural details can

Figure 2: Typical characterization approaches performed on ADC therapeutics.



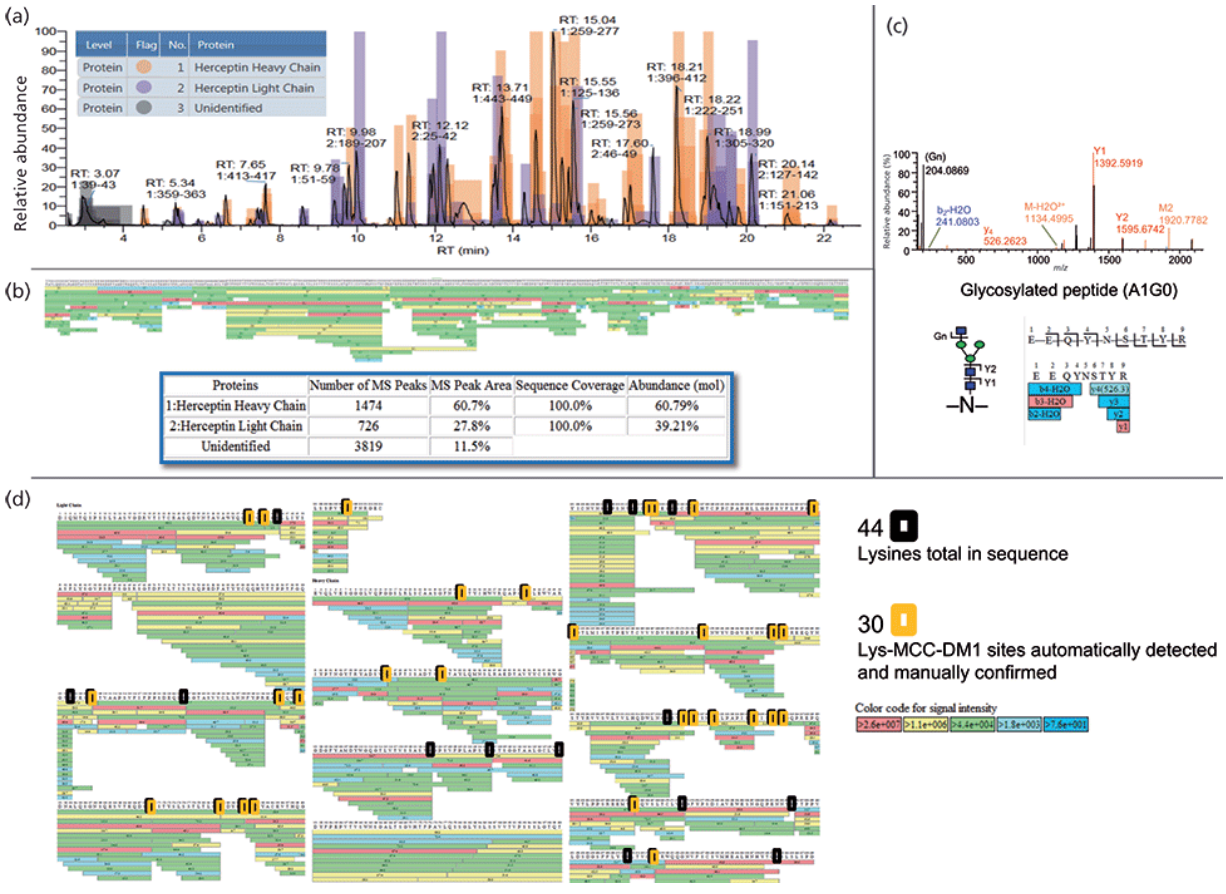
be ascertained by breaking down the intact ADC; both peptide mapping using reversed-phase chromatography and released glycan analysis with hydrophilic-interaction chromatography (HILIC) are deemed essential tools. Each of these analytical approaches reveals different CQAs of the ADC—from primary amino acid sequence and associated modifications (peptide mapping) to the presence of higher order aggregated structures (SEC) that could impact product efficacy and safety. In addition to the standard cohort of small molecule and large biomolecule characterization methodologies, a whole set of tests must be performed to interrogate the level of drug conjugation and the levels of unconjugated mAb, payload, and linker (as shown in **Figure 2**).

Monoclonal Antibody Primary Sequence Analysis

As a technique, peptide mapping is well established in the biotechnology industry with roots lying in protein characterization, proteomics, and de novo peptide sequencing. In recent years, advances in sample preparation (protein digestion), peptide separation, HRAM MS capabilities, and bioinformatics have enabled the biotech industry to confidently apply peptide mapping workflows in routine, high-throughput environments.

Peptide mapping can reveal many CQAs of a protein. In the case of ADCs, peptide mapping is fundamental in confirming not only the sequence of the mAb, but also the site and level of drug

Figure 3: Trastuzumab emtansine lysine-conjugation mapping (26). (a) Color-coded base peak ion chromatogram (BPI) showing heavy and light chain peptides. (b) Coverage map showing 100% sequence coverage, number of MS peaks, and relative abundance of heavy and light chain peptides detected. (c) Example higher energy collisional dissociation (HCD) MS/MS spectrum of a glycopeptide showing fragmentation of both peptide and glycan. (d) Identification of lysine conjugated MCC-DM1 at the peptide level.



conjugation (**Figure 3**). The accuracy with which this information can be determined is based on the method of protein digestion and fidelity of the subsequent UHPLC and MS analysis. The type of fragmentation used within the MS system should also be carefully considered because standard collision-induced dissociation (CID) experiments often fail to reveal the precise site of drug conjugation or glycosylation. Alternative or additive fragmentation techniques such as higher energy collisional dissociation (HCD), electron transfer dissociation (ETD), and ultraviolet photodissociation (UVPD) are becoming increasingly important in the elucidation of site-specific modifications and can generate informative fragmentation patterns, even at the subunit level (23–25).

Chromatographic Techniques for the Determination of Antibody Variants, Fragments, DAR, and Payload Mapping

Hydrophobic Interaction Chromatography

HIC separates proteins by the interactions between hydrophobic pockets present on the surface of the protein and the hydropho-

bic ligands on the HIC resin. Proteins are loaded onto the column in relatively high salt concentrations to induce hydrophobic interactions and are eluted by reducing the salt concentration of the mobile phase during the chromatographic separation. The binding of the proteins is dependent on the inherent surface hydrophobicity, which is influenced by the conformation of the protein. Changes in protein conformation can be characterized by this mode of chromatography, and several publications exist that indicate that common modifications of mAbs, such as oxidation and deamidation, can be seen using HIC (27). With the conjugation of hydrophobic payloads to the mAb to form ADCs, the use of HIC for DAR analysis has become increasingly popular (6,28).

With each additional linkage of the drug to the mAb the retention of the ADC species on the column increases, thus allowing quantification of drug load on the ADC and resolution of isomeric configurations of the same DAR (**Figure 4**).

Figure 4: Comparison of synthesized Cys-conjugated ADC mimics with different drug load (29): (a) unconjugated mAb (5 mg/mL), (b) Cys-conjugated ADC mimic (low load, 5 mg/mL), (c) Cys-conjugated ADC mimic (moderate load, 5 mg/mL), (d) Cys-conjugated ADC mimic (high load, 5 mg/mL). Column: 100 mm × 4.6 mm, 5-µm dp Thermo Scientific™ MAbPac™ HIC-Butyl; mobile-phase A: 95:5 (v/v) 1.5 M ammonium phosphate (pH 7.0)–isopropanol; mobile-phase B: 80:20 (v/v) 50 mM sodium phosphate (pH 7.0)–isopropanol; gradient: 0% B for 6 min, 0–100% B in 14 min, hold at 100% B for 5 min; temperature: 25 °C; flow rate: 1.0 mL/min; injection volume: 5 µL (5 mg/mL); detection: UV absorbance at 280 nm.

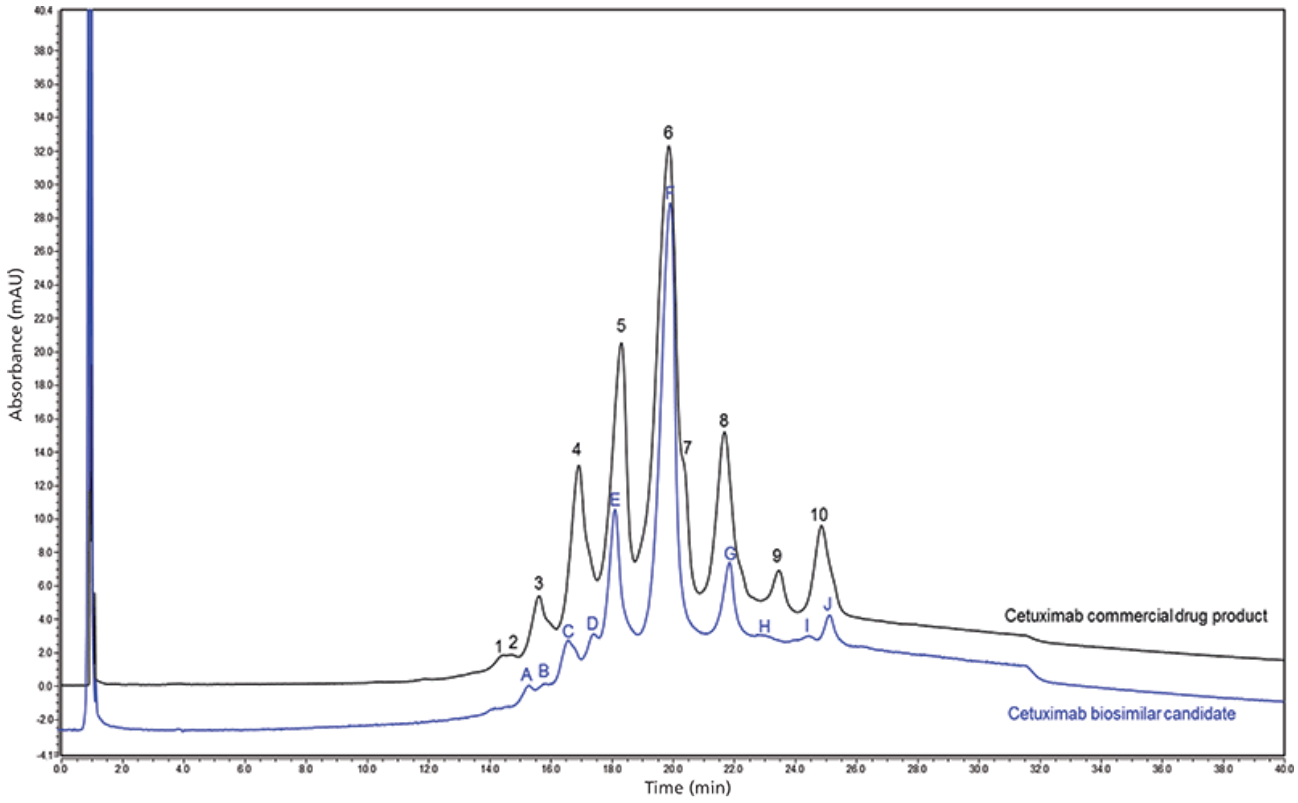
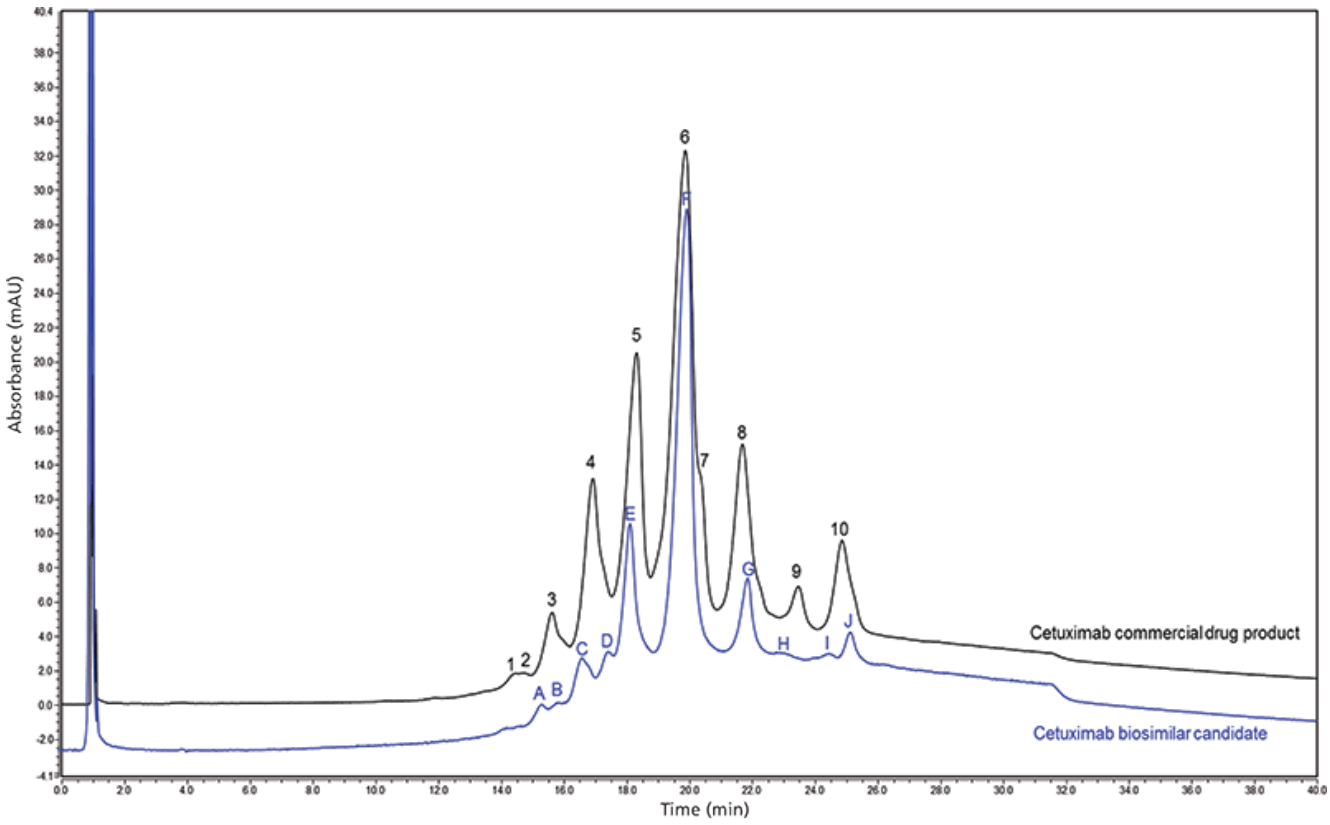


Figure 5: Charge variant chromatographic profile comparison of commercial chimeric IgG1 mAb (black trace) and cetuximab biosimilar candidate (blue trace) obtained with cation-exchange chromatography in pH-based gradient mode (31). Peak labeling corresponds to the number of peaks in each trace and does not indicate peak identification.



Ion-Exchange Chromatography

IEC involving cation-exchange column chemistries is a standard method used to separate and monitor the charge-variant profile of mAb-based therapeutics (30). Charge-variant separations have been further developed with the use of pH gradients that provide ease of use and a more global approach to the method development process (**Figure 5**) (31). There are several PTMs that can alter the charge or conformation of a protein and can, therefore, be characterized using IEC. Glycan variants, deamidation, oxidation, and even aggregation are among them.

The specific charge-variant profile that is obtained from a mAb is closely monitored at each stage in the production to ensure the product quality remains the same. In the case of ADCs, mAbs may not provide an informative charge-variant profile—if the drug or linker is charged, or linkage occurs through a charged amino acid (such as lysine), the underlying mAb charge heterogeneity is difficult to assess because conjugation affects the overall charge of the conjugated molecule. In such cases, the "charge profile" is often more of a "conjugation profile." Despite this, measuring the distribution

of charged species can be a good way to demonstrate process consistency and thus should be included in an ADC comparability toolkit.

Reversed-Phase Chromatography-MS

MS analysis of ADC drug distribution provides insights into the relative concentration of different drug-linked forms, which may elicit distinct pharmacokinetic and toxicological properties. MS analysis of ADC drug distribution is particularly advantageous for conjugates produced using linkage through surface-accessible lysine residues, which are not easily separated by chromatography alone because of their high degree of heterogeneity.

Reversed-phase LC–MS can be used to elucidate the positional isomers of ADCs. Reversed-phase LC–MS following IdeS proteolytic digestion facilitates the subunit analysis of ADCs and enables rapid comparison of the ADC samples, for instance for batch assessment (**Figure 6**). Indeed, IdeS proteolytic digestion has been proposed as an analytical reference method at all stages of ADC discovery, preclinical and clinical development, for routine comparability assays, formulation, process scale-up and transfer, and to define CQAs in a QbD approach (32).

Chromatography and Native Mass Spectrometry

The ADCs currently approved for use utilize naturally occurring lysine side chain amino groups or the cysteine thiol groups, which are formed upon partial reduction of IgG intramolecular disulfide bonds, for conjugation of the drug load (34).

Cysteine-linked ADCs present a unique challenge for characterization because proper intact analysis requires native MS conditions to preserve structurally critical noncovalent binding between antibody chains.

ADCs exhibit significant heterogeneity resulting from the number and distribution of drug molecules across the antibody. This level of molecular complexity and heterogeneity presents a considerable challenge for current analytical techniques.

Native MS of intact proteins allows direct observation of molecules that rely on noncovalent interactions to preserve critical structural features, such as interchain associations that hold together cysteine-linked ADCs. The use of 100% aqueous and physiological pH buffers in native MS analysis produces decreased charge states (increased *m/z*) and improves mass separation of heterogeneous mixtures.

An orbital trap native MS workflow has recently been developed that is compatible with SEC, allowing online desalting and sample delivery, to observe intact proteins at high m/z ranges. This strategy reduces mass interference in complex protein spectra by increasing peak capacity in the m/z space. This workflow has recently been applied to the analysis of Adcetris and Kadcyla, cysteine-linked and lysine-linked ADCs,

Figure 6: Denaturing LC–MS analysis of the ADC brentuximab vedotin (Adcetris) (33). (a) Unmodified sample (1 µg) was analyzed by reversed-phase chromatography coupled to an orbital trap MS system produced several peaks. (b) The resulting averaged MS spectrum is a complex mixture of charge state envelopes as well as a vcM-MAE-specific reporter fragment ion at m/z 718. (c) Data analysis with ReSpect deconvolution and Sliding Window integration show roughly six covalently-structured forms of unraveled cysteine-linked ADC.

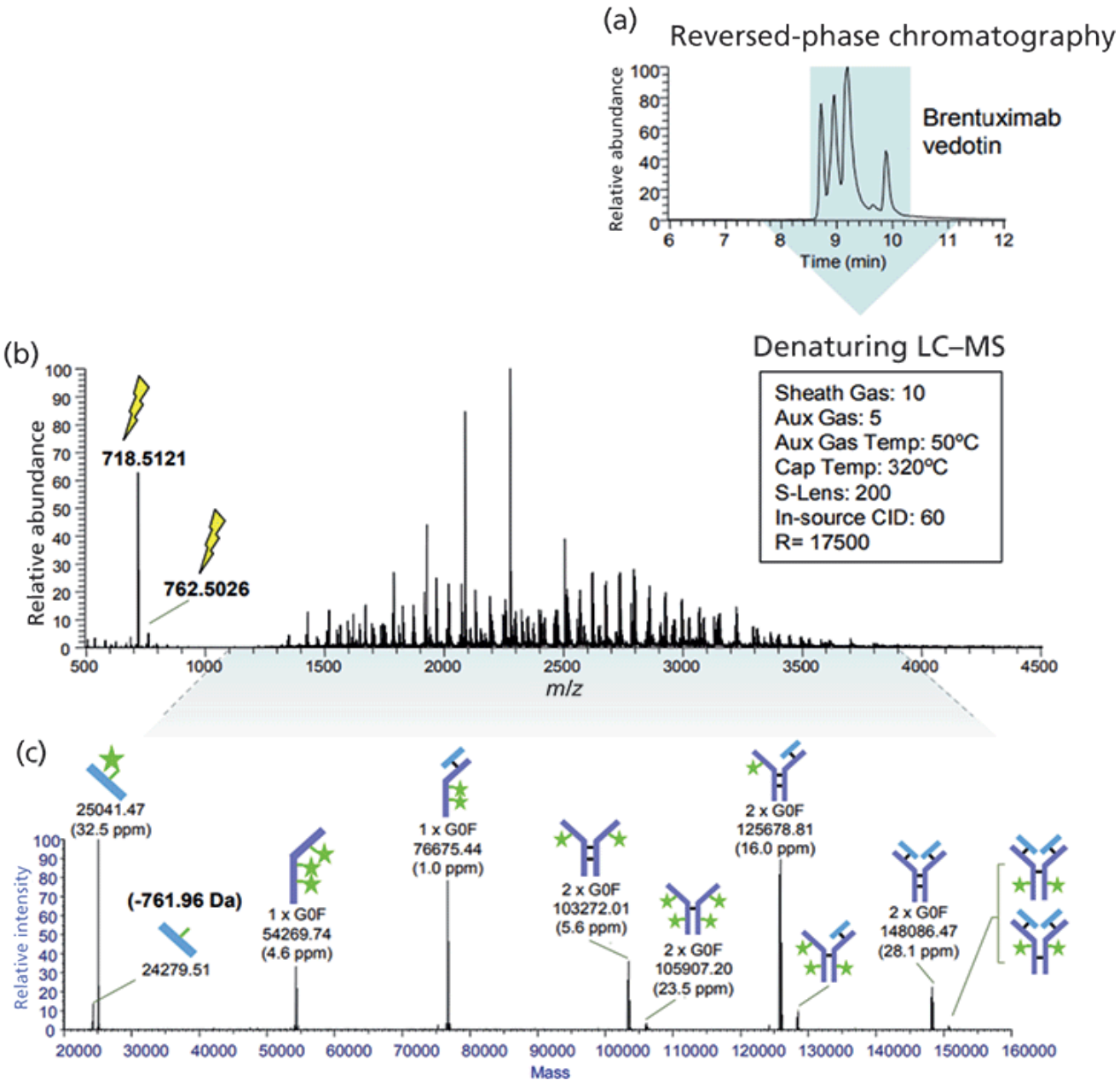
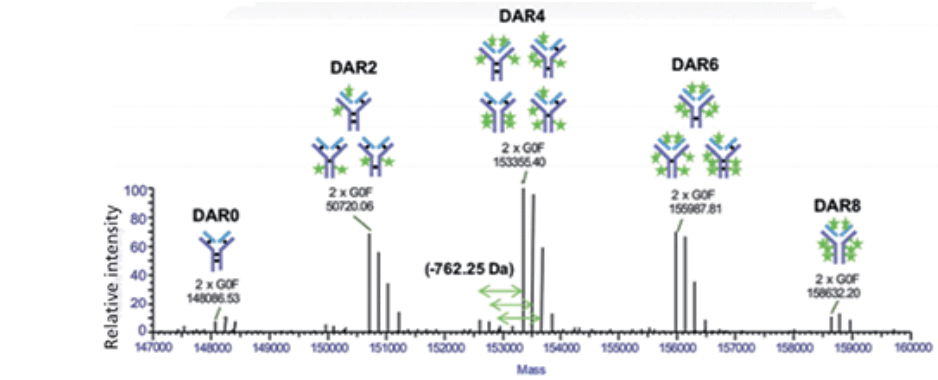


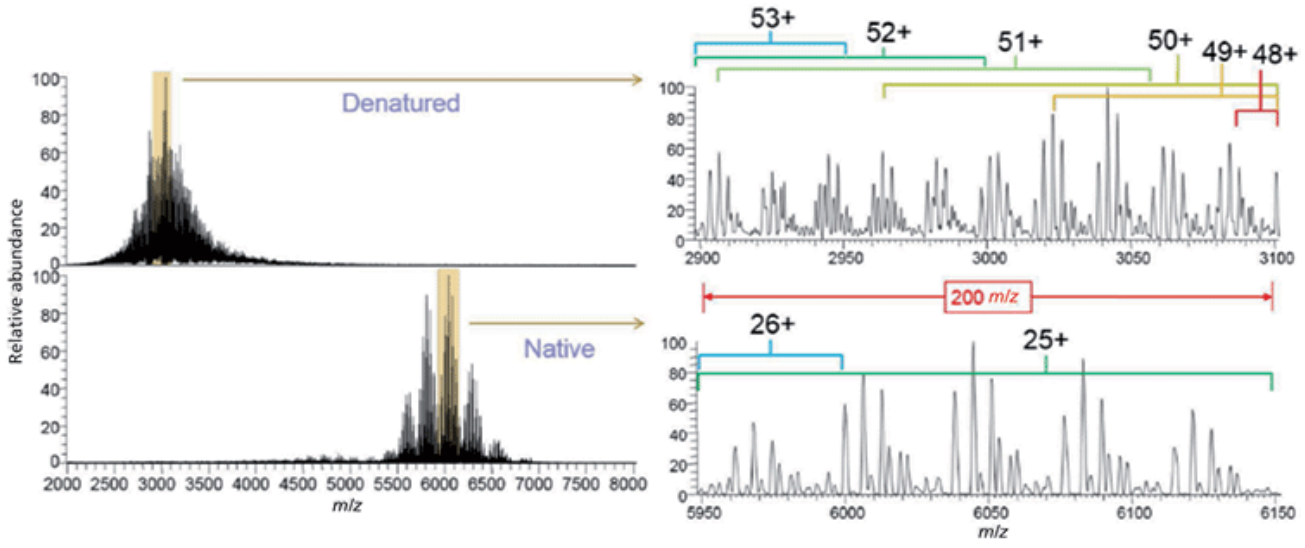
Figure 7: Desalting SEC–MS DAR of Adcetris and Kadcyla (35). (a) Desalting SEC is compatible with a native MS approach and allows preservation of noncovalent interactions which support the structure of cysteine-linked ADCs. Based on the individual deconvoluted abundances of the G0F/G0F glycoform, the authors calculated an average DAR value of 4.07 (32). (b) Denaturing MS spectra (from reversed-phase LC) are observed at lower *m/z* ranges while native MS spectra from online SEC are observed at higher m/z ranges. A detailed view shows that 2–3 sequential charge state envelopes overlap compared to an overlap of 0– charge state envelopes in the native MS spectrum.



G0F/G0F DAR	Mass Accuracy (ppm)	Relative Abundance
DAR0	11.7	6.77
DAR2	23.1	69.23
DAR4	22.4	100.00
DAR6	40.5	69.75
DAR8	17.6	10.61

Average Drug-to-Antibody Ratio (DAR)

4.07



respectively, and the accurate calculation of DAR (**Figure 7**).

This work built on a similar approach that was first applied to the study of Adcetris using an orbital trap mass spectrometer equipped with a high-mass quadrupole mass selector (36).

Higher Order Structural Analysis

Hydrogen–deuterium exchange (HDX)-MS is a powerful tool for studying the dynamics of higher-order structure of protein-based therapeutics. The rate of hydrogen-to-deuterium exchange within the amide hydrogen on the backbone of biotherapeutics provides solvent accessibility information, and thus protein structure and conformation can be inferred.

Although HDX-MS cannot be used to define an absolute structure in the manner of X-ray crystallography, it can be used to directly assess the native structure in a comparative fashion. Proteins in solution are highly dynamic, and the stability and functionality of any protein therapeutic are closely associated to a specific conformation.

The manufacturing of ADCs involves additional processing steps during conjugation, and it is important to evaluate how the drug conjugation process impacts the conformation and dynamics of the mAb intermediate. The ability of HDX-MS to monitor conformational changes at the peptide level makes the technique well-suited for providing detailed insights into the impact of drug conjugation processes on the higher-order structure of mAbs.

Orbital trap–based HDX-MS has previously been used to probe the conformation and dynamics of interchain cysteine-linked ADCs (37). In this publication, a side-by-side HDX comparison of ADCs, mAbs, reduced mAbs, and partially reduced mAbs was used to identify minor local conformational changes and confirm that these were because of the partial loss of interchain disulfide bonds in ADCs. These findings were used to indicate that ADC manufacturing processes that involve partial reduction of mAb interchain cysteine residues followed by conjugation with drug linkers do not significantly impact the conformational integrity of the mAb. A similar approach has been used to study the antibody structural integrity of site-specific ADCs (38). Together these results highlight the utility of HDX-MS for interrogating the higher-order structure of ADCs and other protein therapeutics.

TABLE I: Impurity dose based on the level of conjugatable impurities in the linker-drug intermediate (table adapted with permission from reference (39)

Impurity Level in Linker-Drug	Maximum Impurity Level in DS (wt/wt%)	ADC 5 mg Dose		ADC 50 mg Dose		ADC 50 mg Dose	
		Maximum Impurity Level	Maximum Daily Impurity Level	Maximum Impurity Level	Maximum Daily Impurity Level	Maximum Impurity Level	Maximum Daily Impurity Level
3%	1.5 µg/mg DS (0.15%)	7.5 µg/dose	0.36 µg/day	75 µg/dose	3.6 µg/day	0.75 mg/dose	36.0 µg/day
1%	0.5 µg/mg DS (0.05%)	2.5 µg/dose	0.1 µg/day	25 µg/dose	1.2 µg/day	0.25 mg/dose	12.0 µg/day
0.5%	0.25 µg/mg DS (0.025%)	1.25 µg/dose	0.06 µg/day	12.5 µg/dose	0.6 µg/day	0.125 mg/dose	6.0 µg/day
0.1%	0.05 µg/mg DS (0.005%)	0.25 µg/dose	0.01 µg/day	2.5 µg/dose	0.12 µg/day	0.025 mg/dose	1.2 µg/day

Residual Free Drug Analysis and Control Strategy for Small-Molecule Impurities in ADCs

On the analytical front, one approach to conducting free-drug analysis for ADC drug substance and drug-product preparations is to precipitate the proteins (along with protein-bound drug) and analyze the resulting supernatant using a method that is effective for detecting the small molecule such as those using UHPLC–MS or UHPLC with ultraviolet (UV) detection.

Residual Solvents and Volatile Organic Impurities in ADCs

It is uncommon that residual solvent analysis is conducted for post-production quality assurance of conventional protein-based biopharmaceuticals such as mAbs. Organic solvents are not typically used in cultured cell trains and seldom form part of the risk profile of the drug.

In contrast, the conjugation reaction to form ADCs generally involves a site-selective enzymatic or chemical reaction of antibody to linker to small-molecule drug warhead, where the hydrophobic warhead and linker are solubilized in solvents such as N,N-dimethylacetamide (DMA), N,N-dimethylformamide (DMF), dimethyl sulfoxide (DMSO), or propylene glycol (PG). The conjugation process is followed by protein purification techniques to remove process-related contaminants (unconjugated toxin and residual solvents). However, strategies must be in place to monitor for such impurities. For the analysis of these residual solvents, one possible approach is to use a direct gas chromatography (GC) technique (43) after removal of the proteins rather than the traditional headspace GC approach in *USP* <467> (44). Because of the low levels expected for residual solvents in ADC samples, an alternative GC–MS method (particularly using the selected ion monitoring mode) is likely to yield higher sensitivity as well as provide identification information on unknown peaks, as shown in the example in **Figure 8.**

Bioanalysis of ADCs

ADCs are complex heterogeneous mixtures resulting from differences in glycosylation of the antibody, the number of small-molecule drug moieties attached to the antibody, and the location of the conjugation sites. This situation is further complicated as the drug undergoes in vivo changes such as spontaneous deconjugation of the small-molecule drug and differential clearance rates of

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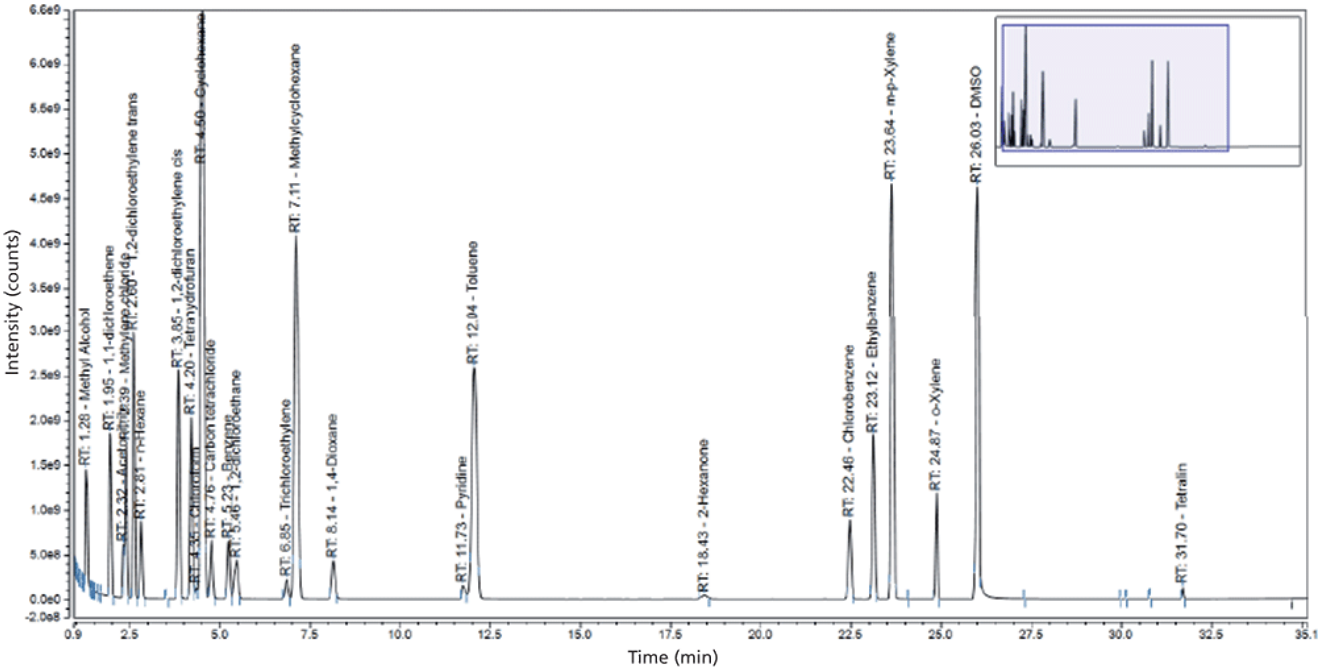
Quick Screening of Intact Antibody and Antibody Drug Conjugates with Integrated Microfluidic Capillary Electrophoresis and Mass Spectrometry

Figure 8: GC–MS of residual solvents following analytical headspace GC conditions similar to those in *USP* <467> that may provide higher sensitivity under single ion monitoring mode as well as information for unknown peak identification.



ADC components as a result of their different DARs. These changes, as well as other attributes of ADCs, contribute to the unique challenges in their bioanalysis. Furthermore, it is becoming clearer that the data required by the bioanalytical scientist is also dependent on the phase of the ADC development. The early discovery phase requires in vivo stability of ADC candidates based on monitoring average DAR or presence and integrity of the drug moiety at a specific conjugation site, while in the clinical development phase, it is important to establish a correlative relationship between one or more components of the ADC and various safety and efficacy indicators. Therefore, to address these bioanalytical challenges both ligand binding assays (LBAs) and LC–MS have been used. For instance, measurement of total antibody to assess antibody pharmacokinetic (PK) behavior and measurement of conjugated antibody (DAR ≥ 1) is typically performed using LBAs, with unconjugated drug monitored by LC–MS. However, a hybrid of the two approaches, referred to as hybrid LC–MS, is becoming more actively developed and applied in ADC bioanalysis. This platform uses the affinity capture of the LBA to retain sensitivity and LC–MS for detection to provide greater specificity and improved characterization of the ADC component being monitored. Therefore, the hybrid LC–MS approach provides benefits of both the LBA and LC–MS, enabling scientists to better address some

of the unique challenges of ADC bioanalysis and to allow for the use of a single platform to generate the data required for ADC bioanalysis (45).

Summary

ADCs are an increasingly important class of biotherapeutics. As the list of the first-generation ADCs entering the clinic grows, new generations of ADCs will benefit from their insights. The future looks set to see ADCs that have higher levels of cytotoxic drug conjugation, lower levels of unconjugated antibodies, more-stable linkers between the drug and the antibody, and increasing analytical challenges. The stability of linkers in circulation is critical to ensure patient safety and to mitigate the side effects caused by the off-target release of toxic payloads.

Today's ADCs pose unique analytical challenges requiring increasingly powerful approaches, consisting of small- and large-molecule techniques for their comprehensive characterization. The complexity of their analysis is matched only with their potential to become the "magic bullet" of anticancer treatment.

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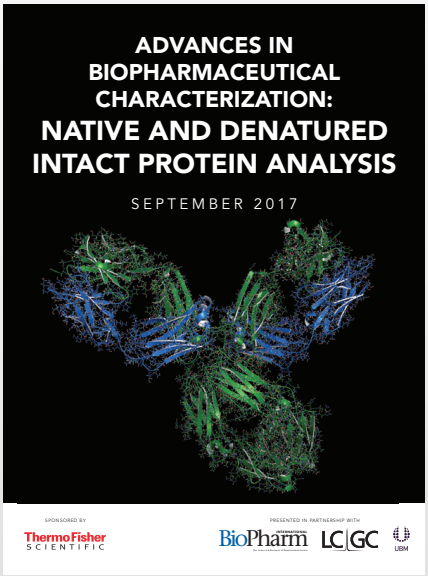

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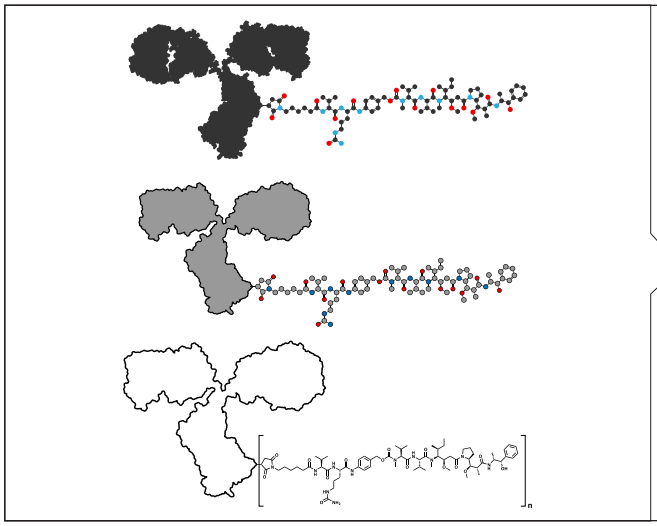

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Update: Decomplexifying ADCs with Native Intact Mass Analysis

An interview with Dr. Krisztina Radi

The complexities of therapeutic proteins present numerous challenges to analytical laboratories. Analysts are increasingly finding that native mass spectrometry provides several benefits over traditional techniques, including faster, more complete information. Here, Dr. Krisztina Radi, BioPharma Manager at Thermo Fisher Scientific, and previously an antibody-drug conjugate (ADC) Analytical Scientist with MedImmune, describes the current state of this important and growing area of mass spectrometry.

Biopharmaceuticals, and in particular ADCs, are very complex. What are some challenges and pain points that laboratories face when characterizing these molecules?

Radi: Whereas traditional small molecules are synthesized chemically, larger biopharmaceutical molecules are produced through complex bioprocesses. Numerous possible structural modifications can be created during the production of biologics, which can lead to product impurities and variants in the drug profile. All of these factors can influence the safety and the efficacy of the drug product.

Confirmation of the primary structure and post-translational modifications (PTMs) of large molecules are necessary quality control mechanisms. For instance, charge heterogeneity must be analyzed for monoclonal antibodies, their aggregates and fragments (which might cause immunogenic responses) should be controlled, and higher order structure must be investigated (which can affect the biological function). Other areas that must be looked at include biological functions, bioassays, binding assays, and other multi-level analyses.

One must also look at process-related impurities, which are not as straightforward to control as they are for chemical synthesis small molecule drug products. Biopharmaceuticals may contain host cell proteins, which may be retained. Thus, process-related impurities should be eliminated during process development.

Antibody–drug conjugates (ADCs) are even more complex than monoclonal antibodies, due to the additional heterogeneity resulting from conjugating a cytotoxic drug with a chemical linker to the protein. This necessitates analysts to look at additional unique quality attributes related to drug load/drug distribution and drug-to-antibody ratio. Several orthogonal analytical techniques—heavily based on liquid separation techniques and mass spectrometry (MS) techniques—are often applied for the quality control of biologic molecules.

What have you seen in terms of developments in LC–MS instrumentation or techniques to keep up with some of these challenges?

Radi: Mass spectrometry is the one technique that is constantly evolving and seems to be developing quicker than any other technique and with more impressive updates.

Thermo Fisher Scientific has focused the development of its recent high-resolution accurate mass MS system to the needs of the analysts in the biopharmaceutical characterization laboratory. The Thermo Scientific™ Q Exactive™ Biopharma platform of mass spectrometers have recently been updated to include the Thermo Scientific™ Q Exactive™ HF-X mass spectrometer, capable of 10 times greater sensitivity under native conditions than previous models. The biopharma option available on these instruments offers three operational modes to cover different level protein characterization (peptide mapping, intact, and native MS characterization). Orbitrap-based MS instruments have long been the technique of choice for peptide mapping; but now they are offering exceptional intact and native protein analysis capabilities too.

Intact level subunit analysis (LC, HC and conjugate chains for ADCs, IdeS digested mAb subunits) is the first level strategy for ADC laboratories, is readily available in Protein mode, and intact mass analysis under native and denaturing conditions in the High mass range mode.

What is really new and exciting in the field of MS is that native mass spectrometers are becoming a readily accessible and suitable tool for looking at extremely complex biopharmaceuticals. These instruments provide the opportunity to look at large molecules in their own true, native structure, which is very useful. For instance, this direct view allows analysts to assess heterogeneity instantly, like drug load distribution varieties for ADCs. Legacy analytical methods required the use of several orthogonal analytical techniques

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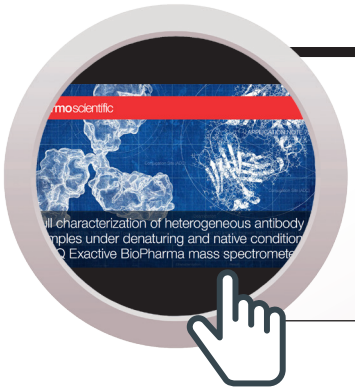

Full Characterization of Heterogeneous Antibody Samples under Denaturing and Native Conditions on the Q Exactive Biopharma Mass Spectrometer

to answer key characterization questions. Now, with native MS capabilities on the Q Exactive BioPharma platform, which allows mass-to-charge ratios of up to 8000 *m/z*, the antibody samples can be analyzed in non-denatured form with the charge envelope shifted to a mass range of *m/z* 5000-7000. This simplifies spectral interpretation and allows small features in the data to be identified, that would not be detectable under denaturing conditions.

"With native MS capabilities on the Q Exactive BioPharma platform, which allows mass-to-charge ratios of up to 8000 *m/z*, the antibody samples can be analyzed in non-denatured form with the charge envelope shifted to a mass range of *m/z*, 5000–7000."

This feature rich data demands flexible and powerful software tools to support intact analysis; Thermo Scientific™ BioPharma Finder™ software has two deconvolution algorithms for intact protein analysis to turn a charge state series into a molecular mass. One algorithm deconvolves the isotopically resolved high-resolution spectra to provide monoisotopic masses while another deconvolves the unresolved lower resolution mass spectra to provide average masses to help analysts look at the native state and immediately acquire important information. So, you can get a much more straightforward view of the native state, and a higher spatial separation of your ions in reduced charge states.

Native MS started as a research or academic kind of tool under static flow conditions using gold-coated borosilicate capillaries. Now, we have chromatographic techniques coupled to the mass spectrometer, which allows us to look at more native-like structures as these chromatographic techniques allow to maintain that kind of structure. For example, size-exclusion chromatography separation is now readily coupled to the native MS instrument. This provides a very quick analysis without major sample preparation because the buffer exchange is readily done through the chromatographic step, which saves time. This analytical process can be used as a quick screening method.

Another key thing about native MS is that it maintains non-covalent interactions, which are very helpful to look at. For example,

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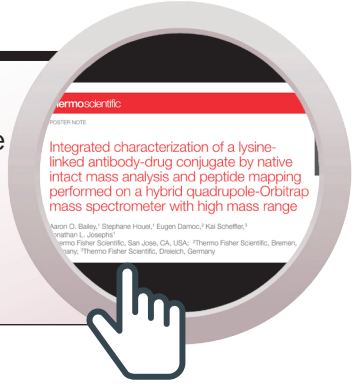
Integrated Characterization of a Lysine-Linked Antibody-Drug Conjugate by Native Intact Mass Analysis and Peptide Mapping Performed on a Hybrid Quadrupole-Orbitrap Mass Spectrometer with High Mass Range



some ADCs are conjugated through cysteines after reduction of interchain disulphide bonds. Usually with an intact denatured MS analysis these molecules would simply fall apart into their subunits, not maintaining non-covalent binding between antibody chains and then you would never really be able to look at its true form. Here, you can maintain non-covalent interactions, and assess the drug-to-antibody ratio on the whole antibody scaffold, which is very much a critical quality attribute for these types of conjugated biomolecules.

You mentioned that this technique started out as a research tool. Do you see drug companies now using this equipment in the discovery stage or are they carrying it through the development process and checking for quality throughout their processes?

Radi: I think this could become a screening tool for both. In both discovery and development phases, you need really good analytical tools that can give you good structural insight as quickly as possible. Scientists at LifeArc are using the Q Exactive BioPharma platform as they look at the conjugation chemistry to help screen and select molecules to take into the next development steps.

This technique is really good for getting a quick answer during the discovery phase. For instance, you can quickly assess how a conjugation condition affects the drug-to-antibody ratio or the drug load with the native MS.

It is also a good technique when you get to development or later phases and you need a quick snapshot overview of the structure for QC purposes. While it is developing area for native MS, having a good method for delivering good results very quickly is a key tool in biopharmaceutical development because it's important to ensure process consistency with various analytical assessments in a timely manner.

So overall, native MS definitely has potential for moving into later phases of the development.

Is the technique well understood by analysts? Is it something that they can use without a lot of training?

Radi: Definitely. Customers like LifeArc were up and running with this equipment in days and can confidently use the instrument to routinely analyze their complex biological molecules in the different characterization workflows.

Our equipment has several pre-optimized tune settings and the Thermo Scientific™ Chromeleon™ Chromatography Data System (CDS) Software helps laboratories get into this kind of easily simplified workflow setting, in a fully GMP compliance-ready environment. These molecules are very complex and although the analytical tools have great complexity behind them, end users can easily use them with the characterization workflows following an initial familiarization training. Users really like that kind of integrated software and workflows to simplify their processes and get answers as soon as possible.

What other advances have you seen in native MS?

Radi: We recently released the Thermo Scientific™ Q Exactive™ UHMR ultra-high mass range MS instrument. This very

unique instrument gives structural biologists and biotherapy companies the opportunity to look at complex protein–protein interactions. For instance, you could look at ADCs or antibodies actually bind to their antigens while you are still maintaining the very high resolution. It has a mass range up to 80,000 *m/z* allowing the study of ever-larger molecules, as well as protein-protein interactions, such as aggregation.

The combination of high mass range and high resolution is a key advantage and is a really good way of helping to get more structural insight about complex molecules, which increases product knowledge and improves analytical method development.

What might the future hold for analytical technologies with regard to ADC characterization?

Radi: Equipment suppliers want to make analytical technology easier for analysts to use, such as providing really good workflows, developing excellent instruments, and supporting the whole process with the necessary software.

There is a lot of effort going into software development. We have integrated software solutions for analytical process like the Chromeleon software, which can be compliance ready. People really appreciate having compliance-ready data integrity as well as tools to look at the data with the Biopharma Finder. The two together provide a ready data control and interpretation tool for users, which is key. Years ago, Native MS was a technique for specialist mass spectrometrists. Today, native mass spectrometry is becoming ever more democratized; putting deep structural insights into the hands of drug developers, from discovery through to manufacturing.

That's one direction we definitely go in the analytical lab—not just concentrating on the technical developments, but also supporting the method development with integrated software and data interpretation tools. For example, we can use the sliding window algorithm, which eliminates the need to look at individual chromatographic peaks. This algorithm in the software makes it possible not just to combine certain regions, but also to produce a list of "component peaks" for individual retention times. It identifies the components directly, rather than looking at the peaks. That might give you an additional level of information that you might not otherwise get, for example low abundance DAR species or ADC variants with drug related impurities being conjugated to the mAb.

In addition, we are expanding the boundaries of Native MS by coupling it with ion-exchange chromatography and other chromatographic techniques that are traditionally not coupled with mass spectrometers because they use high concentrations of non-volatile salts. These chromatographic techniques may better separate the different DAR-species further decomplexifying the MS analysis of ADCs.

In summary, instrument suppliers are working to support hardworking biopharmaceutical scientists by helping them get the answers as soon as possible as best as possible, and to provide reliable, high-quality data.

Tackling Analytical Method Development for ADCs

Cynthia A. Challener

The targeted therapy possible with antibody-drug conjugates (ADCs) makes them an attractive class of drugs. Their composition--a biologic linked to a small-molecule cytotoxic, which enables targeted delivery—also creates challenges for analytical method development, both for characterization and lot release purposes. Expertise in both chemical and biological analysis is required, and techniques for both types of molecules must be used. In addition to performing typical characterization studies, the linker chemistry and its impact on heterogeneity and applicable analytical techniques must be understood. The influence of conjugation on antibody binding must also be evaluated, and a wide range of stability studies must be conducted. Fortunately, both improvements in analytical technology and increased experience with ADCs are leading to improved strategies for analytical method development and validation.

Both Small and Large

As for any drug, analytical methods for the characterization of ADCs must be developed, and separate quality control (QC) tests must be established for lot release of the final drug substance. Because ADCs are both small drug and biologic compounds, characterization and validation need to be appropriate for both types of products. "Testing requirements will still be identity, purity, impurities, activity, concentration, and stability as outlined in the International Conference on Harmonization's ICH Q5C (1) and ICH Q6B (2) biologics guidelines; however, the testing must cover both functional and physiochemical properties, including process control methods and release and stability-indicating assays for both the large and small molecule," says Lisa McDermott, principal scientist at SAFC.

Because ADCs are made of three different components, McDermott believes it is crucial to have an advanced control strategy for each of the intermediates, with testing profiles determined as if each of

the components is being developed as a stand-alone drug substance. "With this approach, many of the quality parameters can be controlled in the release of these intermediates and allow the final release strategy to focus on the quality of the ADC."

Main Methods

"Conjugation usually results in a mixture of ADCs with different drug-to-antibody ratios (DARs), free drug, and naked antibody concentrations," says Harpreet Kaur, synthetic chemistry team leader with Dalton Pharmaceutical Services. This increased heterogeneity associated with all ADCs, even site-specifically linked ones, requires the development of robust methods with sufficient resolution to characterize and measure the diversity of product-related species and potential impurities, according to Fred Jacobson, staff scientist and Kadcyla technical development leader with Genentech.

The critical properties for ADC characterization include target site-specificity and binding properties, stability of the linker and drug species, drug potency and free drug, site(s) of conjugation, DAR, heterogeneity, and solubility. In general, Jacobson notes that most modern chromatographic, electrophoretic, and spectroscopic (ultraviolet-visible [UV-vis] and mass spectrometry [MS]) methods have proven adequate to the task.

UV-Vis spectrophotometry has traditionally been used to measure ADC and free drug concentrations and average DAR. The challenge with this method, according to Kaur, is that the extinction coefficient (λ_{max}) of the drug may change when conjugated to the antibody or in a different buffer. In addiiton, the drug and antibody should have different λ_{max} values.

Chromatographic methods based on hydrophobicity (hydrophobic interaction chromatography [HIC]) and size (size-exclusion chromatography [SEC], SEC-multi-angle laser light scattering (SEC-MALLS) can provide information about the number and location of conjugation sites, average DAR, and free drug content, although these methods are not suitable for purification and characterization of ADCs produced using linkage through lysine residues due to their high degree of heterogeneity, according to Kaur.

Mass spectrometry (MS) methods are often introduced early in the development process to better understand the product and conjugation or to act as an orthogonal tool to standard chromatographic techniques that will ultimately be required for product release, according to Allan Davidson, analytical development manager for Piramal Healthcare. "While processes can be developed to provide a consistent and accurate drug load, most products remain highly heterogeneous and as such there is the need to better understand the complex picture of antibody structure and drug distribution," he says.

Electrospray ionization (ESI)-MS, liquid chromatography (LC)-MS/MS, and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) have been used extensively to analyze the DAR, free drug

and metabolite concentrations, and linker stability. The validation of MS methods can be a challenge, however, due to differences in ionization of ADCs with different DAR values and ADC linker hydrolysis under acidic LC-MS conditions or by the acidic matrices used for MALDI, according to Kaur.

Finally, bioanalytical immunoassays, such as enzyme-linked immunosorbent assays (ELISA) are used for quantitation of the ADC, naked antibody, and free drug content, determination of the extent of ADC binding to the target antigen, and to establish the stability of linker and drug and the immunogenicity, while cell-based mechanism of action assays are used to assess the target-killing ability of the drug. "The biggest challenge with these methods is that the binding of the antibody to the target antigen can be altered by the site and degree of conjugation," says Kaur.

Analysis of the small-molecule components (linker and drug) is relatively straightforward with well-defined expectations in line with what would be required for an API, according to Jacobson. "One important difference, however, is the requirement for understanding the impact of impurities in the small molecule on the process and quality of the ADC," he observes.

In addition, bioanalytical methods are required to determine ADC potency, both with respect to and target binding (ELISA-based). Characterization should also cover the impact of conjugation on antibody binding properties towards specific Fc-receptors, because they may affect pharmacokinetic (PK) or secondary mechanisms of action, according to Jacobson.

Priority assays, according to McDermott, include determination of the potency, drug load and distribution, and size variants. Early development of cytotoxicity assays and chromatographic assays such as HIC, reduced reverse phase high-performance liquid chromatography (RPHPLC), and SEC is therefore important. She adds that additional analytics are often used to underpin the accuracy of these methods in early development stages. LC-MS, for example, is often used to assign structural information to the individual components of a mixture for confirmation of drug load and distribution.The presence of hydrophobic linkers and drugs occasionally leads to problems, however.

As an example, Jacobson points to the difficulty associated with charge-based assays such as ion exchange HPLC (IEX). "Although several new stationary phases have appeared, none have proven great for ADCs due either to nonspecific interactions or inadequate resolution. Capillary isoelectric focusing (cIEF), such as imaged cIEF, has been a reasonable substitute, but experience with monoclonal antibodies (mAbs) suggests that there may be differences in the information obtainable by each method," he comments. Jacobson also notes that it is difficult to do preparative collection of charge variant species from capillary electrophoresis (CE) for characterization.

Bioanalysis in Biological Fluids

From an immunoassay perspective, the development and validation of methods for the bioanalysis of ADCs require the

consistent availability of high affinity, high specificity, anti-toxin antibody reagents to ensure appropriate selectivity in the analysis, according to Michael Brown, director of ICON's Bioanalytical Laboratories. "Generation of these types of antibodies to small molecular weight toxins is considered an art, and often times their availability can be limited," he observes.

Given the potency of the toxins, it can be a challenge getting an MS method developed and validated with the required lower limit of quantitation (LLOQ). Identifying the metabolites of the toxin can be even more difficult because they are generally present in even lower concentrations than the parent. In addition to the determination of very low concentrations of released drug in physiological fluids in the presence of relatively high ADC levels, interaction of reactive intermediates with albumin or other biomolecules, changing DAR values, and the inability to use assays developed for the parent antibody for the corresponding ADC with a different architecture are also issues that must be considered when evaluating the systemic exposure of ADCs as part of drug pharmacodynamic and pharmacokinetic analyses, according to Kaur from Dalton.

One approach is to use hybrid techniques such as affinity capture LC-MS/MS, but that adds complexity to the analysis. A combination of immunoassay and LC-MS/MS techniques can also be used in addition to the hybrid techniques. "There is also increasing interest in the use of high resolution accurate mass spectrometry to support various facets of ADC bioanalysis given the complex and dynamic nature of these molecules," says Mario Rocci, senior vice-president of ICON Bioanalytical Laboratories.

Generally, five different analyses are required to characterize the *in-vivo* performance of an ADC, according to Paula Jardieu, senior vice-president and general manager of ICON's Bioanalytical Laboratories, including quantitation of the time course of the intact ADC, the total antibody, and the toxin with and without linker, plus the immunogenicity of the ADC. Changes in the DAR over time in vivo, as well as the stability of the ADC in the matrix, also must be evaluated. It is also important to establish the stability of the ADC through the analytical procedure, because instability could introduce artifacts, according to Jardieu.

Free drug issues are typically monitored using reverse-phase chromatography with UV detection, or if the levels of detection must be very low, using LC-MS/MS against a drug linear curve, according to Dan Peckman, biochemistry manager with Eurofins Lancaster Laboratories. He also notes that confirmation that the DAR does not change with time as a function of molecular stability is typically achieved using LC or UV approaches. Mass spec analysis of the intact ADC and the naked antibody can give relevant information about the stability of the ADC.

The Platform Approach

For many small-molecule drugs, a platform of generic screens is often used for drugs

based on their structures and mechanism of action. For ADCs, however, it is difficult to generate "platform" methods that are suitable to a variety of ADCs because there are many non-specific interactions with stationary phases post-conjugation, according to Davidson. McDermott adds that while many of the analytical methods for ADCs are based on similar techniques, each construct is unique and requires an understanding of the basic chemical or physical properties that must be assessed. "Asking the right question is as important as getting an answer," she says.

Even given these challenges, SAFC has been able to develop screens for drug load using HIC and reverse-phase chromatography, and a screen for monomer purity using SEC. "These platform methods allow us to move quickly through the development phase and focus on more challenging assays early in the project," she explains. Other techniques such as iCE, CGE, ELISA, cytotoxicity and methods for residuals are developed by subject matter experts and optimized for each product. Testing for safety and quality attributes (bioburden, endotoxin, pH, osmolality, excipients, and appearance) are either verified using compendial-based platform methods or developed per product.

Analysis through the Development Process

As ADCs move through the drug-development process, different analytical methods are often required. Much of the early-stage development is driven by methods required to support development of the conjugation process, according to Davidson, and only a few methods may be required to support early conjugation process development, for example, SEC for aggregation, HIC for DAR, and RPC for free drug. Once the conjugation process has been established, additional analytical methods are required to ensure that the functionality of the antibody and potency are effective, and other characterization methods are then introduced (e.g., cytotoxicity, binding, charge heterogeneity, residual solvents, excipient testing, etc.).

For the production of early-phase clinical supplies, in fact, McDermott notes that multiple orthogonal methods are required to ensure method accuracy and process consistency. Then, as the phase of development advances further and multiple lots are produced, the number of methods can be reduced to the ones determined to provide information around critical quality attributes. Methods are often optimized as the project progresses, and further understanding of the chemistry is achieved. "A good example for ADCs is the assay for monitoring any residual-free drug equivalents," she comments. "Typi-

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cally, this method is first developed for either the drug or drug linker used in production. As stability information or multiple lot information is available, further compounds that are drug-related may be detected, and the method will be modified to track these impurities. Continual monitoring of release and stability data is necessary to ensure adequate methods are available for validation."

It is important to realize, Jacobson adds, that sometimes knowledge from characterization studies will result in the need for a method for lot-release or may be used to justify its omission. "The availability of analytical methods and product-specific knowledge that may lead to the addition of new assays (or replacement of older technologies) as a clinical candidate approaches licensure," he says.

Building a Bridge

ADCs present a complex bioanalytical problem that requires leveraging large- as well as small-molecule analytics. Recent developments in LC-MS/MS technology and the use of orthogonal analytical and bioanalytical methods combined with process knowledge support the idea that manufacturing and analysis of ADCs with consistent quality and efficacy is achievable, according to Kaur. He also notes that with the move to adopt a quality-by-design (QbD) approach for defining critical quality attributes for ADC, it is becoming easier to demonstrate the reproducibility of conjugation and ADC analysis. Meanwhile, McDermott asserts that "Advances in method development require subject matter experts in both fields to forge integrated techniques that will bridge the knowledge gap and blend pharma and biotech platforms, and the interface of these analytical techniques allows real understanding of the ADC construct to be achieved."

Importantly, sharing of information is taking place not only between different groups within pharmaceutical manufacturers, but also between companies. "Cross-fertilization within the biopharmaceutical industry, particularly driven by the proliferation of ADC-focused conferences, workshops, instrument company webinars, etc., has lead to an increased convergence in the methods being used," observes Jacobson. "While there are clearly some differences, for example those that might result from the specific requirements imposed by a particular company's unique conjugation technology, in general good ideas are being picked up and incorporated widely as they are introduced," he continues. Peckman agrees. "I think the most helpful resource is the sharing of information from technical presentations and

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technical papers. We are at a point where many groups are experiencing the same challenges with characterizing ADCs, and evaluating the research results obtained by others inspires new ideas for method changes in the future."

Evolving ADC Chemistry

Adding to the complexity of ADC method development and validation is the growing diversity of bioconjugates being advanced into the clinic. "Traditional cysteine and lysine chemistry is still a significant part of the ADC regime, but we are seeing a greater breadth of linkers, toxins, mAbs, and classes of conjugation chemistry. New analytical methods are needed for each of these new classes, both for characterization of the chemistry and release and stability testing," says McDermott.

In addition, as more products make it into the clinic and then into the market, Jacobson expects that regulators will become more familiar, and more comfortable, with the ability of current analytical tools to demonstrate the robustness of ADC manufacturing. "One consequence may be a better definition of what really needs to be controlled by product testing. In such a rapidly evolving area, the regulatory requirements are clearly evolving as more knowledge makes it into health authority submissions and into the peer-reviewed literature," he says.

On the other hand, as the ADC industry continues to expand at pace and current understanding of the complex interactions between drug payloads and antibodies increases, Davidson notes that new problems are constantly being uncovered that often require analytical solutions. "It can therefore be expected that more issues will be identified and there will be more challenges to come," he concludes.

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Cynthia A. Challener, PhD, is a contributing editor to BioPharm International.

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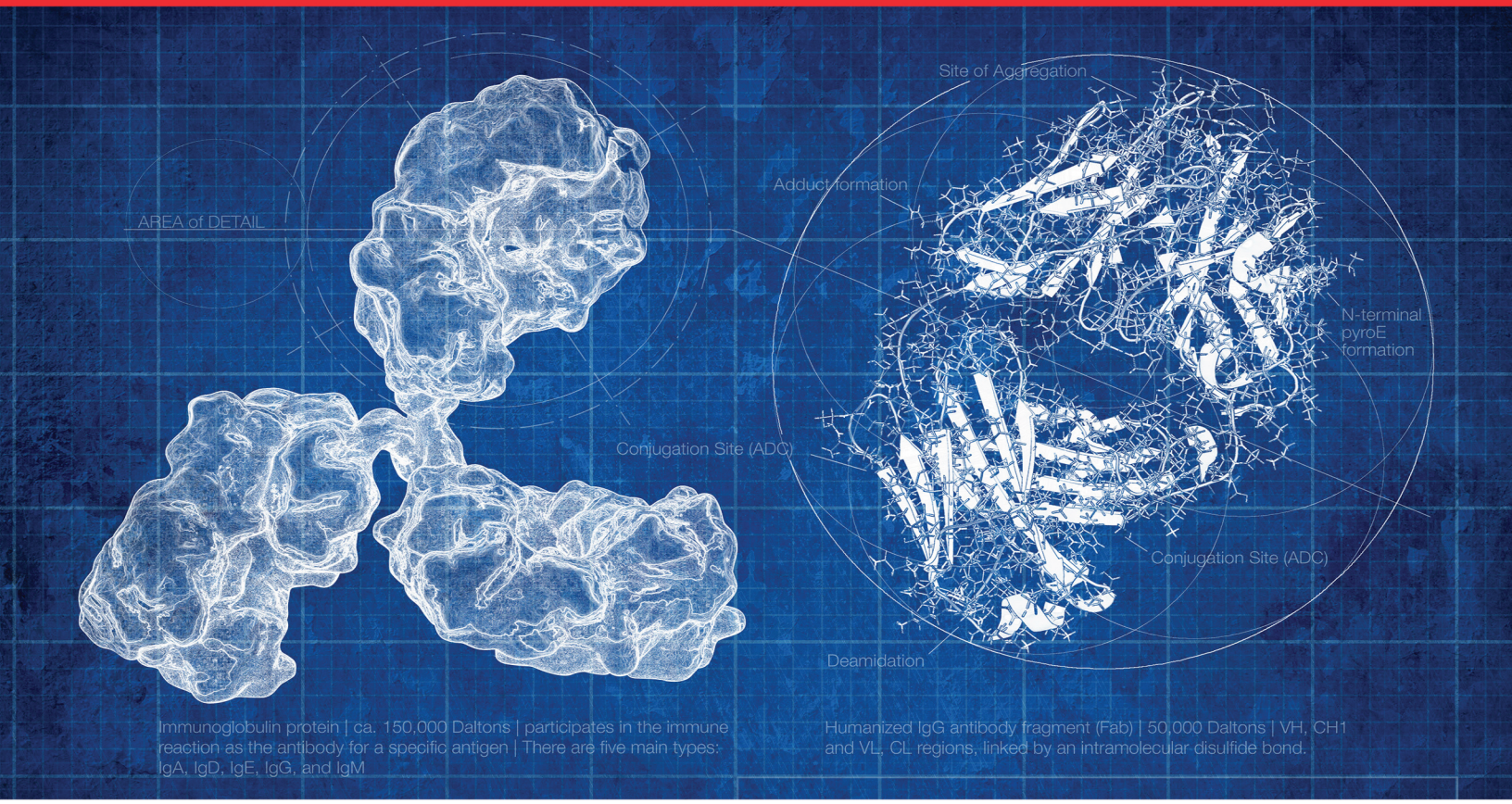

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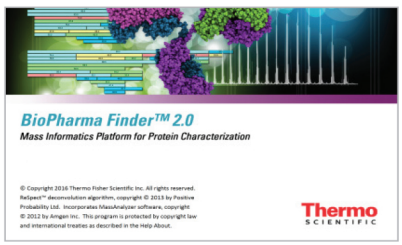

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