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To cite this article: Anil Wagh, Hangtian Song, Ming Zeng, Li Tao & Tapan K. Das (2018): Challenges and new frontiers in analytical characterization of antibody-drug conjugates, mAbs, DOI: 10.1080/19420862.2017.1412025

To link to this article: https://doi.org/10.1080/19420862.2017.1412025

Accepted author version posted online: 02 Jan 2018.

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Challenges and new frontiers in analytical characterization of antibody-drug conjugates

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KEYWORDS: Antibody-drug conjugates; analytical; quality attribute; stability; characterization; CQA, DAR

Abbreviations:

Analytical ultracentrifugation, AUC;
Anion exchange chromatography, AEX;
Capillary electrophoresis, CE;
Capillary electrophoresis sodium dodecyl sulfate, CE-SDS;
Cation exchange chromatography, CEX;
Critical quality attributes, CQAs;
Drug-to-antibody ratio, DAR;
Enzyme-linked immunosorbent assay, ELISA;
Hydrophobic interaction chromatography, HIC;
Imaged capillary isoelectric focusing, iCIEF;
Ion-mobility mass spectrometry, IM-MS;
Mass spectrometry, MS;
Reversed-phase-HPLC, RPC;
Size-exclusion chromatograph, SEC-UV;
Two-dimensional liquid chromatography, 2D-LC;
US Food and Drug Administration, USFDA;

ABSTRACT
Antibody-drug conjugates (ADCs) are a growing class of biotherapeutics in which a potent small molecule is linked to an antibody. ADCs are highly complex and structurally heterogeneous, typically containing numerous product-related species. One of the most impactful steps in ADC development is the identification of critical quality attributes to determine product characteristics that may affect safety and efficacy. However, due to the additional complexity of ADCs relative to the parent antibodies, establishing a solid understanding of the major quality attributes and determining their criticality are a major undertaking in ADC development. Here, we review the development challenges, especially for reliable detection of quality attributes, citing literature
and new data from our laboratories, highlight recent improvements in major analytical
techniques for ADC characterization and control, and discuss newer techniques, such as two-
dimensional liquid chromatography, that have potential to be included in analytical control strategies.
INTRODUCTION

The development of monoclonal antibodies (mAbs) has introduced new curative strategies with improved efficacy and novel treatment approaches for many life-threatening diseases, including different types of cancers.\(^1\) Over 40 therapeutic antibodies have been already approved for the treatment of various diseases based on their remarkable effectiveness over the standard chemotherapeutic agents and more than 450 molecules are currently at different stages of clinical development.\(^2\) The rapid late-stage clinical development of antibody therapeutics, partly assisted by creative regulatory pathways for therapies with high clinical potential for unmet medical needs (such as introduction of the Breakthrough Therapy designation by US Food and Drug Administration in 2012, and the PRIority MEdicines designation by the European Medicines Agency in 2016), has led to an increase in the number of regulatory approvals.\(^3\)

Similar to antibody therapeutics, mAb-based antibody-drug conjugates (ADCs), a new class of therapeutics, is also getting attention for the treatment of various cancer types.\(^4\)\(^-\)\(^6\) This is primarily attributed to a significant improvement in the therapeutic index of the conjugated chemotherapeutic agents owing to targeted delivery of drugs to cancer cells over normal cells. Gemtuzumab ozogamicin (Mylotarg\(^\text{®}\), from Pfizer) was the first ADC approved by the US FDA in 2000 for the treatment of patients with acute myelogenous leukemia (AML). Later, the product was voluntarily withdrawn from the US market (marketing continued in Japan) in 2010 due to its safety concern and failure to demonstrate clinical benefit to patients relative to its safety profile.\(^7\) In 2017, the drug was again approved by US FDA for treatment of newly-diagnosed CD33-positive AML.\(^8\) Since the first approval of Mylotarg\(^\text{®}\), three more ADCs have received FDA approval: brentuximab vedotin (Adcetris\(^\text{®}\), marketed by Seattle Genetics and
Millennium/Takeda) for the treatment of Hodgkin and anaplastic large cell lymphoma,\(^9\) trastuzumab emtansine (Kadcyla\(^\circledR\), marketed by Genentech and Roche) for the treatment of metastatic breast cancer,\(^10\) and inotuzumab ozogamicin (Besponsa\(^\circledR\) marketed by Pfizer) for the treatment of acute lymphoblastic leukemia\(^11\) (Table 1). There are over 60 ADCs currently at different stages of clinical development for various indications.\(^12\)-\(^16\)

Abbreviations: B-Cell Maturation Antigen, BCMA; Epidermal growth factor receptor, EGFR; Human epidermal growth factor receptor, HER; PSMA, Prostate-Specific Membrane Antigen

ADCs are constructed from three different components: 1) an antibody that targets a specific antigen, usually on the surface of cancer cells, 2) a highly potent cytotoxic agent (drug),\(^17\) and 3) a linker that enables covalent attachment of the cytotoxin to the antibody (Fig. 1).\(^18\) An antibody offers a number of functional groups that can be used for the covalent conjugation of the drug molecules. The primary conjugation site is the amino groups from the lysine residues or the native cysteine residues derived after reduction of inter-chain disulfide bonds.\(^19\) Well-known examples are Kadcyla\(^\circledR\) and Adcetris\(^\circledR\) in which the drug molecules are coupled to the lysine and cysteine residues of the antibody, respectively. Both lysine and cysteine conjugation chemistries are inherently random in nature, as they rely on the non-specific electrophilic reactions between the solvent-exposed amino acids and the linker molecule. Because of polyvalent nature of antibody, the resulting ADC is a highly heterogeneous mixture containing species with varied drug-to-antibody ratios (DARs) as well as variable conjugation sites. This heterogeneity may affect drug safety and efficacy (due to the presence of undesired species). The target product profile of an ADC must include a robust definition of DAR and degree of homogeneity. Therefore, the ultimate goal of process development is to develop a
manufacturing process based on solid understanding of the conjugation chemistry, accessibility of the conjugation sites on the mAb, stoichiometry of reactants, characterization of side products and undesired species, and purification steps to reduce heterogeneity. The manufactured drug substance of an ADC must be of consistent quality between early clinical stages and commercial development.

Site-specific conjugation approaches are being developed using antibody engineering to mitigate the heterogeneity issue.\textsuperscript{20,21} For example, McDonagh \textit{et al}. substituted the cysteine residues involved in formation of the interchain disulfide bonds with serine residues to achieve the desired stoichiometry and targeted sites of drug attachment.\textsuperscript{22} A similar approach, THIOMAB\textsuperscript{®}, in which antibody was engineered at targeted positions on the light and heavy chains to provide two free cysteine residues for drug conjugation, was subsequently developed.\textsuperscript{23} An alternative approach to site-specific conjugation involves the creative use of enzymatic reaction. One such example is the covalent bond formation between an engineered glutamine residue on an antibody and an amine group on the linker/drug by using microbial enzyme transglutaminase.\textsuperscript{24-27} Overall, these site-specific conjugation techniques help to reduce product heterogeneity and optimize the therapeutically beneficial properties of the ADC candidates in terms of their conjugation sites, linkers, and payload characteristics.\textsuperscript{28} As highly potent cytotoxic drugs are used in the development of ADCs, there are significant challenges in regard to safety during handling, manufacturing and storage of these cytotoxic materials.\textsuperscript{29} Any type of direct human contact with these cytotoxic entities, especially free drug, can be highly unsafe. Therefore, attention must be paid to establishing safe handling procedures with appropriately defined band levels for occupational exposures, as well as using dedicated
containers/equipment/laboratory if necessary. Use of appropriate personal protective equipment is paramount for operator safety, and proper signage must be posted to alert all personnel entering these areas.

Given the complexity of ADCs resulting from the addition of the drug payload to an already structurally-complex antibody via a linker molecule, characterization of ADCs presents a substantial challenge from an analytical development perspective. Apart from the quality attributes of each component (i.e., mAb, linker and drug), there are several ADC-specific critical quality attributes (CQAs) that need to be defined beforehand. One of the most important of these attributes is DAR heterogeneity, which necessitates determination of total drug load, drug load distribution, level of unconjugated antibody/linker/drug, and sites of conjugation. Here, we discuss various analytical techniques that are used for defining the CQAs (Table 2) for development and production of ADCs, in the context of its physical and chemical stability, and their control strategy. Furthermore, we discuss recent improvements in existing analytical techniques, as well as promising new techniques that can be used for advanced ADC characterization and control. Published literature data, as well as our own observations, are included in the discussions of analytical characterization and control strategies. We also provide a brief overview of persistent analytical challenges that will require disruptive technologies for solution, and future directions in ADC development.

**MAJOR QUALITY ATTRIBUTES OF ADC**

**Determination of Drug-to-Antibody Ratio**

DAR, which represents an average number of drugs conjugated to an antibody, is an important quality attribute that is directly linked to efficacy and safety of ADC because a low
drug loading (low DAR) can reduce potency of ADC, while high drug loading (high DAR) can alter pharmacokinetics and toxicity of the ADC molecule.\textsuperscript{34} Therefore, control of the conjugation reaction where changes in reactant concentrations can result in variable DAR (especially in the case of random conjugation) is the most critical step in development. The manufacturing and post-manufacturing processing/handling controls must be in place to prevent occurrence of unconjugated antibody, as well as low/high DAR species, to consistently achieve the desired DAR target.\textsuperscript{35} From a product stability perspective, the extent of drug loading can alter the stability and aggregation propensity of ADC.\textsuperscript{36} Hence, the drug load and drug distribution are CQAs that should be controlled in the manufacturing of ADCs. Various analytical methods utilizing spectroscopic,\textsuperscript{37} radiological,\textsuperscript{38} chromatographic,\textsuperscript{39,40} and mass spectrometric techniques,\textsuperscript{41,42} have been reported for the determination of DAR. The suitability of these methods is based on the conjugation chemistry and physiochemical characteristics of the linker or drug molecules. Methods used for the DAR determination including their strengths and limitations are discussed in the following sections.

Among all of these methods, UV-Visible spectroscopy is a relatively simple and frequently used method for DAR determination. Prerequisites for the method are: 1) drug should contain a UV-Visible chromophore; 2) the drug and the antibody should exhibit distinct and separate absorption maxima in their UV-Visible spectra; and 3) the presence of the drug should not affect the light-absorbing properties of the antibody moiety in the ADC sample and vice versa. Based on the measured absorbance and extinction coefficient, the individual concentrations of the protein and drug can be calculated according to the Beer–Lambert
principle. The average DAR (moles of drug per mole of antibody) can be determined following equation 1:

\[
Avg \text{ DAR} = \frac{C_{\text{drug}}}{C_{\text{antibody}}} \quad (1)
\]

where \(C_{\text{drug}}\) and \(C_{\text{antibody}}\) refers to molar concentration of drug and antibody, respectively.

Since at any given wavelength, total absorbance is the sum of individual absorbance values for each component, the absorbance value of the antibody and drug at their respective wavelengths (\(\lambda_{\text{max}}\)) must be corrected to limit overestimation of the payload. Owing to its simplicity, the UV-Visible spectroscopic method has been routinely used in the determination of DAR for various ADCs. For example, trastuzumab emtansine (Kadcyla\textsuperscript{®}) is an ADC in which the humanized anti-HER2 IgG1 antibody trastuzumab (Herceptin\textsuperscript{®}) is covalently conjugated to the cytotoxic agent DM-1 using 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (MCC) as a linker molecule.\textsuperscript{37} Free DM-1 has an absorption maxima at 252 nm, which is significantly different from the absorbance maxima (at 280 nm) of the major aromatic chromophores (tryptophan and tyrosine residues) of trastuzumab (Fig. 2). The successful conjugation of DM-1 to trastuzumab is evidenced by the presence of an additional peak at 252 nm in the conjugate and the differentially located absorbance bands (at 252 nm and 280 nm), which are used for calculation of the DAR.

Other conjugated drug molecules that have been analyzed by UV-Visible spectroscopy for the determination of DAR are summarized in Table 3. Despite its simplicity and ease of use, the utility of the UV-Visible spectroscopic method is limited to UV-Visible active drugs that possess different absorption bands than the aromatic residues (tryptophan or tyrosine) of an antibody. In addition, the presence of free drug in an ADC sample can potentially interfere with
the measurement and, therefore, lead to overestimation of the DAR value. Furthermore, the UV-Visible spectroscopic method does not provide any information on drug load distribution.

To circumvent some of these limitations associated with the UV-Visible spectroscopic method, other approaches such as labeling of the drug or antibody molecules have been explored. For example, Cohen et al. used a dual labeling approach wherein both the drug moiety (tubulysin variants) and the antibody (trastuzumab) were distinctively conjugated to two different radioactive labels before the conjugation reaction. Simply by measuring the radioactivity, this approach facilitates the monitoring of ADCs during the conjugation reaction and purification processes. Additionally, radioactive label can help in studying in vivo behaviors of the conjugate (¹³¹I-TUB-OMOM-⁸⁹Zr-trastuzumab conjugate⁴³) to predict the pharmacokinetics and tumor targeting potential of an ADC.

In another study, a new fluorescence-based approach involving the derivatization of two maytansinoids, DM1 and DM4, using BODIPY FL fluorophore is reported.⁴⁴ The derivatization involves conjugation of BODIPY to thiol-containing drug in 1:1 stoichiometry. The advantages of this approach are that it can be used for quantification of drug analytes at microscale (50 µg scale), and therefore can be used during early research phase when accurate weighing is often challenging owing to material scarcity. Also, theoretically, a simple fluorescence measurement may facilitate its potential use for the quantification of DAR for ADCs, especially for the compounds where absorbance of drug and protein is overlapped. However, the application may be limited due to the inherent issues of photobleaching and fluorescence quenching.
Some of the newer approaches of DAR measurement include enzymatic cleavage of the drug from the antibody and subsequent qualification using chromatographic techniques. The rate and extent of drug cleavage by cathepsin B enzyme from vcMMAE-based ADCs conjugated at reduced disulfide bonds has been shown to be independent of the drug location and monoclonal antibody carrier. Based on a similar concept, a novel approach using cathepsin B enzyme has been reported for the quantification of DAR for random lysine-conjugated ADCs. This method is applicable to either random or site-specific ADCs conjugated using hydrolytically stable cathepsin-B cleavable linker. It involves a series of treatments with enzymes and reductant to allow complete release of the conjugated drugs followed by chromatographic separation. First, the ADCs are cleaved by IdeS protease at a site below the hinge region to yield F(ab’)2 and Fc fragments followed by reduction with 2-mercaptoethylamine (2-MEA) to allow further fragmentation to create Fd’, Fc and Lc fragments (Fig. 3). This pretreatment of IdeS and 2-MEA ensures access to the cleavage sites and thus aids in complete release of the conjugated drug. Following the pretreatment, the samples are treated with cathepsin B enzyme to facilitate release of the drug from antibody fragments. The released drug is chromatographically separated from the protein components by RP-HPLC with quantitation by UV absorbance. The quantification of cleaved drug allows precise and accurate determination of the total DAR for cathepsin B cleavable linker-based ADCs irrespective of the conjugation chemistry. This method circumvents most common limitations associated with other DAR-determining methods, such as the UV method requiring differential absorption wavelengths for drug and antibody, mass spectrometric methods in which mass signal is affected by the hydrophobic nature and net protein charge, and the hydrophobic interaction chromatography (HIC) method that often results.
in low resolution efficiency for random-conjugated ADCs. However, similar to the UV-Visible method, the cathepsin B method does not provide the drug load distribution profile because the technique relies on the sum of total absorbance of all cleaved drug molecules. Furthermore, breakdown of the protein into smaller fragments by the use of IdeS and 2-MEA prohibits direct quantification of protein concentration. The cathepsin B method involves many pretreatment steps prior to drug quantification, therefore method transfer and implementation in a quality control environment can be challenging.

Mass spectrometry (MS) is another frequently used technique for the characterization of ADCs. Currently available MS spectrometers using electrospray ionization (ESI) coupled with time-of-flight (TOF) or Orbitrap with extended mass range can routinely differentiate heterogeneous molecular species of ADC with sufficient resolution. For example, a complete mass profile of ADC molecules can be obtained with intact mass measurement under reducing and non-reducing conditions. Molecular species such as ADC molecules with different numbers of payload and null linker (linker without a payload), as well as unconjugated antibody molecules, can be separated, identified, and quantified. In a mass spectrum, peak area or peak height represents the relative contents of each DAR species. Therefore, the average DAR value can be derived from the weight-average of detected species with different DAR numbers. As examples, Fig. 4 (A) shows the deconvoluted mass spectrum of a cysteine-conjugated antibody, and Fig. 4 (B) shows the deconvoluted mass spectrum of a lysine-conjugate from LC-MS. Mass spectrum of an ADC molecule is much more complex than that of the antibody due to additional layers of heterogeneity introduced by conjugation. To obtain more ADC-specific structural information with less interference from antibody features, it is beneficial to treat the ADC
molecules with a glycosidase such as PNGase F to remove heterogeneity brought forth by N-linked glycans, to reveal detailed structural features of the ADC, including the conjugated species and linker related heterogeneities.

MS-based methods are especially useful for characterization of non-specific conjugation through Lys, since, of about 80 Lys residues on a typical antibody, more than 20 Lys residues are surface exposed and thus have greater propensity to conjugation. Chromatography-based methods are not very effective in separating random conjugated ADCs with different drug load and quantifying DAR accurately, since the high degree of conjugation heterogeneity overwhelms chromatographic separation. The intact mass measurement, on the contrary, is much less affected by the heterogeneity of different conjugation sites for the same DAR species, and can separate and quantify ADC molecules with different drug loads in a straightforward manner. In practice, deglycosylation by PNGase-F treatment prior to intact mass analysis can simplify the mass spectra significantly. Another way of simplifying the mass spectra is to reduce the ADC molecules into heavy chain and light chain species, or cleave the ADC molecules into its Fc and Fd components by a combination of enzymatic treatment and reduction. This approach allows for more detailed structural information and detection of low levels of variants such that the DAR can be determined with high accuracy and sensitivity. The strategy of analyzing ADC molecules at the fragment level and combining information together afterwards improves mass resolution and sensitivity significantly. Another benefit from fragment analysis is the data obtained on fragments can be used to cross examine those obtained on the whole molecules for DAR measurement.
Usually ADC samples are presented in matrices that are not compatible with mass spectrometers. Therefore, sample treatment is necessary prior to MS analysis. If ADCs need to be enriched from biological samples such as plasma or serum, immunocapture is a powerful technique. ADC samples could be desalted and separated by reversed-phase chromatography or size-exclusion chromatography (SEC), or directly infused by nanospray. Alternatively, capillary electrophoresis (CE) interfaced with MS provides an orthogonal option for separation.

For cysteine conjugated ADCs, where inter-chain disulfide bonds are reduced and employed as conjugation sites, antibody molecules dissociate into light chains and heavy chains under the reversed-phase chromatography condition used in LC-MS analysis. Therefore, native spray MS without high levels of organic solvent ought to be used to obtain comprehensive intact mass profile for cysteine-conjugated ADC molecules. In native spray MS, samples could be directly sprayed by the nanospray source or desalted and separated by SEC with MS-compatible mobile phases, such as ammonium acetate (~200 mM) at neutral pH. An example of an intact mass spectrum of ADC with inter-chain disulfide cysteines as the conjugation sites is shown in Fig. 4. It is interesting to note that, for this class of ADCs, the predominant species are molecules with an even number of drug conjugation, which indicates that nearly all free thiols on cysteine residues are conjugated.

MS signal intensity depends on the ionization efficiency of the analyte. In the positive-ion mode of MS analysis, proteins are usually ionized by protons preferentially attached to the side chains of basic amino acid residues. For lysine-conjugated ADCs, ionization efficiency decreases by the attached hydrophobic drug, especially with larger load number, likely because
conjugation reduces the total number of charged lysine residues available for protonation. Such correlation is consistent with the observation that the DAR measured by MS is usually lower than DAR by UV-based method. Ionization efficiency naturally is affected by the degree of hydrophobicity. Chen and coworkers reported that after enzymatic removal of the hydrophobic portion of the drug load (PABC-MMAE), the DAR measured by MS (based on remaining MC-VC linker) matched well with the DAR measured by HIC.\textsuperscript{64}

The majority of the cytotoxic drugs used in creating ADCs are hydrophobic in nature, and therefore the conjugation of these drugs to an antibody alters the overall physiochemical properties, such as hydrophobicity-hydrophilicity balance on the surface. The alteration in hydrophobicity of the molecules can be used for determination of DAR and drug load distribution.\textsuperscript{40,68} One such method is HIC, which relies on the separation of conjugates based on the number of attached hydrophobic drugs to antibody. Therefore, the drug-loaded species are resolved based on increasing hydrophobicity, with the least hydrophobic unconjugated antibody eluting first from the column and the conjugate with the highest DAR eluting last. Importantly, the species resolution provided by a HIC column can be sufficient to capture the differences in payload, and therefore the level of conjugation in an ADC can be analyzed. The area percentage of the individual peaks represents the relative distribution of drug loaded conjugates. The average DAR can be calculated by using the following equation 2:

\[
\text{Average DAR} = \frac{\sum (\text{Weighted peak area})}{100} \quad (2)
\]

Where, the weighted peak area is the individual percentage peak area multiplied by the corresponding drug load.
HIC is a method of choice for determination of DAR and drug load distribution for ADCs produced by site-specific conjugation chemistry, including engineered cysteine residues and inserted unnatural amino acid by transglutaminase. The ADCs produced by site-specific conjugation, in which drug is attached to specific locations on the antibody, exhibit relatively low level of heterogeneity compared to ADCs produced by random conjugation chemistry, and therefore are more amenable to analysis using HIC. One such example is THIOMAB in which the site-specific conjugation of monomethyl auristatin E to the engineered cysteine substitutions at positions on the light and heavy chains to yield a composition with high degree of homogeneity that can be easily resolved using HIC-UV. Additionally, HIC can also be used for ADCs synthesized by partial reduction of inter-chain disulfide bonds, which are less heterogeneous relative to lysine conjugation. The conjugates generated by partial reduction of four antibody inter-chain disulfide bonds (such as Mab-vc-MMAE) have variable stoichiometry, ranging from zero to eight drugs molecules per antibody. HIC allows chromatographic separation of these drug-loaded conjugates to yield five predominant peaks that correspond to antibody containing zero, two, four, six, and eight drug molecules. Recently, Zhou et al. reported determination of DAR and drug load distribution by HIC-UV for novel site-specific ADCs generated through glycoengineering, i.e., by targeting the native glycans on Asn-297 of antibody (glyco ADC). First, two sialic acid units are introduced onto the native glycans of an antibody by an in vitro modification followed by mild oxidation of new sialic acids by sodium periodate. Finally, the desired ADC is generated by conjugating the aldehyde functionalized antibodies to aminooxy drug linker via a stable oxime bond. The glyco ADCs synthesized from three different antibodies and two different linker types showed good
separation by HIC (Fig. 5A to 5D) and the resulted DAR values were well correlated with the LC-MS results. Moreover, the authors further demonstrate that these glyco-conjugated ADCs can generate a more homogeneous and well-defined product profile compared to thiol-maleimide-based conjugates (Fig. 5E).

One of the issues with HIC-based methods is its inability to separate the positional isomers of ADC species containing the same number of conjugated drugs, including drugs conjugated to heavy and light chains. Developing a HIC method that is capable of separating different DAR species for lysine-conjugated ADCs is a significant challenge. Use of a high concentration of salts in the HIC method may limit its direct compatibility with MS analysis.

**Determination of Drug Load Distribution**

As described in the preceding sections, the overall drug load distribution can be characterized by intact mass analysis, or by HIC for less heterogeneous ADCs generated through cysteine or other site-specific conjugations. To determine drug load distribution at each conjugation site, more comprehensive analysis, such as peptide mapping, is necessary. Depending on the complexity of each scenario, several strategies can be applied accordingly. For ADCs with site-specific conjugation, it is relatively straightforward to determine or confirm the conjugation sites by peptide mapping with MS detection.

If a good chromatographic separation is achieved, accurate information about conjugation site occupancy can be obtained by LC method. It should be pointed out that the conjugated compounds may affect the efficiency of proteolysis due to steric hindrance and conformational change. Therefore, it is important to achieve full coverage of all related species for accurate determination. Often, it is necessary to use a second enzyme in sequential or in parallel treatment
to capture all species involved. If a satisfactory chromatography separation is not feasible, an MS signal can be used to achieve a relative quantification of the conjugated and non-conjugated peptides with an accuracy heavily dependent on each specific case.

For ADCs with random conjugation, if the conjugated drug exhibits an absorption peak not shown by the unconjugated antibody, peptide maps of the ADC and the unconjugated antibody at this unique wavelength can be used to compare and locate the peptides conjugated with the drug, as demonstrated by Kim and coworkers (Fig. 6A). The peptides conjugated with drug molecules and the locations of conjugation sites can then be determined by LC-MS analysis. A similar approach was used to characterize a lysine conjugate (Fig. 6B). If the typical 214 nm (absorption wavelength of general peptide bonds) is used for peptide mapping detection, identification of individual conjugation sites and their occupancy levels can be more challenging. For example, as reported by Wang et al, a side-by-side comparison of conjugated vs unconjugated antibody usually shows little difference between the two maps due to low abundance of each modified peptide relative to its native unconjugated form. In addition, many of the minor differences observed in a map are potential artifacts arising from method-induced variations. In this case, MS analysis through searching for expected mass increase in the conjugated map, in correlation with the lack of this mass increase in the unconjugated map, is the most efficient way of identifying the conjugated peptides that may lead to determination of the conjugation sites.

The detection limit by this approach can routinely go down to less than 1%. When an MS signal is used in the quantification of conjugated peptides, the effect of attached compounds on ionization efficiency due to loss of primary amine group and introduction of the hydrophobic
moiety should be accounted for to achieve an accurate quantification. However, the impact of conjugation on ionization efficiency is likely to be less prominent for the intact protein. Additionally, endoproteinases trypsin/Lys-C, which is commonly chosen for peptide mapping, does not cleave at modified lysines, making it difficult to measure the site occupancy.

Another difficulty in peptide mapping analysis of ADC is the greater propensity of conjugated peptides to precipitate and adsorb onto the vial surface, although this can sometimes be alleviated by adding acetonitrile or isopropanol alcohol to the sample solution.\textsuperscript{72}

Peptide mapping can be used to assess process consistency of conjugation between batches, therefore it is a very useful technique in supporting process development. Fig. 7 demonstrates site occupancy of a random lysine-conjugated ADC. MS signal intensities of the conjugated peptides illustrate consistency between four batches of ADC manufactured by the same process, at each conjugated lysine residue.

Reversed-phase-HPLC (RPC) is an orthogonal method that provides information about the distribution of drugs on the light and heavy chains. If incorporation of linker or drug induces a detectable increase in hydrophobicity of the antibody, the drug congeners (various DAR species) could be resolved by the RPC gradient. Based on the chemistry and nature of conjugation, untreated samples can be injected onto the column directly or samples can be pretreated with the reducing agent to obtain the information regarding intact and reduced DAR. For example, in case of Cys-linked ADCs where cysteine residues from inter-chain disulfides bonds are conjugated with linker and drug, even a small amount of organic solvent has proven to be too disruptive, and could lead to antibody dissociation.\textsuperscript{40} Therefore, the samples are usually
treated with reductants such as dithiothreitol (DTT) before analyzing on RPC. Wiggins et al. reported the characterization of Cysteine-linked IgG1 and IgG2 drug conjugates by using RPC-UV, wherein each of the conjugates was reduced by DTT followed by reverse phase separation.\textsuperscript{73} In another study, Xu et al. developed a RPC-UV method for DAR characterization of site-specific non-natural amino acid-based ADC, which was applicable to both reduced and intact conjugates (\textbf{Fig. 8}).\textsuperscript{74}

One of the limitations of a RPC-based method is that it relies on hydrophobicity differences among the species with various levels of conjugation; therefore, it may not be suitable for monitoring certain drug conjugates with low hydrophobicity. This technique is applicable to cysteine-conjugated ADCs, but may not be suitable for highly heterogeneous ADCs such as random lysine conjugates. Additionally, the use of denaturing conditions in this method could cause dissociation of antibody molecules lacking inter-chain disulfide bonds.

**Determination of Unconjugated Antibody**

In general, a typical distribution profile of ADC contains a mixture of unconjugated antibody and drug-antibody congeners.\textsuperscript{75} The naked antibody competes with ADC for the target antigen and ultimately decreases the amount of drug delivered to the target cell. The level of unconjugated antibody in an ADC formulation is a critical parameter in process control because it can directly affect the efficacy of ADC. Therefore, the level of unconjugated antibody is an important quality attribute that must monitored throughout the shelf life of the product. Mass spectrometric techniques continue to be versatile tools that have been successfully applied for quantification of unconjugated antibody. MS analysis of huc242 antibody conjugated to
maytansinoid molecules (DM4) showed a typical distribution where the ADC contains a mixture of unconjugated huc242 antibody (D0) and antibody-drug congeners (D1 to D7), with average DAR of 3.6 (Fig. 9). The percentage of unconjugated antibody is calculated based on the area under the curve for each peak and total area. Overall, LC-MS is the best method for complete characterization of ADCs, although MS-based methods for ADCs involve expensive instrumentation and very complex data analysis.

As the conjugation of drug molecules onto the antibody can greatly alter the surface and overall electrostatic profile, charge based separation techniques can be useful for improved characterization including detection of unconjugated antibody. Imaged capillary isoelectric focusing (iCIEF) has attracted substantial attention in recent years as an analytical technique for charge variant analysis of ADCs. Unlike the traditional separation methods such as ion exchange chromatography (IEX) and slab-gel based isoelectric focusing (IEF), iCIEF separates charge variants more precisely based on their isoelectric point (pI). If the antibody and ADC have different pI values, the antibody can be separated from various ADC species and quantified by iCIEF. For example, Lin et al. successfully demonstrated the separation of unconjugated antibody and drug-conjugated antibody based on their different pI values using iCIEF. The drug loaded species (conjugated antibody) are further resolved based on the reduction in surface charge due to conjugation to Lys. With the increase in the number of drugs conjugated to antibody, pI of the drug loaded species shift toward the acidic side relative to the unconjugated antibody (as demonstrated in Fig. 10A). Each individual peak represents a conjugate with a certain payload. The concentration of the unconjugated antibody is determined from a calibration curve. Furthermore, iCIEF may also serve as a quick and convenient fingerprinting technique for
ADCs to monitor any conjugation- or process-related changes. **Fig. 10B** shows lot-to-lot consistency of 4 different ADC batches as determined by iCIEF. However, characterization of drug load distribution by iCIEF can be very challenging due to the fact that conjugation of linker without drug can also shift charge profile toward the acidic side, and thus overestimate the distribution. In summary, iCIEF can be applied to measure the level of unconjugated antibody and drug load distribution, but it cannot distinguish between conjugates, process intermediates and impurities such as antibody with linker only and antibody with linker/drug conjugate. Additionally, iCIEF analysis may not be applicable to other conjugation chemistries, such as cysteine, glycan or site-specific chemistries, that do not result in significant changes in charge and net pI.

Similar to surface charge changes, the conjugation of a (hydrophobic) drug also alters the hydrophobicity of the protein molecules due to the inherent lipophilic nature of the drug or linker. Such changes in surface hydrophobicity can be used to determine the level of unconjugated antibody in ADC samples by hydrophobicity-based chromatography techniques such as HIC, where the least hydrophobic unconjugated antibody elutes first, followed by ADC molecules with increasing surface hydrophobicity. The interactions between the hydrophobic patches on the surface of an ADC molecule and the HIC stationary phase is generally reversible in nature, and is significantly influenced by the presence of salt in the mobile phase. High salt concentration enhances the hydrophobic interactions, whereas a low salt concentration weakens it. Therefore, the retention and elution of ADC molecules from a HIC column can be modulated by salt content.
The hydrophobicity of drug or linker and the heterogeneity of ADCs have a significant impact on the separation of unconjugated antibody from drug-conjugated species. For example, species with different DARs from site-specific or cysteine-based ADCs are usually well resolved in HIC. In contrast, ADC species with different DARs from random conjugation at the lysine residues are rarely well resolved in HIC due to the high degree of molecular heterogeneity. However, it is still possible to at least partially separate unconjugated antibodies from the conjugated ones. Fig. 11 shows the HIC-UV profiles of two batches of a lysine-based ADC on a MAbPac HIC-20 column using sodium phosphate/ammonium sulfate mobile phase. Despite complex HIC-UV profile due to random conjugation, the unconjugated antibody is resolved well enough to differentiate it from the drug-conjugated species. The level of unconjugated antibody can be calculated using equation 3:

\[
\text{% of unconjugated Mab in ADC} = \frac{\text{Unconjugated Mab (mg/ml)}}{\text{Total protein concentration (mg/ml)}} \times 100
\]

Where, the amount of unconjugated antibody is determined based on a standard curve of free antibody at different concentrations.

**Determination of Charge Variants**

The charge variants of a protein therapeutic are important quality attributes because of their potential effects on stability and biological activity. Various forms of charge-related heterogeneity can be observed in an ADC caused/influenced by protein expression, drug conjugation, purification process, and various post-translational modifications including enzymatic or chemical degradation. The subtle charge differences in these variants can be monitored by using either IEX or capillary electrophoresis-based ICIEF. In ADCs, the charge
variants of the parent Mab could be important quality attributes if they affect the binding affinity or activity. However, the attachment of linker or drug through lysine residues results in a decrease of positive charge by one unit for each lysine conjugation, therefore the resulting profiles show the drug/linker load distribution (Fig. 10B) rather than the charge variants of the parent mAb. Charge variant analysis of ADC can be challenging depending on the physico-chemical properties of drug/linker and conjugation chemistry. Additionally, the complex charge variant profile of an ADC may not readily correlate to certain charge-altering chemical degradation pathways, including Asn deamidation, glycation, and Asp isomerization (when forming succinimide intermediate). Thus, IEX or iCIEF may not prove to be highly useful for the determination of comprehensive charge variant profile for lysine-conjugated ADCs. In contrast, charge-based assays can be suitably applied to cysteine-conjugated ADCs.\textsuperscript{36} In conclusion, the applicability of charge-based methods must be evaluated on a case by case basis depending on conjugation chemistry and nature of drug-linker.

**Determination of Size Variants**

Size-Exclusion Chromatography

Size variants (aggregates, particles, fragments) are important quality attributes that can directly affect the efficacy or safety of protein therapeutics. Aggregation, which occurs when two or more monomeric units associate with each other to form higher oligomeric structures with sizes ranging from tens of nanometers to hundreds of microns, is the most common pathway of physical degradation of proteins. ADCs in particular are more prone to aggregation than the parent antibody, primarily due to the presence of drug/linker molecules which impart more hydrophobicity around the local surface of the conjugation sites. Additionally, the drug
conjugation may also have a direct effect on the total surface charge,\textsuperscript{78} as well as thermostability of proteins.\textsuperscript{79,80} Overall, these factors either individually or in combination influence the aggregation propensity of ADCs.

Fragmentation, on the other hand, results from peptide bond cleavage \textit{via} chemical or enzymatic reactions. Although protein backbones have relatively high stability, certain sites may be prone to fragmentation, with several contributing factors, e.g., sequence of certain amino acid combinations, solvent accessibility, structural flexibility, solution conditions (temperature, pH, presence of metals/radicals).\textsuperscript{81} In many cases, fragmentation of proteins may also trigger or aggravate the process of aggregation.\textsuperscript{82} Monitoring both fragments and aggregates is important, because these degradation products may elicit immunogenic responses, such as release of anti-therapeutics antibodies (by aggregates), and may affect pharmacokinetics or potency (by fragments), when drug product is injected into the blood stream. Therefore, the presence of size variants can compromise the purity and shelf life of the drug product. Various analytical techniques such as SEC, capillary electrophoresis sodium dodecyl sulfate (CE-SDS), SEC with multi-angle static light scattering, dynamic light scattering, and particle measurements (e.g., light obscuration, Flowcam, MFI) are commonly used for the characterization and control of ADC size variants.\textsuperscript{82}

SEC is the most widely used technique for size variant analyses. The basic principle of SEC is separation of molecules by size \textit{via} selective exclusion of the sample molecules from inner porous matrix of the column. Typically, the separation of protein is achieved under non-denaturing conditions with an aqueous mobile phase of high ionic strength (e.g., containing salts) to reduce molecule-stationary phase interactions. However, application of the same SEC method
used for the parent antibody to monitor ADC generally would not work well. A standard mAb SEC method applied to ADC may lead to poor peak shape, loss of resolution, shift of retention time and peak tailing owing to the hydrophobic interactions between the conjugated drugs and the SEC column stationary phase. Therefore, a small percentage of an organic modifier such as isopropyl alcohol, acetonitrile, propylene glycol, or dimethyl sulfoxide can be added to overcome the hydrophobic interactions during the SEC separation of ADC. For example, Wakankar et al. reported that addition of 15% v/v of 2-propanol to potassium phosphate/potassium chloride mobile phase completely eliminated peak tailing associated with trastuzumab emtansine conjugate and also improved resolution between the dimer and monomer.\textsuperscript{83} Importantly, no change in the retention time of the conjugate was observed compared with that of the unconjugated antibody, indicating that organic modifier suppresses non-specific interactions between the ADC and the column stationary phase.

Li et al. recently reported a novel SEC application wherein an organic modifier, acetonitrile was added to a sodium phosphate-containing mobile phase to overcome interactions between hydrophobic unconjugated drugs and the stationary phase during SEC-UV separation.\textsuperscript{84} The addition of organic modifier resulted in the separation of unconjugated small molecules (including the linker conjugated drug, the free drug and other impurities) from the ADC without altering the SEC profile (dimer, HMWs, and monomer).

As an example of the use of SEC to monitor aggregation and fragmentation of ADC, Wakankar et al. investigated physicochemical stability of an antibody before and after conjugation with the linker or drug molecules.\textsuperscript{80} In this study, the changes in monomer, dimer, and high molecular weight (HMW) aggregate species were monitored by SEC-UV after the
ADC was subjected to thermal stress (40°C for 7 days). The aggregation kinetics of antibody, antibody-linker conjugate, and ADC under thermal stress was successfully established using the SEC data. Similarly, Beckley et al. reported their study (Fig. 12) investigating the role of DAR on the physical stability of ADC under thermal stress. The SEC-UV data reveal that the high DAR species (6-8 drug molecules per antibody) are relatively less stable at 40°C compared to the low DAR species. Overall, the study showed that aggregation of ADC can be induced by various conjugation-related factors such as DAR, drug distribution, and conjugation chemistry, as well as external stress factors including heat, pH, ionic strength, light, shaking and freeze-thaw (see Table 4 for literature reports of some of these factors).
Despite its frequent use and importance in measuring two CQAs (HMW and LMW), the SEC method has limitations. First, for HMW species, SEC is capable of resolving only so-called ‘soluble aggregates’ (in the size range of ~150 to ~1000 kDa for ADCs) with a quantification limit of approximately 0.1% to 0.5%. Low molecular weight (LMW) species, with a size down to a few kDa, can be quantified with a similar limit. The larger soluble aggregates of ADC may elute at the column void volume, and thus result in no resolution and potentially inaccurate determination of HMW levels. Second, the chromatographic conditions, including the column pressure, column temperature and salt concentration in the mobile phase, may affect the monomer-dimer equilibrium, which may lead to dissociation of aggregates in the column.\textsuperscript{89} Last, compared to CE-based method (discussed below), the resolution of SEC is limited for LMW species. For example, it may not be able to separate various reduced species of ADC such as HL (half-mer, heavy chain-light chain) and HHL (heavy-heavy-light) from the intact antibody (HLHL) peak. To ensure that the SEC method developed for a given ADC is able to produce reliable quantification of size variants, orthogonal verification using analytical ultracentrifugation can be performed.

**Capillary Electrophoresis Sodium Dodecyl Sulfate**

CE-SDS has been used as an orthogonal separation technique for the characterization of size variants in ADCs to distinguish between the LMW species in the range of ~10-150 kDa with improved resolution. Additionally, utilizing a combination of different sample analysis modes (e.g., reduced, non-reduced), CE-SDS can provide critical information regarding the nature of aggregates/fragments with regard to the presence of unexpected disulfide linkages and any nonreducible covalent degradation product. While CE-SDS has valuable for determining overall
protein purity (or impurity), its utility is limited to the study of ADC heterogeneity arising from conjugation chemistry, drug loading, and anchoring sites (e.g., Lys, Cys, glycan) since the technique involves formation of protein-SDS complexes. For lysine-conjugated ADCs, non-reduced CE-SDS analysis usually shows a single prominent peak of full-length conjugated antibody, and a few minor peaks representing LMW and HMW species (Fig. 13A). On the other hand, reduced CE-SDS analysis usually shows prominent peaks corresponding to light (LC) and heavy chains (HC) as well as minor peaks for non-glycosylated heavy chains and nonreducible species (Fig. 13B). Interestingly, reduced CE-SDS is capable of partially separating light chains with different drug loads, namely LC1, LC2 and LC3 corresponding to zero, one, and two drugs molecules, respectively (presumably due to higher percentage changes in mass of LC from drug loading compared to that in HC or full-length antibody).

In contrast to lysine-conjugated ADCs, the non-reduced CE-SDS profile of cysteine-conjugated ADCs look quite different. This is because the cytotoxic drug is conjugated to antibodies via sulfhydryl groups that are activated by partial reduction of interchain disulfide bonds, and the action of SDS detergent to dissociate the ADC depends on how many drugs are linked to the antibody and where they are attached, since the presence of the drug prevents reformation of an interchain disulfide bond.\textsuperscript{90,91} In summary, the utility of CE-SDS for control strategy of an ADC may be limited and needs to be carefully assessed depending on the conjugation type.

\section*{STABILITY OF ADC MOLECULES}

\textbf{Physical stability}
The stability profile of ADCs is significantly more complex than that of the antibodies from which they are derived. As a result of linker-drug conjugation to an antibody, not only do the common degradation pathways of antibodies remain, the added moieties bring more degradation pathways and instability.\textsuperscript{92} For example, the partial reduction step prior to Cys-based conjugation may change the quaternary structure and binding properties of IgG1 antibodies.\textsuperscript{93} The hinge-region cysteine of IgG1 may racemize during storage.\textsuperscript{94} In addition, the lysine activation step introduces a risk of cross-linking of the activated species.\textsuperscript{83} The conjugation step to link a hydrophobic drug molecule to largely hydrophilic surface patches of an antibody often requires addition of significant amounts of organic solvent such as dimethylformamide (DMF) to maintain solubility of the hydrophobic drug and linker. Under these conditions, antibody may aggregate severely, resulting in significant reductions in yield. For example, the conjugation step for Mylotarg requires up to 20% DMF, which causes up to 50% aggregate formation measured by SEC.\textsuperscript{95} This can be alleviated to a certain extent by additives such as glycerol, propylene glycol, and octanoic acid, which have been shown to attenuate the aggregation propensity and permit the use of lower DMF concentrations during conjugation reactions.\textsuperscript{92} Generally speaking, as the DAR increases, the propensity to form aggregates increases, and it is more pronounced in higher ionic strength solutions.\textsuperscript{86,96} Reported results also suggest that partial unfolding of the CH\textsubscript{2} and hinge regions of antibody caused by conjugation is responsible for aggregate formation.\textsuperscript{85} The increased hydrophobicity imparted by higher DARs may negatively affect \textit{in vivo} performance of ADCs due to the higher propensity to aggregate or other structural destabilization effects, as reported in a recent study by Lyon \textit{et al.} showing that the efficacy of
auristatin ADCs can be improved by using more hydrophilic linker design or attachment of polyethylene glycol (PEG) chains.96

The moieties added in an ADC via the drug and linker may introduce more liability to light-induced degradation that are not present in the antibody, the drug, or the linker. Cockrell et al. evaluated light exposure in a model ADC system and demonstrated that aggregate and particulate formation specific to the ADC is due to light sensitivity, and these phenomena were not observed in the starting antibody or the linker payload alone.88 Thermal stability using differential scanning calorimetry was studied by Acchione et al. for a series of lysine-linked, thiol-linked, and carbohydrate-linked model IgG1-biotin conjugates. The primary finding from this work was that the thiol coupling had a greater destabilizing effect on the antibody than lysine coupling. It was also observed that partial antibody reduction using tris-(2-carboxyethyl)-phosphine had very minimal effect on overall thermal stability, suggesting that the observed loss of stability is not directly due to partial reduction of antibody.79

**Chemical stability**

In the context of ADCs, limited reports thus far have been available on the degradation of payload itself or on deconjugation of the payload from ADCs.92 The degradation monitored directly by HPLC or LC-MS shows minimal loss of payload97,98 during incubation in plasma,98 in buffer, or in the presence of human liver lysosomal extracts and proteases.97 Studies also confirmed that the commonly used hydrazone linker is less stable chemically and biochemically than the protease-labile Val-Cit linker. Instability of the hydrazone linker97,99,100 and premature payload release are hypothesized to be the primary reasons for Mylotarg’s poor efficacy.92 In recent studies of site-specific conjugation with monomethyl auristatin D (MMAD) compound,
the terminal dolaphenine group of MMAD was found to be susceptible to cleavage in rodent plasma. The extent of this degradation pathway was shown to be dependent on the site of conjugation. The MMAD cleavage reaction in plasma was also found to be species dependent. In the case of lysine-reactive SMCC linker, 5-6% of drug released from the ADC. In this instance, payload release was a result of unintended side reactions of the original SMCC activation step, rather than direct lysine deconjugation. LC-MS analysis indicated that the SMCC linker reacts with cysteine and tyrosine side chains in addition to lysine. The resultant thioester and ester bonds are less stable than the amide bond formed with the lysine amine.

Most of the reported studies on the chemical stability of ADCs focused on the covalent attachment of the linker payload to cysteines on the antibody, with the thiol-maleimide linkage used most frequently. Reversal of the maleimide-thiol linkage in ADC was observed with concomitant attachment of linker drug to albumin in plasma. Essentially, the Cys residue on the conjugation site of the antibody is replaced by a Cys group from the albumin after the thiol-maleimide reversal reaction. Another degradation pathway for the maleimide-thiol linkage is the “ring-opening” of the maleimide bond to form a more stable succinimidyl-thio structure, which readily occurs at alkaline condition (pH 9.2). In fact, the succinimide ring hydrolysis has been proposed to be a primary solution to the thiol-maleimide reversibility problem by subverting the retro-Michael reaction. Alternatively, other approaches have been used to deal with the reverse-Michael phenomenon. For example, disulfide bridging can be used to protect free thiols from unwanted side reactions and circumvent maleimide reversibility concerns, as the resulting conjugate contains a 3-carbon alkyl bridge to the linker payload.
Similarly, aryl-propionitrile\textsuperscript{109} or substituted phenyloxadiazole methylsulfone\textsuperscript{26} have been shown to replace maleimides in targeting free thiols.

Introduction of a unique conjugation site by antibody engineering represents another direction in replacing maleimide-based conjugation and providing a site-specific linker-payload attachment.\textsuperscript{19,110-115} Other than the chemical approaches described earlier, enzymatic reaction by bacterial transglutaminase\textsuperscript{114,115} has been used as a method for conjugation. Site-specificity is achieved in this conjugation technique by incorporating a unique transglutaminase recognition sequence containing the target glutamine residue to the antibody. Site-specific conjugation can also be realized by introducing non-natural amino acids.\textsuperscript{19,110-113} For example, p-acetylphenylalanine has been engineered into an antibody to form an oxime linkage to auristatin derivatives.\textsuperscript{110} An engineered formylglycine has been linked to a tryptamine-functionalized payload through Pictet-Spengler ligation.\textsuperscript{112} These new processes can be used to conjugate drug compounds to antibodies in order to reduce heterogeneity and improve stability for ADCs. However, it should be noted that more studies are needed to fully assess the benefits and challenges brought by each one of them with regard to developing reliable manufacturing process to produce ADC consistently with high quality to enable and support clinical studies.

\section*{NEW TECHNOLOGIES TO ADDRESS CURRENT CHALLENGES}

Two-dimensional liquid chromatography

Due to the distinct physiochemical properties of the antibody and ADC, two-dimensional liquid chromatography (2D-LC) can be a powerful tool\textsuperscript{54,84,116-119} to overcome the limitations associated with 1D-LC for ADC characterization. As the technique involves two
chromatography techniques, different separation modes, and multiple columns choices, it enhances the resolving power to achieve significantly higher separation efficiency than 1D-LC. Two configuration modes, namely comprehensive and heart-cutting modes, are commonly used in 2D-LC. In the comprehensive 2D-LC, the entire eluent from the first dimension column (1D) is continuously introduced into the second dimension column (2D). In contrast, only the peaks of interest in 1D are selectively redirected to the 2D in the heart-cutting 2D-LC mode, which makes this mode more popular for the separation and characterization of biopharmaceutical molecules that contain a complex mixture of species, such as ADCs.

With the advancement of 2D-LC technology, numerous recent applications have been reported in small molecule and proteomic separations. Likewise, there is growing interest in applying the 2D-LC technique in the characterization of ADCs. For example, Li et al. developed a novel approach using SEC coupled with RP-HPLC in a 2D format for the direct analysis of free drug and related small molecule impurities in ADC samples (Fig. 14). The advantage of SEC-RPC 2D-LC is that the limited resolution of SEC methods for small molecules is overcome by the higher resolution provided by RPC in the absence of interfering protein components. This approach eliminates the need for protein precipitation and provides information on both size variants and free drug related species in a single analysis. This approach demonstrated excellent precision, linearity, and sensitivity, and it was used for monitoring ADC drug product stability at different temperatures and pHs. Li et al. further utilized SEC/RP 2D-LC to identify the root cause of low recovery of the free linker/drug in ADC formulation. Table 5 summarizes the reported applications of 2D-LC in the characterization of ADC attributes.
Mass spectrometry in higher mass range

Dyachenko et al. recently characterized ADCs in a high-mass range. Their mass spectrometer (Thermo Orbitrap Exactive Plus EMR) was designed to achieve better transmission efficiency and better detection of higher-mass ions than other Orbitrap instruments. The authors demonstrated they can probe the heterogeneity and stoichiometry of the drug conjugation in ADCs. They could also retrieve data on the site-specific location of the drugs in the antibody. Moreover, tandem mass spectrometry allowed them to unambiguously probe the stoichiometry of antigen binding, even in IgG6-antigen protein assemblies with molecular weights over 1 million Da.121

Ion-mobility mass spectrometry

Ion-mobility mass spectrometry (IM-MS) is an emerging technology to probe protein structure by separating proteins by size, in addition to mass, in the gas phase. Marcoux, et al. demonstrated IM-MS was able to resolve different DAR species of trastuzumab emtansine and positional isomers.63 Additionally, Botzanowski et al. revealed collision-induced unfolding as a powerful technique to elucidate structural differences between a site-specific ADC and its parental mAb.122

Ultra-high voltage capillary electrophoresis

Ultra-high voltage capillary electrophoresis (UHV-CE) is an electrophoretic technique where high electric field strength has been applied for separation of many closely related species that are difficult to resolve using traditional CE instrumentation.123 Unlike the commercial CE instrumentations, UHV-CE is capable of applying at least 120 kV with electric field strength over 2000 V/cm. UHV-CE was originally used for the separation of peptides, glycans, nucleotide
and hyaluronic acid. Recently, Henley et al. successfully demonstrated application of UHV-CE in separation of mAb charge variants, drug conjugates and disulfide isomers with better resolution and short analysis time.\textsuperscript{124}

**CONCLUSIONS AND FUTURE DIRECTION**

Over the past few years, ADC development has undergone significant improvements enabled by scientific innovation in several areas, including conjugation chemistry, manufacturing and product stability. The identification of CQAs is an important aspect of the development of ADCs because the identification process enables thorough understanding of quality attributes and the assignment of criticality in terms of the potential impact on safety and efficacy. For example, the DAR of an ADC is a highly critical quality attribute because it can affect both safety and efficacy, and solid understanding and control of this attribute need to be in place in the early stages of development such that the molecule identified at the selection stage is faithfully reproduced for first-in-human (FIH)-enabling safety assessment as well as FIH studies. Therefore, early investment in establishing a reliable conjugation process coupled with comprehensive product characterization is essential to avoid major delays in mid-phase/pivotal clinical trials due to drift in product quality attributes, especially DAR, drug load distribution and linker-related heterogeneities/instabilities. For ADCs, late-stage development activities directed towards establishing commercial process and control strategy would typically focus on robustness of process and formulation, as well as development/optimization of any additional analytical techniques for control strategy suitable for commercialization.
The complexity of ADC therapeutics brings new challenges to analysis. Analysis of the antibody requires a number of analytical/biochemical/biophysical techniques. ADC characterization is a level higher in degree of complexities, and a multitude of advanced analytical techniques is required for these highly heterogeneous molecules to achieve a strong control of product quality and consistency. MS continues to be one of the most widely utilized techniques in analysis of ADCs at both process and product development levels. MS-based approaches have been used for structural integrity, stability, and higher order structural determination. Moreover, continuous technical advancements in MS-based instruments using ESI coupled TOF or Ion Trap with extended mass range permit more accurate characterization of DAR, drug distribution, and identification of conjugation sites especially for more complex lysine-based ADCs. MS-based methods for ADC characterization is expected to further evolve with new capabilities of MS techniques emerging.

Among chromatographic techniques, RPC is a robust separation technique due to its simplicity and versatility. It is routinely used in structural elucidation and stability testing for both ADC and the drug/linker parts. Recently, RPC-based analytical methods coupled with novel biochemical approaches such as Cathepsin B digestion have permitted accurate determination of DAR, overcoming limitations associated with UV-based methods. HIC is another chromatographic technique that regained a lot of interest for the analysis of ADCs. New method development strategies and the availability of new HIC columns for a variety of chemistry and particle sizes further broaden its usefulness. HIC has been a method of choice to determine drug load distribution in site-specific ADCs. In recent years, a promising development in liquid chromatography is the application of 2D-LC to improve the separation power to decipher key
information on ADC structures. Different combinations of RPC, HIC, and SEC have been successfully used in two-dimensional setup for analysis of different attributes of ADCs.

In addition to liquid chromatography, next-generation capillary electrophoretic separation techniques such as CE-SDS and iCIEF have potential as orthogonal methods with relatively high resolution separations to provide size and charge characterization data for ADCs.

Despite these advances, several analytical challenges remain to be solved for robust control of ADC manufacturing and quality, especially for lysine-based ADCs. Developing a quantitative method for charge variants for random-conjugation ADC continues to be problematic due to the high degree of surface charge heterogeneity; therefore, assessing the potential impact of charge heterogeneity on product performance remains a high hurdle. Residual antibody (unconjugated) can compete with ADC for target binding, thereby impacting the efficacy of the ADC. Analytical methods for quantification of residual antibody needs further improvement. Lack of a quantitative and high-throughput analytical method to separate the positional isomers of ADC species with the same number of conjugated drugs, as well as separating different DAR species, continues to be a major issue in ADC development. In conclusion, disruptive ideas are needed for the ADC conjugation process, as well as reliable analytical methods that will enable manufacturing of ADC products with consistent quality, safety, and efficacy for clinical and commercial use.

ACKNOWLEDGMENTS

The authors thank Dr. Thomas Slaney of Bristol-Myers Squibb for some of the analyses reported here, and Dr. Reb Russell, Bristol-Myers Squibb for encouragement and support. The authors
also thank Dr. Janice M. Reichert, Editor-in-Chief of *mAbs*, for help refining the list of clinical stage ADCs, and an anonymous reviewer for providing some of the literature references used here.

**DISCLOSURE OF INTEREST**

The authors report no conflict of interest. The authors are employees of Bristol-Myers Squibb, a global biopharmaceutical company whose mission is to discover, develop and deliver innovative medicines that help patients prevail over serious diseases. Additional information on its research and pipeline of development candidates can be found at the company website.
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FIGURE LEGENDS

Figure 1. Schematic representation of ADCs with different conjugation chemistries.
Figure 2. UV-Visible absorbance spectra of (A) free emtansine (DM-1), (B) Trastuzumab, and (C) Trastuzumab emtansine conjugate. The molar concentrations of antibody and drug are determined based on measured absorbance and extinction coefficients (Reprinted with permission, from reference 37)
Figure 3. Graphical representation of IdeS cleavage, 2-MEA reduction, and cathepsin B cleavage of ADC. Reprinted with permission, from reference 55.
Figure 4. (A) Deconvoluted mass spectrum for the deglycosylated mcMMAF conjugate, ADC-A, and the corresponding parent material, Mab-A (reference 67) (B) Deconvoluted mass spectrum of a deglycosylated mc-vc-tubulysine conjugate.
Figure 5. Hydrophobic interaction chromatography (HIC) of Glyco ADCs prepared with 3 different sialylated antibodies and 2 different aminooxy drug-linkers (AO-MMAE and AO-PEG8 Dol10). (A) Trastuzumab conjugated with AO-MMAE. (B) Trastuzumab conjugated with AO-PEG8-Dol10. (C) Anti-fibroblast activation protein (FAP) antibody B11 conjugated with AO-MMAE. (D) Anti-FAP antibody G11 conjugated with AO-MMAE. (E) Trastuzumab conjugated with MC-VC-PABC-MMAE (thiol conjugate). Numbers below each peak indicate the number of drugs conjugated. AO-MMAE: Aminooxy-Cys-MC-VC-PABC-MMAE; AO-PEG8-Dol10: Aminooxy-Cys-MC-VCPABC-PEG8-Dol10. (Reprinted with permission, from reference 70).
Figure 6. (A) Overlaid UV traces at 252 nm of trastuzumab emtansine and Herceptin sample tryptic digests separated by reversed-phase HPLC. An example pair of diasteriomer peaks, corresponding to the same conjugation site, is annotated. Only the portions of each chromatogram showing the elution region of the drug-containing peptides are presented in the figure. (reference 46). (B) Overlaid UV traces at 252 nm of a lysine mc-vc-tubulysine conjugate and the correspondent antibody tryptic digests separated by reversed-phase UPLC.
Figure 7. Peak areas in the extracted ion chromatograms of all detected conjugated peptides in tryptic digest of a lysine mc-vc-tubulysine random conjugate. Four lots produced by the same process were analyzed side-by-side to demonstrate process consistency.
Figure 8. (A) DAR for complete conjugation. Using the reduced RP-HPLC method as a monitoring tool, a range of drug–antibody molar ratios from 1:4 to 10:1 were used for MMAF conjugation on HC-F404. The conjugations were performed at room temperature overnight. (B) Intact DAR characterization by RP-HPLC. RP-HPLC chromatograms revealed baseline resolution of DAR0, DAR1, and DAR2 species for unconjugated (black), partially conjugated (red) and fully conjugated (blue) HC-F404-MMAF. (Reprinted with permission, from reference 74)
A

Unconjugated

LC

HC

Conjugated

Drug:Ab = 1:4

Drug:Ab = 1:2

Drug:Ab = 1:1

Drug:Ab = 2:1

Drug:Ab = 4:1

Drug:Ab = 6:1

Drug:Ab = 8:1

Drug:Ab = 10:1

Time (mAU)

B

- Unconjugated
- Partially conjugated
- Fully conjugated

DAR0

DAR1

DAR2

Time (min)

A280 (mAU)
Figure 9. Mass spectrum of deglycosylated huc242-DM4 conjugate. The peak labels indicate the number of DM4 molecules attached to the antibody: D0 is the unconjugated antibody, while Dn corresponds to antibody molecules carrying n DM4 molecules (for n = 1, 2, ..., 8) (Reprinted with permission, from reference 41)
Figure 10. (A) Electropherogram of a model Lysine based ADC. The amount of unconjugated antibody was determined by using calibration curve. (B) ICIEF as a fingerprinting technique to monitor the batch-to-batch process consistency.
Figure 11. HIC profile of lysine-conjugated ADCs lot 1 (black) and lot 2 (blue), yielding one prominent peak corresponding to unconjugated antibody (red) and several unresolved peaks for drug-conjugated antibody species. The estimated level of unconjugated antibody was around 4%.
Figure 12. SEC chromatograms (at 280 nm) of T=0 through 8 weeks at 40°C DAR 3.5 ADC stability samples with DAR shown (dotted lines) on the right axis for the HMWS and main peak. Reprinted with permission, from reference 85.
Figure 13. Analysis of lysine-conjugated ADCs by CE-SDS under non-reduced (A) and reduced (B) conditions. Peak identification: Internal standard (10 kDa), Light chain (LC), Nonglycosylated heavy chain (NGHC), Heavy chain (HC), 2 Heavy chains (HH), 2 heavy 1 light chain (HHL), IgG monomer.
Figure 14. Analysis of unconjugated small molecules in ADC by 2D-LC. (a) SEC on 1st D that separates the unconjugated small molecules from the ADC, and (b) RP-HPLC on 2nd D that separates the three unconjugated small molecules. Reprinted with permission, from reference 84.
Table 1: Antibody-Drug Conjugates – Approved in US or EU, and in various stages of clinical development [primary sources: U.S. National Library of Medicine, clinicaltrials.gov; sponsor websites]

<table>
<thead>
<tr>
<th>Antibody-drug conjugate</th>
<th>Target</th>
<th>Most advanced phase</th>
<th>Company</th>
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</thead>
<tbody>
<tr>
<td>Gemtuzumab ozogamicin (Mylotarg®)*</td>
<td>CD33</td>
<td>US approval/EMA review</td>
<td>Pfizer</td>
</tr>
<tr>
<td>Brentuximab vedotin (Adcetris®)</td>
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<td>US/EU approvals</td>
<td>Seattle Genetics/ Takeda (Millenium)</td>
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<td>HER2</td>
<td>US/EU approvals</td>
<td>Roche (Genentech)/Immunogen</td>
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<tr>
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<td>CD22</td>
<td>US/EU approvals</td>
<td>Pfizer</td>
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<td>Delta-Like Protein 3 (DDL3)</td>
<td>Phase 3</td>
<td>AbbVie</td>
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<td>Tyrosine-protein kinase Met (c-Met) or hepatocyte growth factor receptor (HGFR)</td>
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*Withdrawn from the US market in 2010; marketing continued in Japan; approved again in the US in September 2017 for the treatment of CD33-positive acute myeloid leukemia (AML).*

** Phase 3 start date is pending
Table 2. Critical Quality Attributes of ADCs

<table>
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<tr>
<th>Quality Attributes*</th>
<th>Analytical Techniques/Methods</th>
<th>Analyte</th>
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<tbody>
<tr>
<td>Drug-to-antibody ratio</td>
<td>• UV-Visible spectroscopy</td>
<td>ADC</td>
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<td></td>
<td>• Cathepsin-B enzyme-based digestion method</td>
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<td></td>
<td>• Hydrophobic interaction chromatography (HIC-UV)</td>
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<td></td>
<td>• Reversed-phase-HPLC (RPC-UV)</td>
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<td></td>
<td>• Mass spectrometry (MS)</td>
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<td>Drug load distribution</td>
<td>• Mass spectrometry (MS)</td>
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<td>• Hydrophobic interaction chromatography (HIC-UV)</td>
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<td>• Capillary electrophoresis (CE)</td>
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<td>• Reversed-phase-HPLC (RPC-UV)</td>
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<tr>
<td>Residual Antibody (unconjugated)</td>
<td>• Imaged capillary isoelectric focusing (iCIEF)</td>
<td>Antibody</td>
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<td>• Enzyme-linked immunosorbent assay (ELISA)</td>
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<td>• Mass spectrometry (MS)</td>
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<td>• Size-exclusion chromatography (SEC-UV)</td>
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<td>• Two-dimensional liquid chromatography (2D-LC)</td>
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<td>Size Variants (e.g., HMW, monomer, LMW)</td>
<td>• Size-exclusion chromatography (SEC-UV)</td>
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<td>• Capillary electrophoresis sodium dodecyl sulfate (CE-SDS)</td>
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<td>• Analytical ultracentrifugation (AUC)</td>
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<td>Charge Variants (e.g., acidic, main, basic)</td>
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<td>ADC, Antibody</td>
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<td>• Imaged capillary isoelectric focusing (iCIEF)</td>
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*Most of these quality attributes are likely to be critical for ADCs; however, experimental determination is needed to confirm criticality for a given ADC.
Table 3. List of ADCs with their $\lambda_{\text{max}}$ value for the antibody and drug molecule used for the determination of DAR

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<th>Targeting Moiety</th>
<th>Antibody Molecule</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
<th>Therapeutic Moiety</th>
<th>Drug Molecule</th>
<th>$\lambda_{\text{max}}$ (nm)</th>
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<td>Vinca Alkaloid</td>
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*DC1: Synthetic analogue of CC-1065
Table 4. Effect of intrinsic and extrinsic factors on aggregation propensity of ADCs

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<td>IgG1-Auristatin, Maleimide-thiol chemistry</td>
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<td>Drug loading and</td>
<td>IgG1-Auristatin, Maleimide-thiol chemistry</td>
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<td>Trastuzumab-Eosin (T-EO) Conjugate, UVA</td>
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Table 5. Applications of 2D-LC in the characterization of ADCs

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