CHAPTER

1

LC-MS characterization of antibody-based therapeutics: recent highlights and future prospects

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The first monoclonal antibody therapy, Muromonab-CD3 to treat kidney rejection, was approved for use in the US and Europe in 1986 and marked the beginning of a major shift in the pharmaceutical industry away from small molecule drugs toward antibody-based therapies. Today, there are well over 100 approved antibody-based therapies on the market with approximately 60 in late stage clinical review and hundreds more in phase 1 and 2 clinical study [1,2]. Unlike small molecule drugs, antibody therapeutics are large, complex, heterogeneous and dynamic proteins that require sophisticated analytical strategies to thoroughly characterize them and to ensure good quality control and patient safety.

Liquid chromatography coupled with mass spectrometry (LC-MS) is a powerful technology that is at the core of many assays for the characterization of antibody-based therapeutics. With a small amount of material and minimal sample preparation, LC-MS can generate detailed information about multiple antibody features that inform on their purity, heterogeneity, function and stability. Few other technologies can claim to match LC-MS for sensitivity, specificity and throughput. Even fewer have the flexibility of LC-MS that enables it to keep pace with the rapid development of new biotherapeutic modalities. LC-MS characterization of antibody-based therapeutics encompasses a wide range of assays including molecular weight determination, amino acid sequencing, the identification of modifications, host cell protein analysis, antibody-drug conjugate analysis, stability testing, PK/PD studies as well as higher order structure analysis (Fig. 1.1).

Each year, hundreds of research articles are published describing new applications and technological advances relating to the characterization of antibody-based therapeutics by LC-MS. This continual development is driven by the needs of a growing biopharmaceutical sector that is continually developing more effective and safer antibody-based therapeutics.

Approaches to the Purification, Analysis and Characterization of Antibody-Based Therapeutics
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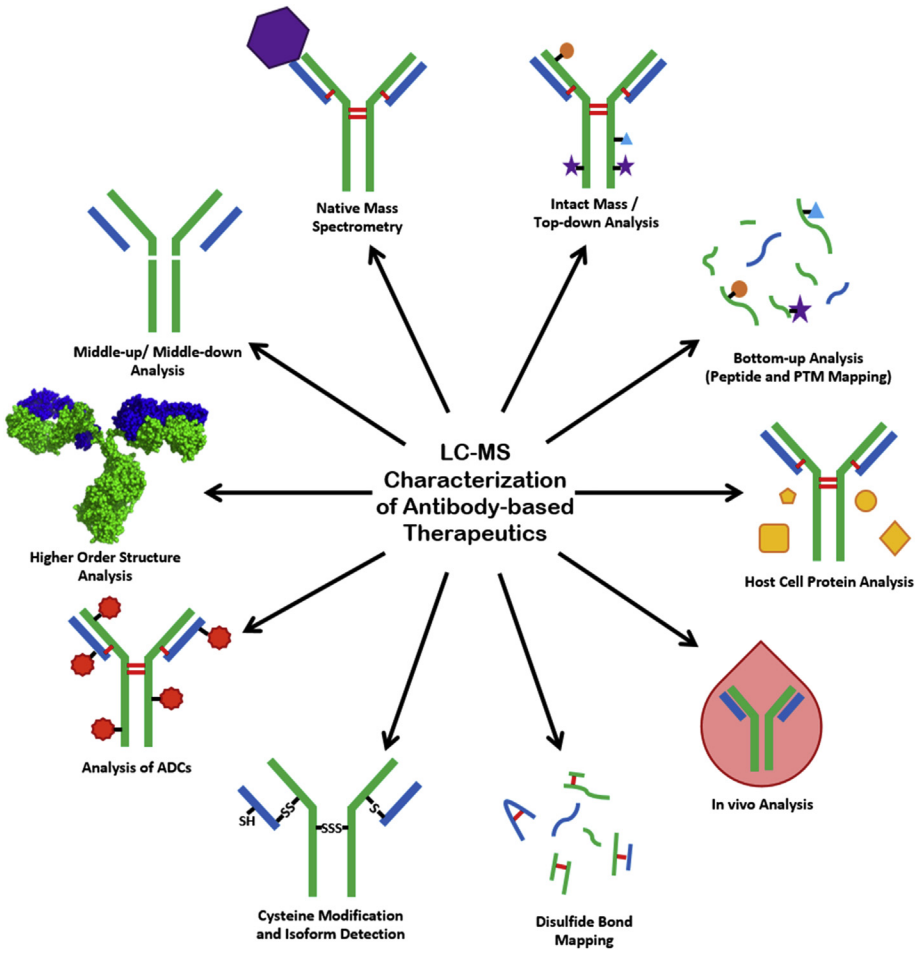

FIG. 1.1 Schematic illustration of some of the many applications of LC-MS for the biophysical characterization of antibody-based therapeutics. "Higher Order Structure Analysis" image is PDB 1IGT [156] rendered using Pymol [157].

This trend is likely to continue for some time as LC-MS gains greater acceptance within the biopharmaceutical community and with regulatory authorities, and as new application areas are explored.

This chapter will cover current applications and recent advances in LC-MS-based biophysical characterization of antibody-based therapeutics. Section 1 will briefly describe some of the more established LC-MS techniques in this field, while Section 2 will focus on the LC-MS analysis of specific attributes, including lesser known post-translational modifications (PTMs) and drug conjugates. Finally, Section 3 will explore recent innovations and trends in LC-MS-based analysis that are likely to significantly impact the manner in which antibody-based therapeutics are analyzed in the future.

Section 1 − LC-MS technologies frequently used for the characterization of antibody-based therapeutics

In this section, a brief overview will be provided of the more established LC-MS technologies for the characterization of antibody-based therapeutics.

Analysis of intact antibodies

Intact mass analysis is a simple, rapid MS technique where the mass of an antibody is determined, with little or no sample preparation. It can be used as a high throughput assay for monitoring the identity, purity and PTMs of recombinant antibody-based therapeutics. Electrospray ionization-MS (ESI-MS) is well-suited for analyzing large proteins, such as antibodies, because it generates multiply charged ions that can be analyzed using mass spectrometers with a relatively limited scanning range but capable of producing high resolution mass spectra, such as Orbitrap and quadrupole time-of-flight (Q-TOF) mass spectrometers [3]. ESI-MS can be connected in-line with separation techniques, such as reverse phase liquid chromatography (RPLC), to remove salts and other buffer components that interfere with ionization and detection, and can be automated to run unattended. Although unit mass resolution of intact antibodies has been achieved [4,5], intact mass analysis is usually done at a lower resolution because ultra-high resolution results in a significant drop in signal intensity with minimal benefit. Even at unit mass resolution, an antibody is so large that the monoisotopic ion is too minor a component to be observed within the dynamic range of current mass spectrometers [6]. As a result, intact mass analysis measures the average mass of an antibody instead of the monoisotopic mass.

The different "proteoforms" of an antibody can be quickly distinguished by intact mass LC-MS based on the molecular weights observed. "Proteoform" is a term coined by the Consortium for Top-Down Proteomics referring to one of "all of the different molecular forms in which the protein product of a single gene can be found, including changes due to genetic variations, alternatively spliced RNA transcripts and post-translational modifications" [7]. The amino acid sequence and modifications of each proteoform can often be inferred from the observed mass. Intact mass analysis has its limitations: it cannot detect modifications that result in zero mass change (e.g., disulfide scrambling, aspartic acid isomerization) and can struggle to distinguish modifications resulting in small mass changes (e.g., deamidation and oxidation), especially when these are present in low abundance. Highly heterogeneous proteins can be challenging to analyze by intact mass LC-MS as the complex spectra are often difficult to deconvolute and interpret. However, it is often possible to remove common sources of heterogeneity such as N-linked glycosylation or C-terminal lysines prior to intact mass analysis so that other more pertinent or low-level features such as glycation, drug conjugation or the heterodimer/homodimer ratio of bispecifics can be more easily detected.

Middle-up analysis

In a typical "middle-up" analysis workflow, an antibody is selectively cleaved and/or reduced into "large" (typically > 7 kDa − refer to Lermyte et al. for a discussion of terminology [8]) polypeptide fragments prior to mass analysis. The antibody is usually enzymatically

cleaved close to the hinge and the inter-chain disulfide bonds are chemically reduced. For example, the most popular preparation for "middle-up" analysis of an antibody involves cleavage below the hinge with the enzyme IdeS followed by reduction of disulfide bonds with dithiothreitol (DTT) treatment, resulting in a heavy chain Fc/2 fragment, a heavy chain Fd fragment and the light chain. The mass of each fragment is measured by intact mass LC-MS analysis, often after chromatographic separation. Since smaller in size, the mass resolution attained for the fragments is greater than the resolution attained for the intact antibody. Furthermore, the molecular weight profiles for the fragments are usually less complex and easier to interpret. "Middle-up" analysis is useful for defining the regional location of a modification or sequence error and for differentiating and characterizing Fc and Fab glycosylation. This technique has seen a large growth in popularity in the last 10 years as more specific enzymes for cleaving at the hinge have become commercially available. A more detailed description of the enzymes available for specific cleavage at the hinge region of an antibody and applications is provided in Section 3.

Bottom-up analysis

"Bottom-up" analysis involves cleaving an antibody into small peptides (typically <3 kDa) with one or more proteases and resolving the peptides chromatographically (typically by RPLC) prior to analysis by MS and MS/MS. Specific proteases such as trypsin (most common), LysC, AspN or GluC (also called *Straphylococcus aureus* Protease V8), or non-specific proteases such as pepsin can be used. Protease cleavage can be performed on antibodies free in solution or immobilized in gels or on stationary phases [9], resulting in release of peptides into solution. "Bottom-up" LC-MS is used in antibody characterization to confirm amino acid sequence, to identify and locate modifications and determine their relative abundance at each site, and to perform detailed comparisons between samples [10]. MS/MS spectra of the peptide ions generated from gas-phase fragmentation techniques, such as collision-induced dissociation (CID), can be matched to the expected antibody amino acid sequence using database searching and sequence match algorithms to confirm identity. Failing this, unidentified MS/MS spectra can be interpreted manually to determine novel amino acid sequences or the presence of a modification. Finally, "bottom-up" analysis of antibody digests prepared without reduction can provide information on the disulfide bond linkages present [11].

The term "extended bottom-up" has been used to describe LC-MS analysis of peptides intermediate in size to those typically generated for "bottom-up" or "middle-up" analysis [12]. In an extended bottom-up assay, antibodies are incubated for a limited period with secreted aspartic protease 9 (Sap9), a non-specific protease, at slightly acidic pH to generate peptides with an average size of approximately 3.5 kDa [13]. Standard "bottom-up" LC-MS conditions are used, though the standard C18 RPLC column is substituted for a C8 column to compensate for the increased hydrophobicity of the larger peptides. The advantages of "extended bottom-up" are improved sequence coverage and more confident identification of IgGs, especially when distinguishing between similar IgGs, because of the increased length of the identified peptides and increased likelihood of identifying unique peptides [13].

Higher order structure analysis by mass spectrometry

Higher order structure mass spectrometry (HOS-MS) analysis is used to study antibody-antigen interactions and to probe changes in the three-dimensional structure of antibody-based therapeutics that result from formulation in different buffers [14], degradation processes [15], post-translational modifications [16] or drug conjugation [17]. HOS-MS can evaluate the structural similarity of lots of the same biotherapeutic or of a biosimilar with an originator molecule [18]. HOS-MS has the advantage of being able to detect antibody structural changes with medium to high resolution. As such, it complements other HOS analysis techniques such as circular dichroism, X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy [19].

In a typical HOS-MS experiment, an antibody or antibody-antigen pair in the native state is labeled at solvent-exposed residues, digested into peptides and analyzed by LC-MS. Peptides containing the exposed residues are identified by the mass increase due to the label. Labeled peptides are mapped onto the 3-D model (usually a homology model) of the antibody to determine how the structure is being impacted by the experimental conditions. For example, upon antigen binding, labeling will decrease at the binding site and may increase or decrease at peripheral sites due to allosteric effects. The most popular HOS-MS assay is hydrogen/deuterium exchange mass spectrometry (HDX-MS, also called HX-MS) where solvent-exposed backbone amide hydrogens are exchanged with deuterium from the buffer [20]. This technology has the ability to detect and determine the location of dynamic changes in the orientation of the protein backbone. In covalent labeling mass spectrometry (CL-MS), specific amino acid side chains are covalently modified with chemical labels [21]. This approach has the advantages of detecting changes in the orientation of side chains (which in some cases may be more relevant to function than changes in the protein backbone) and producing permanent labels that can be more easily identified and quantified by well established "bottom-up" assays. Finally, cross-linking MS (XL-MS) uses multifunctional reagents to form covalent bonds between spatially proximal amino acid side chains, allowing for the determination of the distance between two interacting proteins or two residues of the same protein [22].

Host cell protein analysis

Host cell proteins (HCPs) are derived from the cell line used to express a therapeutic antibody and are released into the culture media via secretion or cell lysis. LC-MS/MS studies have identified thousands of HCPs present in the spent culture media [23,24]. Downstream purification protocols will remove many HCPs but it can be challenging to completely remove all of them. Many factors influence the composition and abundance of HCP impurities in purified antibody products including HCP initial abundance in the cell media and their tendency to co-elute or associate non-covalently with the therapeutic protein during the purification process [23,25–28]. Even after multiple steps of purification, HCPs can be present in a protein drug substance at concentrations of up to 100 ppm. Therefore, setting and achieving acceptable limits for HCP contamination in the final product is an important requirement for all biotherapeutics.

HCP abundance is usually monitored by enzyme-linked immunosorbent assay (ELISA) using polyclonal antibodies raised against the host cell proteins. ELISA is a simple, quantitative measure of total HCP content but provides no information about the identity of HCPs present and is dependent on HCPs being reactive to the polyclonal antibody used. LC-MS, on the other hand, can identify and quantify individual HCPs and is not dependent on immunogenicity. The challenge for LC-MS based assays lies in the fact that HCPs are typically many orders of magnitude less abundant than the biotherapeutic product. The majority of LC-MS strategies for HCP analysis exploit the impressive separation capabilities of two-dimensional chromatography (2D-LC) to resolve the antibody/HCP digests prior to MS analysis [24,29–38]. Most employ high pH RPLC in the first LC dimension and low pH RPLC in the second. HCP identification is done using MS/MS or MSe analysis coupled with database searching. Ion mobility mass spectrometry (IM-MS) can add an additional dimension of separation to aid in the detection [33]. HCP quantification can be achieved with MRM using isotopically labeled peptide internal standards, using the Hi3 fragment ion strategy on a Q-TOF instrument or by spectral counting in data-independent acquisition mode [29,39]. Detection and quantification of HCPs in NISTmAb reference material (see Section 3) down to 1 ppm has been reported [33].

MS-based methods for HCP detection and quantification are evolving but currently still suffer from lengthy analysis times and poor inter-lab reproducibility [33]. However, innovative techniques, such as using sample concatenation [38], are being explored to shorten 2D-LC-MS analysis times. ELISA will likely remain the primary assay for HCP monitoring and product release for some time to come but, ideally, a combination of ELISA and LC-MS would be more effective for HCP monitoring and control [40].

Section 2 – LC-MS characterization of antibody attributes

Antibody-based therapeutics may be modified in many ways over the course of production, purification and storage. In this section, we will describe some of these modifications as well as possible ways to detect and quantify them. Some modifications are typically only present on a minority of the antibody proteins but may still be functionally significant. Table 1.1 lists the mass difference and location associated with post-translational modifications known to occur to antibodies and antibody-based therapeutics.

Confirmation of primary sequence and detection of cleavages

LC-MS is routinely used to confirm the identity of antibody-based therapeutics. A comprehensive workflow for amino acid sequence confirmation includes both intact mass LC-MS analysis and "bottom-up" LC-MS/MS sequencing of the peptide digest. The mass observed for an antibody by intact mass LC-MS analysis on a medium to high-resolution mass spectrometer should be within ~ 20 ppm of the theoretical average mass for the predicted amino acid sequence plus known PTMs (such as disulfide bonds or glycosylation) [3]. A discrepancy between the observed and calculated masses may be indicative of an amino acid sequence error, proteolytic cleavage, poor disulfide bond formation or unexpected

TABLE 1.1 Masses of post-translational modifications observed in antibody-based therapeutics.

Modification	Mass difference (monoisotopic)	Location
Loss of Lys	−128.0950	C-terminal Lys
Dehydroalanine formation	−33.9877	Cys
Thioether bond formation	−31.9721	Disulfide bond
Succinimide formation from Asp	−18.0106	Asp
Succinimide formation from Asn	−17.0265	Asn
Pyroglutamic acid formation	−17.0265	N-terminal Gln
Disulfide bond formation	−2.0145	2 Cys residues
Deamidation	+0.9840	Asn (or Gln)
Kynurenine formation (Trp oxidation)	+3.9949	Trp
Methionine sulfoxide formation (Met oxidation)	+15.9949	Met
Hydroxytryptophan/oxindolylalanine formation (Trp oxidation)	+15.9949	Trp
Hydroxyproline formation	+15.9949	Pro
Trisulfide formation	+31.9721	Disulfide bond
N-formylkynurenine formation (Trp oxidation)	+31.9898	Trp
Sulfation	+79.9568	Ser, Thr or Tyr
Phosphorylation	+79.9663	Ser, Thr or Tyr
Cysteinylation	+119.0041	Cys
O-xylosylation	+132.1161	Ser
Citric acid imide modification	+156.0059	N-terminus
Glycation	+162.0528	N-terminus or Lys
Citric acid amide modification	+174.0164	N-terminus
Glutathionylation	+305.0682	Cys
O-linked HexNAcHexNeuAc	+656.2276	Ser or Thr
O-linked $HexNAcHexNeuAc_2$	+947.3230	Ser or Thr
N-linked glycan (example: $HexNAc_4Hex_3Fuc$ (G0F))	+1444.5339	Asn of NXS or NXT (or occasionally NXC)

PTMs. "Bottom-up" LC-MS/MS is the preferred method for confirming amino acid sequence and it is common practice to perform multiple LC-MS/MS experiments using two or three different proteases in order to attain LC-MS/MS coverage of the entire sequence. If a sequence error is suspected, "middle-up" analysis is often used to narrow down the location of the mass difference in order to facilitate the identification of the amino acid change by "bottom-up" LC-MS/MS.

Unexpected cleavages may occur in antibodies, especially near the N- and C-termini, at the solvent-exposed flexible loops connecting β-strands or in the hinge region [41]. Mass separation assays such as CE-SDS or SDS-PAGE can detect extensive cleavage but LC-MS is superior at detecting cleavages resulting in loss of only a few amino acids. Some protein cleavages are only apparent under reducing or denaturing conditions because cleaved portions of the protein are held together by disulfide bonds or non-covalent associations. Minor cleavages near the termini may not affect antibody activity (though loss of tags may cause purification problems) but do increase antibody heterogeneity and can be indicative of longer term stability problems. Partial loss of the C-terminal lysine is a very common cleavage. Other common cleavages include loss of terminal glycine (N- or C-terminal) and loss of one or more histidines from a His-tag. Cleavage is often catalyzed by the side chains of certain amino acids and is influenced by pH (with antibodies typically most stable at pH 5–7), temperature, the presence of metal ions (especially Cu^{II}) as well as protein structure [41]. Peptide bonds especially prone to non-enzymatic fragmentation include Xaa-Ser (where Xaa is any amino acid), Asp-Xaa (at pH < 5, particularly Asp-Pro), Asn-Xaa and Gly-Gly [41]. β-elimination of a disulfide bond can cause cleavage at Xaa-Cys. If the dehydroalanine resulting from β-elimination of a disulfide bond is hydrolyzed (Fig. 1.2C), cleavage will occur N-terminal to the former Cys (an alternative end-product of β-elimination is a thioether bond). In the hinge region of IgG1, the dominant sites of cleavage are Asp-Lys at pH < 5 and at Ser-Cys due to β-elimination at pH > 7 [41].

Cleavage may also be enzymatic and caused by host cell proteases [42]. Co-purification of host cell proteases may only become apparent during a stability stress test. It is often possible to inhibit enzymatic cleavage with protease inhibitors but improved protocols to remove the proteases during production and purification is more desirable.

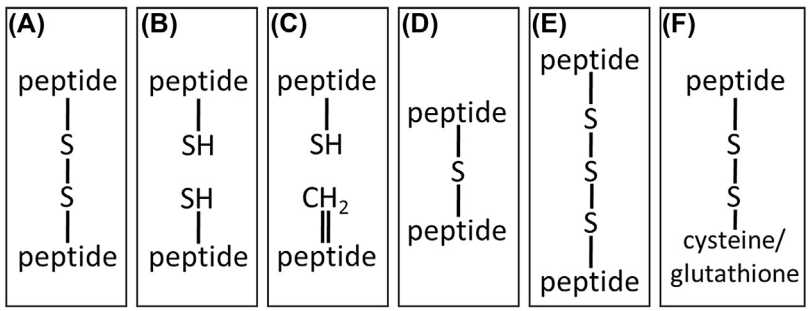

FIG. 1.2 Schematic of different cysteine modifications: (A) a disulfide bond, (B) two free sulfhydryls resulting from reduction of a disulfide bond, (C) a free sulfhydryl **(top)** and dehydroalanine **(bottom)** resulting from β-elimination of a disulfide bond, (D) a thioether bond, (E) a trisulfide and (F) cysteinylation or glutathionylation.

Cysteine modifications

Ensuring that disulfide bonds are formed correctly is an important step in characterizing recombinant antibodies. A typical IgG has 1 intra-chain disulfide bond for each of the 12 domains, 2 disulfide bonds attaching the light chains to the heavy chains and 2 to 11 (subtype-dependent) hinge disulfide bonds connecting the two heavy chains [43]. Scrambling, reduction or modification of these disulfide bonds may negatively affect the biotherapeutic. For example, the reduction of an antibody disulfide bond to free sulfhydryls has been observed to decrease antigen affinity [44], increase propensity for aggregation [45–48], and/or decrease thermal stability [49]. Some common modifications of cysteine residues are shown in Fig. 1.2.

Free sulfhydryls (Fig. 1.2B) have been observed in recombinantly expressed antibodies and in antibodies extracted from serum [48–50]. The free sulfhydryl abundance of a protein can be measured spectroscopically using the Ellman's reagent method [51] or free sulfhydryl-reactive fluorescent labels [52] but these methods can suffer from poor sensitivity and precision and are unable to provide information about the location or distribution of free sulfhydryls. The reduction of a disulfide bond only increases the mass of a protein by 2 Da but free sulfhydryls can be easily and sensitively detected by intact mass LC-MS if they are labeled with a maleimide derivative, such as maleimide-PEG2-biotin, so that the presence of a free sulfhydryl is observed as a significant mass shift [53]. Robotham and Kelly determined the locations of the free sulfhydryls in antibodies by first labeling the free sulfhydryls with d_0-NEM, then labeling the remaining cysteines with d_5-NEM after reduction [53]. Protease digestion was performed under mildly acidic conditions to prevent label loss and scrambling and LC-MS/MS was used to identify peptides containing a free sulfhydryl based on their high d_0/d_5-NEM ratio. In instances where a free sulfhydryl is solvent exposed, it may become cysteinylated and glutathionylated, resulting in characteristic mass shifts of +119 Da and +305 Da, respectively (Fig. 1.2F) [46].

The disulfide bond connecting the light chain to the heavy chain in IgG1 is prone to thioether bond formation (Fig. 1.2D), particularly when exposed to elevated temperature or basic conditions [54,55]. Formation of a thioether bond decreases the mass of an intact antibody by 32 Da (the mass of sulfur). The thioether bond is created when the dehydroalanine and cysteine formed from β-elimination of the disulfide bond react to form a covalent bond by Michael addition [55]. Since this bond is not reducible, the thioether-linked light chain-heavy chain pair is observed under reducing condition during "bottom-up" or "middle-up" LC-MS analysis. Thioether formation reduces Fab flexibility and could affect bivalent binding [55]. The heavy-light chain disulfide bond, as well as the hinge interchain disulfide bonds, are also susceptible to trisulfide formation where a sulfur atom is inserted into the disulfide bond (Fig. 1.2E)[56]. Trisulfides are hypothesized to form during fermentation due to the reaction of disulfide bonds with hydrogen sulfide. Gu et al. determined the location and relative abundance of trisulfides in natural and recombinant antibodies using "bottom-up" LC-MS of non-reduced proteins digested with LysC at pH 6.5 [56]. Trisulfide-linked peptides had a mass 32 Da greater than the predicted disulfide-linked peptides. Although they increase biotherapeutic heterogeneity, there is no evidence that trisulfides affect antibody binding or activity and the majority of trisulfides are rapidly converted to disulfide bonds in vivo [56].

Disulfide bond scrambling may occur with any antibody-based biotherapeutic but is a special concern for IgG2 and IgG4 isotypes. IgG2 can exist as three different isoforms of disulfide bond arrangements: IgG2-A, IgG2-B and IgG2-A/B (Fig. 1.3)[43]. The isoforms differ in terms of higher order structure, which influences thermal stability and binding efficiency [57,58]. IgG4 is also prone to disulfide bond scrambling with the hinge cysteines switching between inter-chain and intra-chain disulfide bonds. As a result, the IgG4 heavy and light chains may be non-covalently associated and Fab arm switching with endogenous IgG4 antibodies in vivo has been observed [59]. The relative abundance of different disulfide bond isoforms can be determined by "bottom-up" LC-MS/MS analysis of non-reduced antibody digests. MS/MS fragmentation with electron transfer dissociation (ETD) or electron capture dissociation (ECD) selectively cleaves the disulfide bond to release the linked peptides whereas collision-induced dissociation (CID) results in preferential fragmentation of the peptide backbone [60]. These techniques can be used in sequence (i.e., ETD followed by CID) to identify disulfide bonded peptides with greater confidence [60].

N-linked glycosylation

N-glycosylation is a common post-translational modification of antibodies. Virtually all antibodies are N-glycosylated at a single asparagine residue located in the Fc region (Asn-Ser-Thr) and a smaller proportion of them are N-glycosylated at additional asparagines on the heavy or light chain (e.g., cetuximab)[61]. An N-glycosylation sequon (Asn-X-Ser, Asn-X-Thr or, occasionally, Asn-X-Cys, where X is any amino acid other than proline) is a pre-requisite, though not a guarantee, for N-linked glycosylation. N-glycosylation can be analyzed by intact mass analysis to determine the overall glycosylation of an antibody, by "middle-up" analysis to distinguish N-glycosylation in the Fc and Fab regions and by "bottom-up" analysis to determine the glycosylation at each N-glycosylation site. Standard RPLC is often used for "bottom-up" analysis but alternatively, hydrophilic interaction chromatography (HILIC) and other glycopeptide-enriching chromatographies can be used to separate glycopeptides from non-glycosylated peptides [62]. N-linked glycans can also be released from the protein by PNGaseF treatment, labeled and analyzed by matrix-assisted laser desorption/ionization time of flight mass spectrometry (MALDI-TOF-MS) or by HILIC-fluorescence [63].

Generally speaking, the popular cell lines used for protein expression, such as CHO or HEK293, express antibodies with well-defined N-glycans composed of a restricted set of sugar moieties (i.e., N-acetylglucosamine (GlcNAc), N-acetylgalactosamine (GalNAc), mannose (Man), glucose (Glc), galactose (Gal), fucose (Fuc) and N-acetylneuraminic acid (NeuAc), as well as N-glycolylneuraminic acid (NeuGc) in mouse cell lines). Therefore, it is often possible to infer the number and general composition of the N-glycans present by mass addition alone. Mass spectrometry cannot distinguish between sugar isomers; "HexNAc" is used to indicate that a sugar is GlcNAc or GalNAc and "Hex" to indicate Man, Glc or Gal. Glycans occupying the Fc N-glycosylation site are typically non-sialylated, biantennary complex-type glycans while those occupying the Fab sites tend to be more heterogeneous and sialylated. Typically, each N-linkage site is occupied by a variety of glycan species resulting in a heterogeneous mass profile for glycosylated antibodies. N-glycans

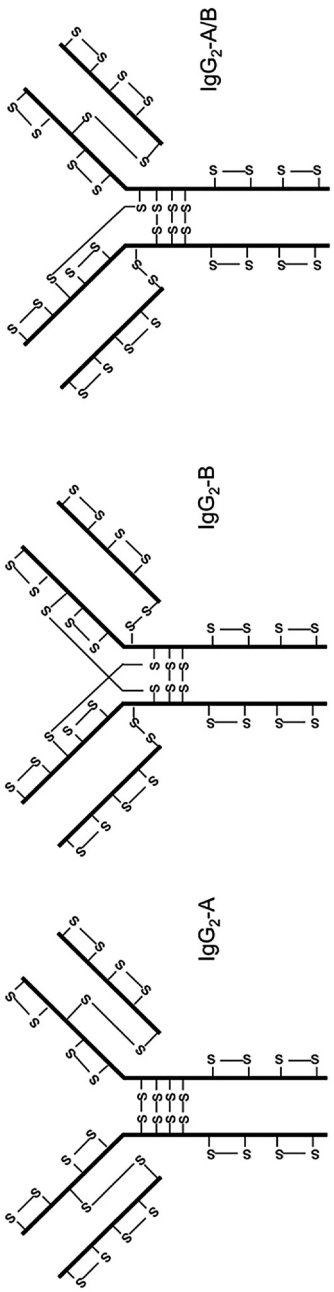

FIG. 1.3 Disulfide bond isoforms observed in IgG2 antibodies. *Adapted from Liu, H. and K. May, Disulfide bond structures of IgG molecules: structural variations, chemical modifications and possible impacts to stability and biological function. mAbs 2012:4(1):17–23 with permission, © 2012 Landes Bioscience.*

are often removed with PNGaseF or trimmed to a common core with exoglycosidases prior to LC-MS in order to facilitate the characterization of other antibody features. Glycosylation analysis by intact mass or "middle-up" LC-MS can be complicated by the presence of O-linked glycosylation or glycation and care must be taken to distinguish these modifications from N-linked glycosylation.

O-linked glycosylation

O-linked glycosylation has been observed on some of the immunoglobulin classes that are not typically used to produce antibody-based therapeutics: IgA1, IgD and IgG3 as well as mouse IgG2b [64–67]. In all of these cases, O-linked glycosylation was located in the hinge region and consisted of mucin-type glycans such as GalNAcGalNeuAc$_2$ (or GalNAcGalNeuGc$_2$ in the case of murine IgG2b). O-linked glycosylation has not been reported on intact IgG1, the most commonly used subclass for recombinant antibodies [68]. However, O-linked glycosylation has been observed on IgG1 Fc fragments expressed alone, on antibody constructs lacking one or both light chains, and on fusion proteins containing an IgG1-type Fc domain (unpublished results). O-linked glycosylation (GalNAcGalNeuAc$_2$) of the Fc fragment of an IgG1 (Rituximab) was observed when the Fc fragment was expressed alone but not when the same fragment was generated from intact Rituximab by papain cleavage [69]. The site of O-linked glycosylation was hypothesized to be located near the hinge because access to this region is greatly improved when the Fab portion of IgG is absent. Other groups have identified unexpected O-linked glycosylation of peptides when they are fused to Fc domains or antibody [70,71] and in some cases, O-glycosylation had a negative effect on the activity of the fusion construct [70].

Intact mass LC-MS analysis can be used to detect mass shifts that are characteristic of O-glycosylation (i.e., +656 Da for HexNAcHexNeuAc and +947 Da for HexNAcHexNeuAc$_2$). O-glycosylation is easier to detect by LC-MS if the N-linked glycans are first removed using PNGaseF. Identifying the site of O-glycosylation is more challenging than for N-glycosylation because there is no consensus sequence for O-glycosylation other than glycosylation occurs at serine and threonine residues. "Top-down" LC-MS analysis has been used to identify O-glycosylation sites [72], however "bottom-up" LC-MS/MS is the preferred method currently. Enrichment using HILIC can aid in the detection of low abundant O-glycopeptides [73,74]. The CID MS/MS spectra of O-glycopeptides are often dominated by glycan oxonium ions (e.g., 204 Da, 366 Da, 292 Da) and by neutral losses of sugar residues from the intact glycopeptide with no evidence of the linkage site. ECD or ETD fragmentation methods are better suited for determining the linking amino acid as these fragmentation methods tend to preserve delicate modifications such as O-glycosylation [75].

Glycation

Non-enzymatic glycation of the N-terminus or the side chain of lysine residues is a common PTM of both recombinant and endogenous mAbs. Glycation of recombinant antibodies typically occurs during fermentation when the expressed protein is exposed to high concentrations of reducing sugars, such as glucose, from the cell feed [76]. Glycation may

also occur during storage if the antibody is exposed to reducing sugars, or to sucrose (a common formulation buffer component) at elevated temperatures and acidic pH [77]. It has even been observed to occur on lyophilized mAbs [78]. Glycation is undesirable because it increases the heterogeneity of a biotherapeutic and may have other negative consequences. A recent study found that glycation in the complementarity-determining region (CDR) interfered with antigen binding [79] and there is mixed evidence as to whether glycation increases mAb aggregation [80,81]. There are also concerns that when exposed to elevated oxidation conditions in vivo, glycated biotherapeutics may further degrade to advanced glycation end products (AGEs) and trigger the expression of AGEs-specific receptors and adverse immune responses [76]. AGEs have also been linked to the discoloration of recombinant antibody product [82]. Given the safety concerns, glycation is an attribute that must be monitored and controlled.

Glycation is detected by intact mass LC-MS analysis as a characteristic mass shift of 162 Da. Glycation is easier to detect if the protein is first deglycosylated, as many N-glycoforms differ from one another by a single hexose (162 Da). Though rare, a 162 Da increase can also be due to O-linked glucosylation and mannosylation where a single glucose or mannose is linked to a serine residue [83,84]. Additionally, two phosphorylations/sulfations also have a similar mass to a glycation (+160 Da). Glycation can be distinguished from these other modifications by determining the site of modification using "bottom-up" LC-MS. Detection can be challenging because glycation abundances tend to be low (<10%) and spread across multiple sites, there is no consensus sequence for glycation other than a solvent accessible primary amine [76], and trypsin and LysC will not cleave at glycated lysine residues. Generally speaking, CID MS/MS spectra of glycated peptides tend to be poor, being dominated by simple losses of water from the hexose residue and with very few informative peptide fragment ions [77]. These challenges can be overcome in a number of ways. For example, boronate-affinity HPLC (BAC-HPLC) can be used to enrich for glycated proteins and BAC-HPLC-UV has been used for determining overall glycation abundance. BAC-HPLC separates glycated and non-glycated forms based on the interaction of the glycated sugar cis-1,2-diol array with the boronate ligands [85]. Treatment of glycated peptide with sodium borohydride or cyanoborohydride stabilizes glycation and generates higher quality CID MS/MS spectra as a result [86]. In addition, alternative MS/MS fragmentation techniques such as electron transfer dissociation (ETD) can yield superior sequence information relative to CID as the modification is not lost during fragmentation, making it possible to confirm the site of glycation [87].

Deamidation and aspartic acid isomerization

Deamidation of asparagine and isomerization of aspartic acid to isoaspartic acid can impact the structure, function and stability of antibody-based therapeutics. These modifications affect antigen-binding if they occur in the CDR region [88,89] and can lead to immunogenicity issues [90]. Asparagine deamidation imparts an additional negative charge to the antibody which can be monitored using charge based assays such as capillary isoelectric focusing (cIEF) or ion exchange chromatography (IEX). "Bottom-up" LC-MS can be used to confirm the site of deamidation. Each asparagine deamidation increases the

mass of a peptide by 1 Da. Conversely, aspartic and isoaspartic acids are isobaric and are more challenging to distinguish by mass spectrometry alone. However, the isomerization of aspartic acid to isoaspartic acid can result in a more hydrophilic peptide and a shorter retention time by RPLC [91]. Using the combination of information provided by both RPLC and MS, sites of deamidation and aspartic acid isomerization can be identified (Fig. 1.4). Special care in sample preparation must be taken during "bottom-up" LC-MS as typical protein digestion conditions can themselves cause deamidation and aspartic acid isomerization. Kori et al. found that asparagine deamidation and isoaspartate artifacts can be reduced significantly by carrying out the digestion procedure in 20 mM Tris buffer, pH 7.8 containing 10% acetonitrile [91]. Aspartic acid isomerization on a model tryptic peptide was 33.8% after an overnight digestion in 20 mM ammonium bicarbonate but only 4.5% in 20 mM Tris 10% acetonitrile. Often, it may be more important to determine which residues are most prone to deamidation rather than the current level of deamidation in a sample as additional deamidation can occur in vivo [92].

Succinimide is a key intermediate in both asparagine deamidation and aspartic acid isomerization and is itself an important modification of antibody-based therapeutics (Fig. 1.5). It is often overlooked as it is unstable at the neutral and basic pH conditions commonly used for "bottom-up" LC-MS sample preparation [93–95]. Cao et al. developed

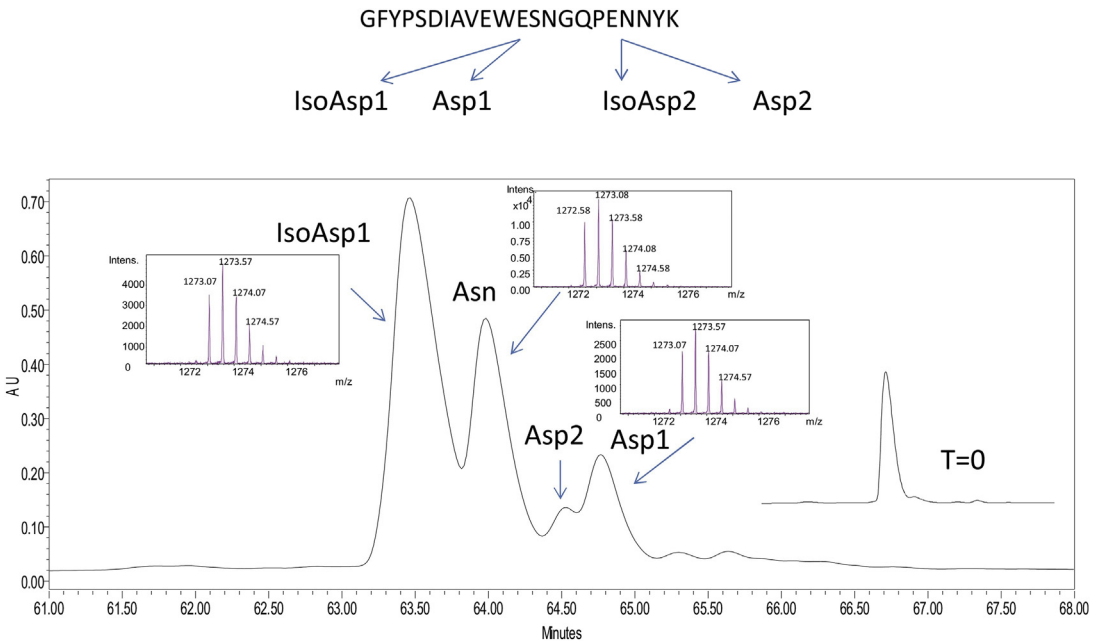

FIG. 1.4 RPLC-UV (214 nm) chromatogram of a synthetic peptide (sequence shown above the chromatogram) after incubation in ammonium bicarbonate, illustrating the separation of multiple deamidation/isoaspartate products. The mass spectra for the major peaks are provided as insets, as is the RPLC-UV chromatogram of the peptide prior to incubation. *Reproduced from Kori, Y., et al., A conventional procedure to reduce Asn deamidation artifacts during trypsin peptide mapping. Journal Of Chromatography B Analytical Technologies In The Biomedical And Life Sciences, 2016;1009–1010:107–113. with permission from Elsevier.*

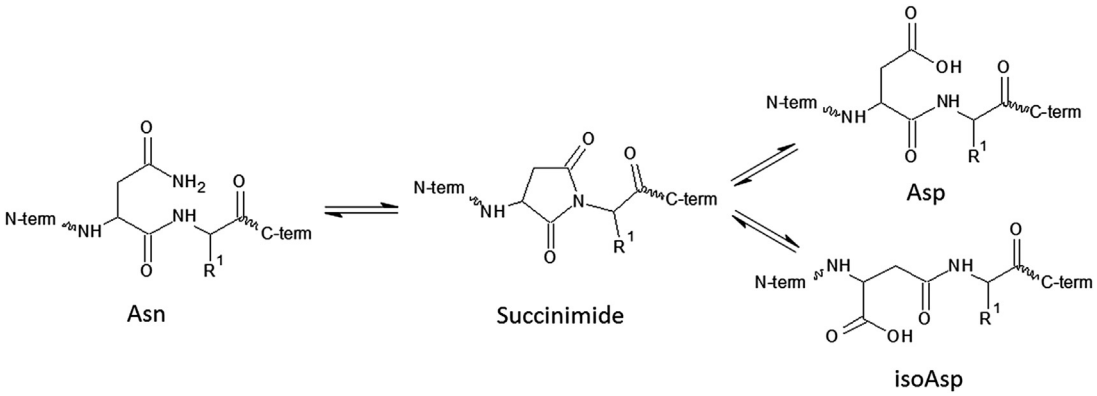

FIG. 1.5 Schematic showing the interconversion of asparagine, aspartic acid, isoaspartic acid and succinimide.

a LC-MS method for detecting and quantifying succinimide that performed reduction, alkylation and proteolytic digestion at pH 5.4–5.8 [95]. The amount of succinimide present on heat stressed antibodies increased with time but was converted almost completely to deamidation products at pH 7.6. Unmodified, deamidated and succinimide forms of an antibody could also be distinguished by hydrophobic interaction chromatography coupled with UV detection (HIC-UV) and the results aligned well with the LC-MS peptide mapping results for the digest prepared under low pH conditions [95].

Oxidation

Oxidation can impact the function and stability of antibody-based therapeutics. Oxidation can be induced by heat, light or the presence of reactive chemical impurities such as reactive oxygen species generated during production and storage [96]. Methionine residues are most susceptible to oxidation [97–99]. Less well appreciated is the fact that other amino acid residues are also prone to oxidation, especially tryptophan. Oxidative degradation products of tryptophan have a chromophore and impart a color to degraded antibody solutions [100]. Oxidation of tryptophan residues in the CDR can have a major impact on antigen affinity [101]. Tryptophans in the CDR may be more susceptible to oxidation given the solvent accessibility and relative flexibility of the CDR loops, though other factors such as side chain orientation and the nature of adjacent residues also contribute to susceptibility to oxidation [101]. Forced oxidation using 2,2-azobis(2-amidinopropane) (AAPH) in the presence of free methionine selectively oxidizes tryptophan residues and allows for the identification of tryptophan residues susceptible to oxidation [96]. Using this technique in combination with "bottom-up" LC-MS, Pavon et al. demonstrated that a single tryptophan in the CDR3 region of two mAbs was susceptible to oxidation [102]. LC-MS identified multiple oxidation products illustrating the complexity of the tryptophan oxidation degradation pathway (Fig. 1.6).

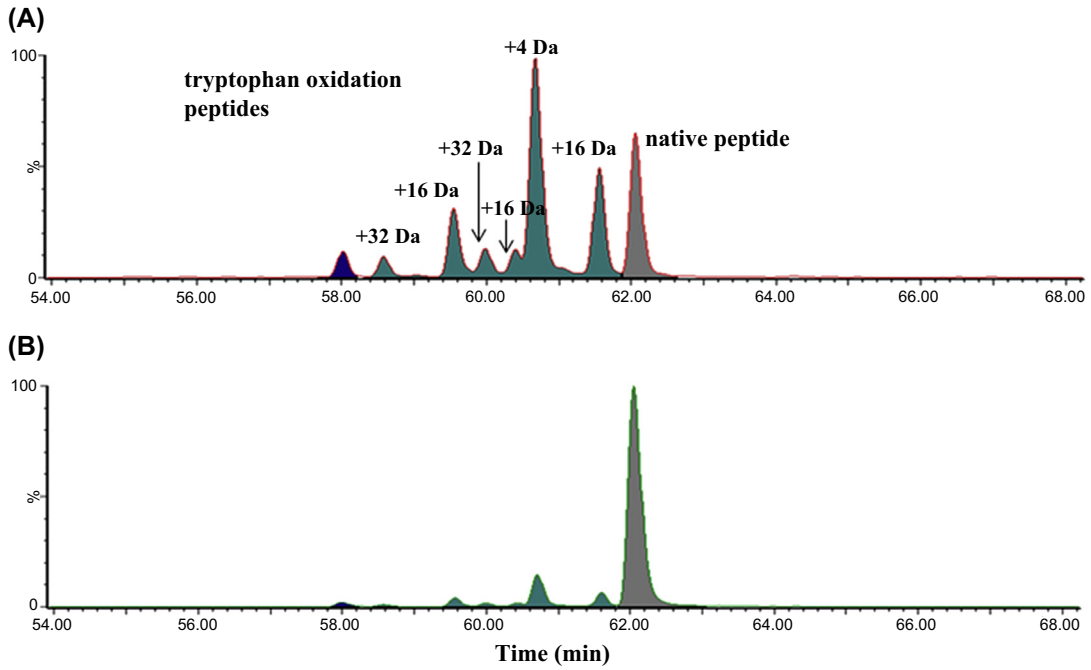

FIG. 1.6 Representative extracted ion chromatogram of the oxidized forms of a tryptophan-containing mAb peptide following forced oxidation with 1.2 mM AAPH. The numbers above each peak indicate the mass difference between the oxidized peptide and the native peptide. The major oxidized peptide peaks correspond to conversion of tryptophan to kynurenine (+4 Da), hydroxytryptophan and/or oxindolylalanine (+16 Da), and N-formylkynurenine (+32 Da). *Adapted from Pavon, J.A., et al., Selective tryptophan oxidation of monoclonal antibodies: oxidative stress and modeling prediction. Anal Chem, 2019:91(3):2192−2200 with permission, © 2019 American Chemical Society.*

Drug conjugates

Antibodies can be intentionally modified with cytotoxic drugs to create antibody drug conjugates (ADCs) - highly specific and potent therapeutics used mainly for cancer treatment. ADCs can be synthesized in many different ways using a variety of linkers and drug payloads. Drug is most commonly attached to the antibody through a linker conjugated to lysine amine groups, free sulfhydryls or modified glycan sugars. Not surprisingly, these unique biotherapeutics can be a challenge to characterize, however, there is a large body of literature demonstrating that LC-MS is well suited for the task [103].

A critical quality attribute of ADCs is their drug to antibody ratio (DAR). Quantitative UV analysis is often used to determine the average DAR of an ADC but can be biased by the presence of free drug in solution. The distribution of drug across the ADC population (i.e. the proportion of antibody molecules having a DAR of 0, 1, 2, 3 … etc.) is more challenging to measure. Hydrophobic interaction chromatography with UV detection (HIC-UV) is frequently used to determine the DAR distribution of ADCs with defined conjugation sites but is challenged by ADCs prepared using random conjugation strategies (i.e. lysine conjugation). On other the hand, intact mass LC-MS analysis can be used to measure the DAR

distribution of almost all ADCs, irrespective of their nature and method of manufacture. Furthermore, LC-MS can identify unusual ADC species (such as antibody with linker but no drug attached) that are not generally detected by other methods.

"Bottom-up" LC-MS is the method of choice for identifying drug conjugation sites; however determining the degree of occupancy at each location can be challenging as the unconjugated and drug-conjugated versions of peptides in ADC digests behave differently by LC-MS. Hill et al. were able to measure site occupancy at 98% of available primary amines on a surrogate ADC prepared by derivatizing the NISTmAb reference material with tandem mass tags [104]. They demonstrated that while many lysines were modified to some extent, the N-terminus as well as Lys187 on the light chain were particularly amenable to conjugation. LC-MS can also be used to measure the amount of free (unconjugated) drug present in ADC preparations [105−107]. For example, Birdsall et al. developed a 2D-LC-MS method using a solid phase extraction (SPE) trap in the first dimension and RPLC in the second [106]. The unconjugated drug-linker products accumulated on the SPE trap whereas the ADC passed through. In this way they were able to measure free drug in ADC preps down to single digit ppm levels - two orders of magnitude more sensitive than equivalent UV methods [106].

In-vivo stability of ADCs is especially important as premature release of drug prior to target engagement can produce undesirable toxic side effects and narrow the therapeutic window. Immuno-enrichment of ADCs from serum followed by intact mass LC-MS analysis has been used to measure DAR and monitor ADC stability in vivo [108−113]. For example, Xu et al. immobilized biotinylated target antigen on paramagnetic streptavidin beads and used it to capture both lysine- and cysteine-conjugated ADCs from the plasma of dosed monkeys [108,109]. Intact mass LC-MS analysis of the immuno-isolated ADCs revealed a gradual reduction of DAR over time, indicating loss of drug during circulation in the blood (Fig. 1.7). Excoffier et al. developed a more universal immune-capture protocol that targeted the Fc portion of ADCs and demonstrated that it could be used to enrich ADCs with both cleavable and non-cleavable linkers from serum [110]. Enriching ADCs from serum at sufficient purity and concentration that the DAR distribution can be determined remains a challenge for ADC analysis.

Other modifications of antibodies and antibody fusion proteins

Tyrosine sulfation is a rare modification of antibodies. Sulfation and phosphorylation both add a mass of approximately 80 Da (79.9568 Da and 79.9663 Da, respectively) and can be distinguished using a mass spectrometer with very high mass accuracy and resolution. Alternatively, sulfation and phosphorylation can be distinguished by their CID fragmentation pattern in MS/MS; sulfation exhibit a 80 Da neutral loss while phosphorylation has a characteristic neutral loss of 98 Da. Zhao et al. observed that a fraction of their CHO-produced human IgG bound to anion exchange resin and had a +80 Da mass difference [114]. Using "bottom-up" analysis, they confirmed that the modification was sulfation. ETD fragmentation was then used to locate the sulfation to a single tyrosine residue. The effect of tyrosine sulfation on antibodies is not known but for many other proteins, tyrosine sulfation recognition is an important driver of protein-protein interactions [115].

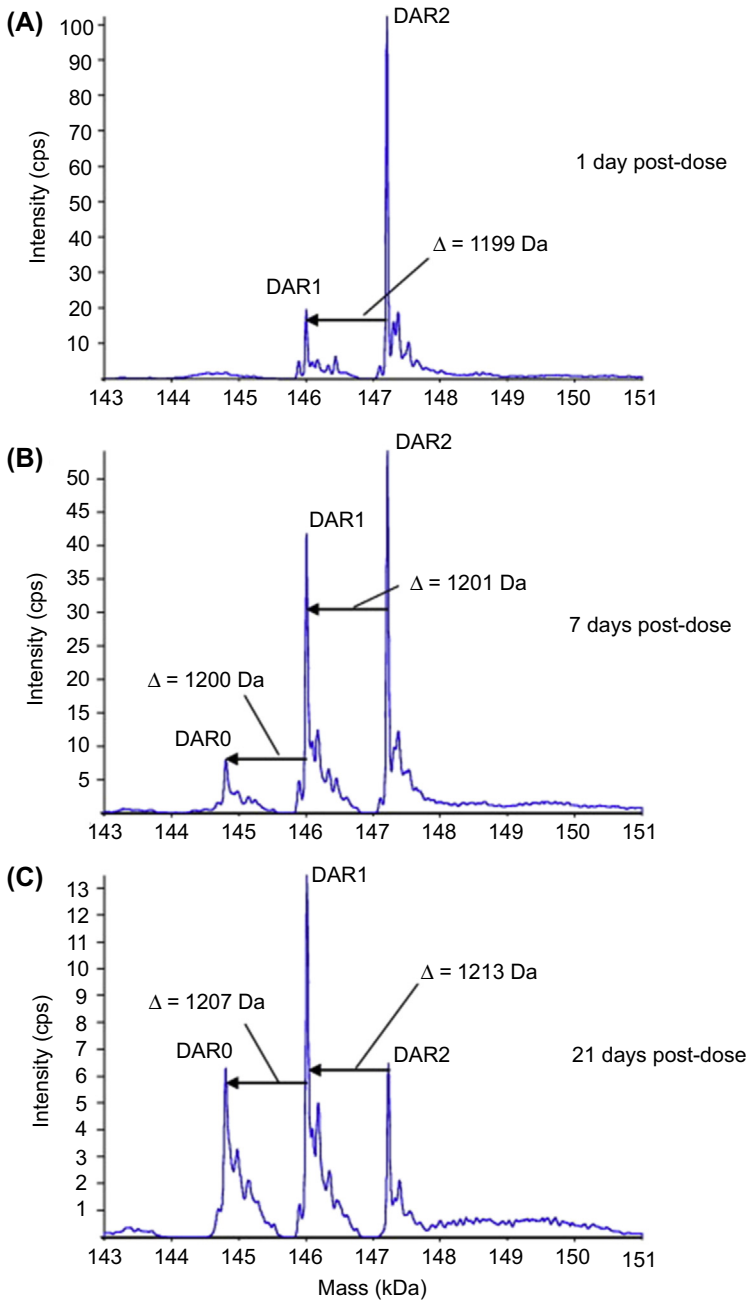

FIG. 1.7 LC-MS spectra showing in vivo drug release from a cysteine-conjugated ADC (anti-MUC16 TDC) in the circulation of cynomolgus monkeys. The ADC had an initial DAR of 2. Using immuno-capture of the ADC from serum, LC-MS monitored the amount of ADC in vivo with a DAR of 0, 1 or 2 at (A) 1 day, (B) 7 days and (C) 21 days post-dose. *Reproduced from Xu, K., et al., Characterization of intact antibody–drug conjugates from plasma/serum in vivo by affinity capture capillary liquid chromatography–mass spectrometry. Anal Biochem 2011:412:56–66 with permission from Elsevier.*

Chumsae et al. have reported covalent modifications of the N-terminus of several antibodies by citric acid resulting in a mass increase of 174 Da or 156 Da [116]. Citric acid is a common buffering agent used in biotherapeutic formulations to maintain the pH between 3 and 6 and is frequently used to elute antibodies from affinity resins during downstream processing. The modifications are hypothesized to be from reaction of citric anhydride with the N-terminal amine, resulting in a +174 Da amide modification and two different +156 Da cyclic imide modifications. Several different N-terminal residues were observed modified but lysine side chain amines were not observed similarly modified. Chumsae et al. hypothesized that other buffering agents with 2 or more juxtaposed carboxylic acids such as adipic acid, malic acid, succinic acid or tartaric acid may also form analogous N-terminal modifications.

The glycine-rich linkers such as $(GGGGS)_n$ or $(GGGGP)_n$ commonly used in antibody-based fusion proteins are prone to modifications such as phosphorylation [117], O-xylosylation [118], xylose-based glycans such as Xyl-Gal-Neu5Ac and Xyl-Gal-Gal-GlcA [119], and hydroxyproline formation [120]. These modifications can be detected during intact mass analysis or "bottom-up" analysis by their characteristic mass changes (see Table 1.1). MS2 or MS3 with CID, HCD and/or ETD may be necessary to confirm the exact site of modification [120]. Since these linker modifications are usually far from the binding region, these modifications are unlikely to interfere with antibody binding but raise potential immunogenicity concerns.

Section 3 — Enabling technologies and future directions for LC-MS-based antibody characterization

New reference materials

In 2016, the NISTmAb reference material 8671 (a monoclonal IgG1κ produced by the National Institute of Standards and Technology, USA) became publicly available to researchers - making available for the first time a highly characterized recombinant monoclonal antibody for method development, system suitability tests and inter-laboratory comparability studies. Prior to 2016, a few recombinant monoclonal antibodies were commercially available (such as Waters' Intact mAb Mass Check Standard and Sigma's SILu™Lite SigmaMAb Universal Antibody Standard) but these antibodies were not well characterized and use was not widespread. Many labs developed their own in-house reference antibodies for method development, which precluded easy comparison of methods developed in different labs. The NISTmAb was thoroughly characterized by a multi-lab, international effort which used multiple methods to assess the identity, purity, aggregation, stability, glycosylation, post-translational modifications and higher order structure. The results of these studies were published in a 3 volume book series "State-of-the-Art and Emerging Technologies for Therapeutic Monoclonal Antibody Characterization" and follow-up papers [19,121–124]. NISTmAb does not replace the need for product-specific standards when developing a new antibody-based therapeutic or biosimilar but it serves as a useful external standard when comparing methods developed in different labs [125].

Enabling enzymes

Over the past 10 years, new antibody proteases commercialized by companies such as Genovis have opened up new options for specific antibody cleavage and "middle-up"/"middle-down" analysis. Previously, partial cleavage of antibodies was done using the proteases papain and pepsin. Papain cleaves above the hinge of IgGs to generate Fc and Fab fragments. Pepsin cleaves below the hinge to generate a $F(ab')_2$ fragment while the Fc fragment undergoes extensive proteolysis. Pepsin and papain protocols need to be carefully optimized for each monoclonal IgG to avoid over-digestion and yields of the desired fragments are typically 45–55% [126]. The introduction of IdeS (by Genovis under the brand name "FabRICATOR"), an IgG-specific cysteine protease isolated from *Streptococcus pyogenes*, made it possible to specifically cleave human IgG1 into $F(ab')_2$ and Fc/2 fragments in an easy, complete and reproducible manner [127]. IdeS cleaves human IgG at one specific location below the hinge (Fig. 1.8) with much greater specificity and efficiency than pepsin and the protocol does not require optimization for individual antibodies. IdeS is now the most commonly used enzyme in "middle-up"/"middle-down" characterization of IgG subunits.

More recently, Genovis has introduced enzymes for cleavage of IgG at one specific site above the hinge to form Fab and Fc fragments. SpeB (FabULOUS, launched in 2014) cleaves a wide range of IgGs but only under reducing conditions, Kgp (GingisKHAN, launched in 2015) cleaves human IgG1 under very mild reducing conditions (2 mM cysteine) and IgdE (FabALACTICA, launched in 2017) cleaves human IgG1 above the hinge under native conditions. Generation of the Fab fragment is of particular interest because this antibody fragment retains heavy-light chain pairing information. Kgp has been used to determine heavy-light chain pairing and to detect light chain swapping during the generation of bispecific antibodies [128,129]. Heterodimer antibodies with swapped light chains are isobaric to those with correct pairing, but at the Fab fragment level, incorrect pairings are easily identified. In addition, a combination of enzymes that cleave above and below the hinge has been used to isolate the hinge region of an antibody and identify reduced disulfide bonds [130].

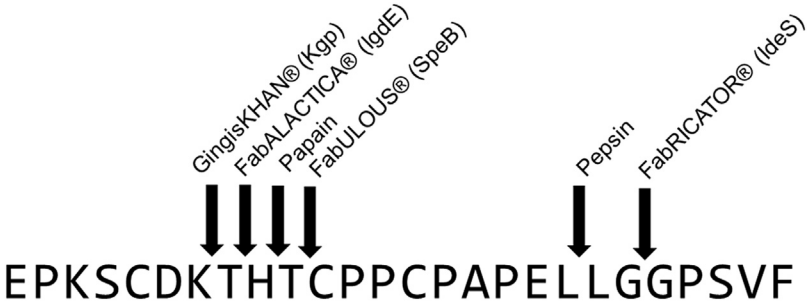

FIG. 1.8 Sites of cleavage in the hinge region of IgG1 for enzymes typically used to generate antibody fragments for "middle-up"/"middle-down" LC-MS analysis.

Top-down and middle-down analysis

Interest has been growing in the potential of "top-down" mass spectrometry for the characterization of antibody-based therapeutics. In a "top-down" experiment, intact antibody ions are selected and fragmented within the mass spectrometer to obtain amino acid sequence and PTM information. A variety of fragmentation techniques can be used in "top-down" assays including ultraviolet photodissociation (UVPD), collision-induced dissociation (CID), electron transfer dissociation (ETD), electron capture dissociation (ECD), higher energy collisional dissociation (HCD) or ETD followed by HCD (EThcD). Like intact mass analysis, "top-down" mass spectrometry requires minimal sample preparation (primarily desalting) so processing artifacts such as deamidation, oxidation and S-thiolation (i.e., cysteinylation) are avoided [3,131]. Also, modifications prone to loss or scrambling during "bottom-up" LC-MS/MS tend to remain in place in a "top-down" analysis. The "top-down" approach provides a more holistic view of an antibody compared with "bottom-up" analysis. For example, "top-down" analysis can detect correlations between modifications, such as any correlations between glycosylation and susceptibility to oxidation; this information is lost in "bottom-up" experiments as the different proteoforms are scrambled during digestion [3,129]. Depending on the fragmentation method used, disulfide bonds may still be intact and branched fragments may indicate chain pairing.

In theory, information about the entire protein (including all modifications) is present in a "top-down" spectrum. In practice, however, "top-down" mass spectrometry has several technical challenges. During MS/MS, the precursor ion signal is split into a large number of fragment ion signals. As the size of the precursor protein increases, the number of fragment ions increases and the signal intensity for individual ions decreases. To attain a sufficient signal-to-noise ratio to detect low abundant fragment ions, many microscans must be averaged, typically by performing multiple replicate runs [6,129]. Additionally, fragmentation methods preferentially cleave the antibody in certain regions, usually mediated by the presence of disulfide bonds [129,132]. Better sequence coverage can be attained by using a combination of fragmentation methods, though this results in multiple datasets to analyze and align [133]. Top-down spectra are very complex due to the presence of canonical N- and C-terminus containing fragments as well as internal fragments and branched disulfide bond-containing fragments, all of which may exist at multiple charge states [129]. Sophisticated software is necessary in order to compile microscans, deconvolute data, match fragments and distinguish false positives [133]. As a consequences, sequence coverage for "top-down" analysis of antibodies lags significantly behind that which can be attained by "bottom-up" methods [6,133].

"Middle-down" analysis of antibodies is showing greater promise than "top-down". "Middle-down" LC-MS/MS is carried out in the same way as "top-down" except the antibody is first cleaved into large fragments (usually Fc/2, Fd and light chain, or Fab and Fc) which are analyzed separately by LC-MS/MS (Fig. 1.9) [6]. Since the cleaved fragments are significantly smaller than the full-sized antibody, the MS/MS spectra are simpler and fragment ion signal is improved. However, the fragmentation data is still complex and complete amino acid sequence coverage continues to be an issue. It remains to be seen whether the technical challenges associated with "top-down" and "middle-down" MS will

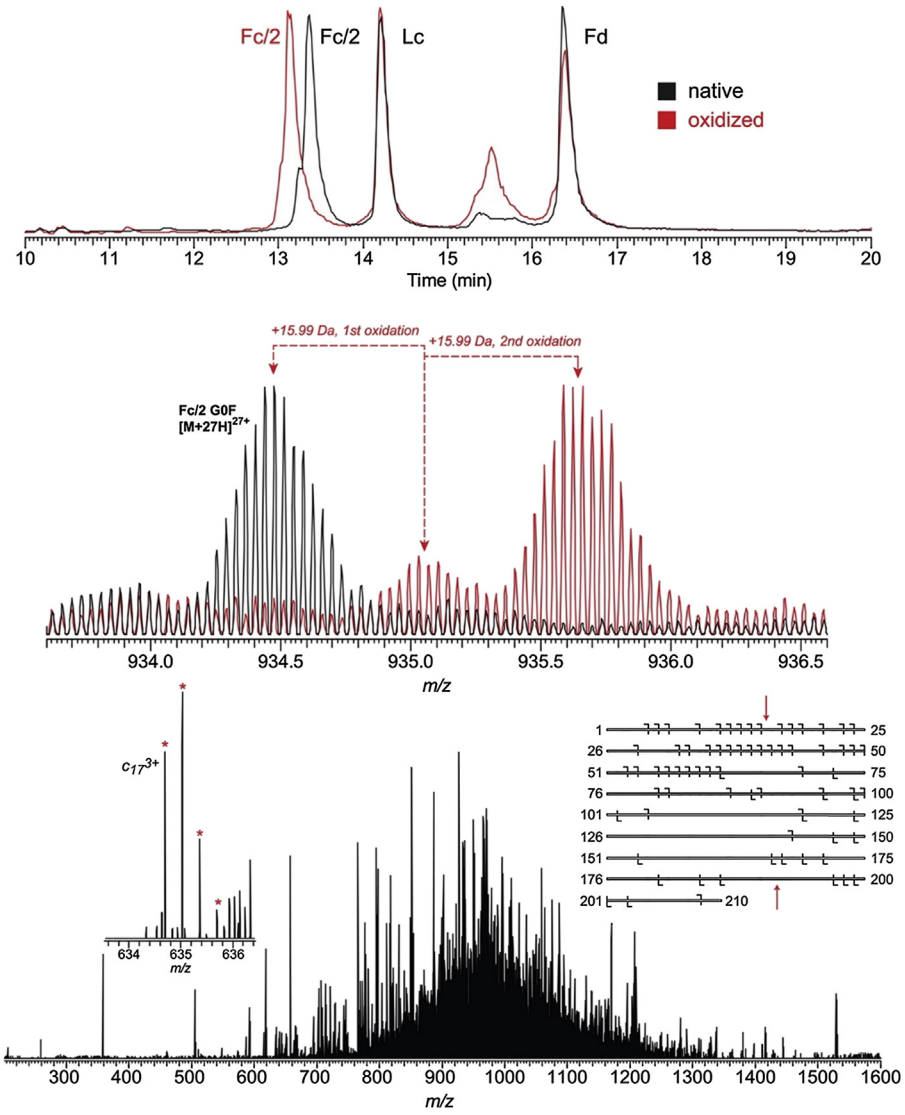

FIG. 1.9 Application of "middle-down" LC-MS for determining the locations of oxidized Met residues in the mAb, Adalimumab. (Top) After IdeS digestion and reduction, the resulting Fd, Fc/2 and light chain fragments were resolved and analyzed by LC-MS on a Orbitrap Fourier Transform mass spectrometer. The oxidized **(***red line (gray in print version)***)** Fc/2 eluted earlier than the control **(***black line***)** Fc/2. (Middle) Expanded view of the overlapped mass spectra for control (*black*) and oxidized Fc/2 (*red* (*gray in print version*)). At isotopic resolution (120,000 resolution at m/z 400), the oxidized Fc/2 is observed with a mass +16 Da and +32 Da greater than control Fc/2, indicating both singly and doubly oxidized form. (Bottom) The "middle-down" ETD fragment ion spectrum for oxidized Fc/2 (single LC run, 3 ms ETD, isolation window 120 Th). The left inset shows a product ion confirming that Met16 is oxidized. The right inset is a scheme of identified product ions, with the positions of oxidized Met indicated by arrows. *Reproduced from Fornelli, L., et al., Middle-down analysis of monoclonal antibodies with electron transfer dissociation orbitrap fourier transform mass spectrometry. Anal Chem, 2014;86(6):3005−3012 with permission, © 2014 American Chemical Society.*

be surmounted in the coming years and use of these promising technologies becomes more widespread.

Native mass spectrometry

One of the more exciting developments in LC-MS in recent years has been the emergence of native mass spectrometry (native MS) for the characterization of antibody-based therapeutics under non-denaturing conditions. Native MS preserves non-covalent interactions and higher order structure normally lost under the denaturing conditions of RPLC-MS and can be used to study antibody-antigen and antibody-antibody interactions [134,135]. Native MS is similar to intact mass LC-MS analysis except that samples are electrosprayed under neutral, near physiological pH conditions in simple solutions consisting of volatile buffers such as ammonium acetate. The resultant protein ions are not highly charged and their charge state envelopes are shifted to a higher m/z range than protein ions generated under denaturing MS conditions. Therefore, a mass spectrometer with a higher mass range than typical for traditional instruments (up to m/z 8,000−20,000) is necessary for native MS. Native MS is particularly useful for studying antibodies, ADCs and bispecifics that are held together non-covalently, such as cysteine-based ADCs where the cysteines that would normally have formed inter-chain disulfide bonds are instead used for drug conjugation [136,137] (Fig. 1.10). Native MS provides a better picture of the true state of an antibody than does denaturing MS.

Different proteoforms are better resolved with native MS than denaturing LC-MS because the separation between ions is greater at the lower charge states typical of native MS. Wohlschlager et al. took advantage of this improved separation and used intact and "middle-up" native MS on an Exactive Plus EMR Orbitrap to characterize the highly complex glycoforms of Etanercept, a 130 kDa Fc fusion protein with 6 N-linked and up to 26 O-linked glycans [138]. Native MS sufficiently resolved the numerous, complex glycoforms of intact Enbrel and enabled lot to lot comparison.

Ion mobility mass spectrometry

In conventional mass spectrometry, gas-phase ions are resolved solely on the basis of their mass to charge ratio (m/z). In ion mobility mass spectrometry (IM-MS), both the m/z of an ion and the time taken by the ion to pass through a drift tube placed in-line with the mass spectrometer (the "drift time") is recorded. The drift time of an ion is dependent on both its charge and shape. IM-MS provides another dimension of separation that can add greater selectivity to an LC-MS assay and offer a means to probe the structure and conformational stability of ions [139]. IM enhances "bottom-up" LC-MS by resolving overlapping peptide ions with different charge states, improving the signal to noise for weaker ions and distinguishing structural isomers of isobaric ions. For example, ion mobility has been coupled with 1D-RPLC-MS to profile lot to lot N-glycosylation compositional heterogeneity in batches of trastuzumab mAb [140] and with 2D-RPLC-MS to identify trace-level host cell proteins in originator and biosimilar mAbs [36].

Ion mobility is a logical match with native MS which maintains the native shape of protein ions and there are numerous publications demonstrating the use of native-IM-MS for

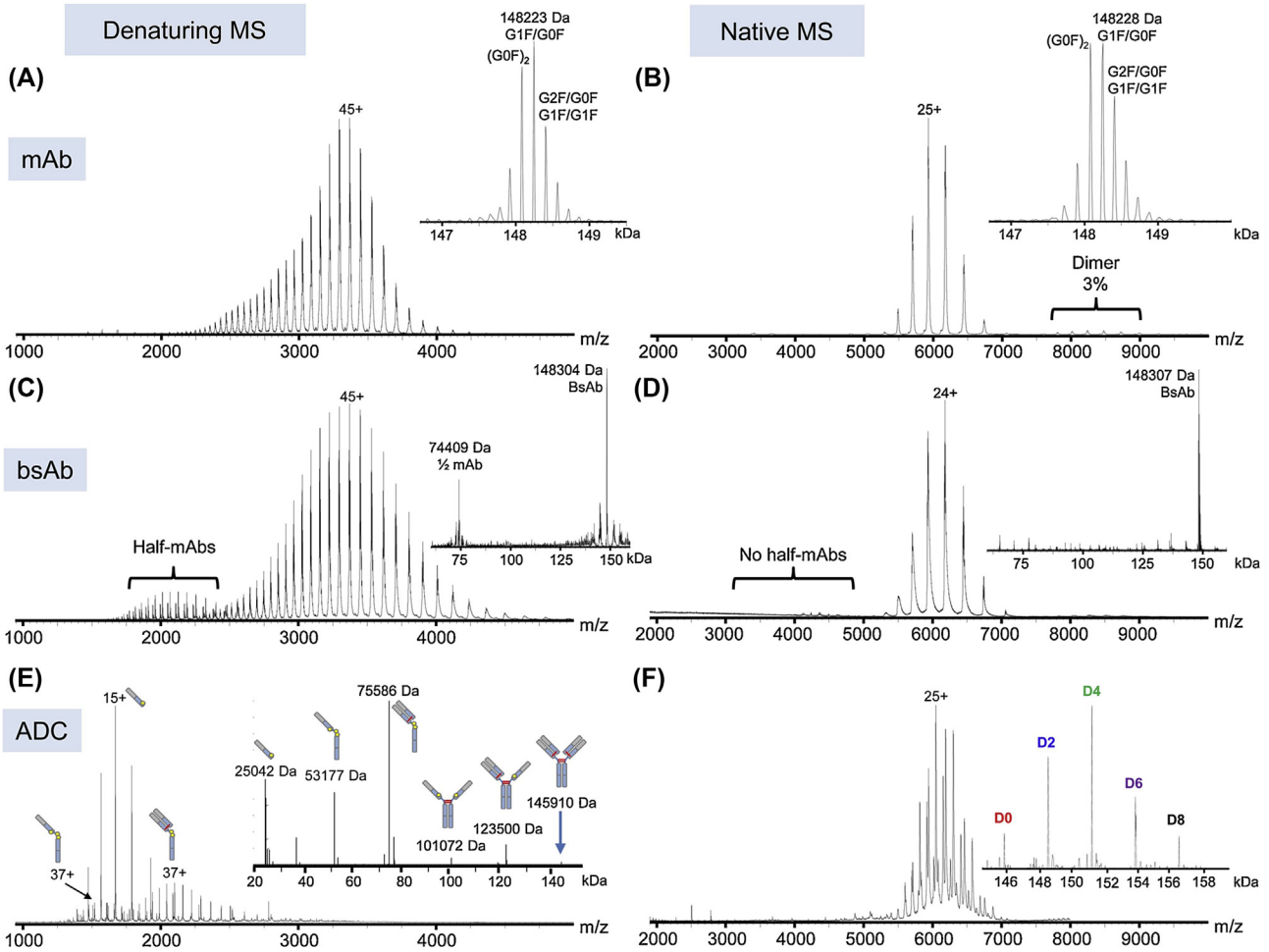

FIG. 1.10 Comparison of denaturing and native LC-MS of antibody-based therapeutics: (A and B) intact trastuzumab, (C and D) a bispecific mAb, and (E and F) the cysteine-conjugated ADC, brentuximab vedotin. The insets show the annotated deconvoluted mass spectra. The intermolecular disulfide bonds in brentuximab vedotin were partially reduced during drug conjugation. The ADC dissociated into fragments under the denaturing conditions but remained intact during native MS analysis. *Reproduced from Terral, G., A. Beck, and S. Cianférani, Insights from native mass spectrometry and ion mobility-mass spectrometry for antibody and antibody-based product characterization. Journal of Chromatography B Analyt Technol Biomed Life Sci 2016;1032:79—90 with permission from Elsevier.*

investigating the structure and non-covalent interactions of antibody-based therapeutics. In one of the first publications, Atmanene et al. used native-IM-MS to study interactions between human JAM-A, a protein expressed in tumors, and anti-JAM-A mAbs [135]. IM-MS resolved on the basis of drift times populations of ions that had alternative disulfide bonding in the JAM-A ectodomain. However, analysis of the antibody/JAM-A interactions by native-IM-MS indicated that ectodomain disulfide heterogeneity did not have a significant effect on antibody affinity or binding stoichiometry. Bagal et al. demonstrated that native-IM-MS could distinguish between the A and B disulfide isoforms of intact IgG2 mAbs. The B isoform had a slightly longer drift time suggesting that it has a larger cross-sectional area relative to the A isoform. More recently native-IM-MS has been incorporated into multi-pronged analytical strategies to compare biosimilars with originator mAbs [141,142].

Native-IM-MS can be coupled with collision induced unfolding (CIU) to study protein conformational stability [139]. In a CIU experiment, the protein ions undergo collisional activation in the gas phase just prior to IM. Unfolding events are detected as changes in the IM drift time. Native-IM-MS CIU was used to compare CBW-03-06, an ADC with engineered sites for drug conjugation, with its parental mAb [143]. The ADC was less prone to unfolding than the parent mAb, suggesting that drug conjugation actually stabilized the conformational structure of the mAb. Native-IM-MS with CIU was also used to study Fab arm exchange in IgG4 antibodies, a process that can be exploited to create bispecifics [144]. Native-IM-MS alone could not distinguish the different IgG4 species but the addition of CIU generated unique signatures for each antibody species present, both native and bispecific. Finally, IM-MS with CIU has been used recently in accelerated stability studies [145]. IM-MS with CIU detected changes in the stressed antibodies indicative of unfolding.

Alternative chromatographies for antibody and ADC analysis

Different chromatographies can be interfaced with native MS to facilitate the separation of proteoforms based on size, charge or affinity prior to MS. For example, Hengel et al. coupled a microscale size exclusion chromatography (SEC) column with native MS for the separation of cysteine-conjugated ADCs immune-isolated from the plasma of dosed rats prior to DAR assessment [146]. The improved separation provided by SEC allowed them to monitor the gradual decrease in DAR occurring over time due to loss of drug-linker and to detect cysteinylation of the deconjugated cysteines. Similarly, Ehkirch et al. demonstrated the potential of coupling SEC with native MS and ion mobility-MS (SEC-native-MS/IM-MS) for the enhanced characterization of antibody-based therapeutics, including ADCs [147]. The same group also developed a four dimensional HIC-SEC-native-IM-MS platform and applied this to the comparison of cysteine-conjugated ADCs in native form and after forced degradation [148]. The addition of HIC separation in the first dimension enabled a greater resolution of all ADC species present, especially those in the degraded samples.

Proteoforms can vary in charge due to the presence of charge-modifying modifications, such as C-terminal lysine, sialic acid and deamidation. Füssl et al. described the development and application of a pH gradient cation exchange chromatography-native mass spectrometry platform for the charge-based separation and identification of antibody species [149,150]. Volatile, low ionic strength buffers were used for the separation so as to be compatible

with native MS. This novel LC-MS platform could distinguish charge isoforms of stressed-tested Adalimumab due to the presence of C-terminal lysine, glycation, deamidation, isoaspartate and possibly succinimide formation [150]. Finally, Chen et al. demonstrated that it is possible to directly interface HIC with electrospray mass spectrometry [151,152]. Normally this would not be possible as the mobile phase uses relatively high concentrations of non-volatile salts which are incompatible with mass spectrometry, however, by replacing the non-volatile salts with ammonium acetate and utilizing more hydrophobic stationary phases, they achieved good HIC separations of antibodies and performed "top-down" HIC-ECD-MS sequencing on the multiply charged antibody ions [152].

Multi-Attribute Method

LC-MS is at the core of a recent technology development called Multi-Attribute Method (MAM) that has generated significant interest within the biopharmaceutical industry. MAM utilizes "bottom-up" LC-MS protocols to simultaneously detect, quantify and monitor multiple features of a protein biologic such as N- and O-glycosylation, oxidation, deamidation, isoaspartate formation and protease cleavage [153,154]. In a conventional biomanufacturing environment, a separate assay is used to assess and monitor each important feature, especially those deemed to be critical quality attributes (CQAs). These assays are based on conventional and well understood analytical technologies such as CE-SDS, CEX-HPLC, cIEF and HILIC, typically coupled with UV and fluorescence detectors that generate a straightforward and quantifiable output. MAM has the potential to consolidate many of these conventional assays into a single LC-MS assay. Moreover, MAM is capable of providing additional information about CQAs such as the location and abundance of individual modifications.

Recently, a white paper was published by a working group with representation from across the biopharmaceutical industry advocating for increased adoption of MAM for identifying and monitoring CQAs, improving control strategies for biomanufacturing and simplifying submissions to regulatory authorities [155]. The potential implications for the industry are enormous. Instrument manufacturers have taken note and are developing LC-MS platforms that are better tailored for MAM and are creating software for the automated control of the entire MAM workflow, from instrument control to data analysis, in a manner that is compliant with regulatory expectations. New peak detection (NPD), the ability to flag new and unexpected features in the LC-MS/MS data, is a critical component of MAM control and analysis software [155]. NPD reduces the risk of false negatives and, as a result, has greatly increased user confidence in the technology. Broad application of MAM in the highly regulated environment of QA/QC labs and GMP manufacturing facilities is still some way off in the future and will require continual improvements in instrument design, improved quality control in the manufacture of HPLC columns, enzymes and other reagents as well as the development of new reference materials suitable for MAM. However, it does appear that MAM, and by implication "bottom-up" LC-MS, will have a major impact on how biotherapeutics are manufactured and characterized in the future.

Conclusion

In this chapter, we have provided an overview of how LC-MS is used for the biophysical characterization of antibody-based therapeutics. LC-MS protein characterization is a broad topic area spanning an enormous body of literature and it is impossible to cover all applications in a single chapter. Instead we have highlighted some current LC-MS applications, particularly for the characterization of different PTMs, and recent developments in the field that illustrate both the scope and future potential of LC-MS for biotherapeutics analysis. In this, we have omitted some other applications of LC-MS for the study of antibody-based therapeutics such as quantitative analysis for PK/PD studies which, though outside the scope of this chapter, also relies heavily on LC-MS.

It is acknowledged that LC-MS capabilities represent a large investment for any organization in terms of upfront capital cost, maintenance and specialized staff. However, LC-MS is unparalleled in terms of specificity, sensitivity and flexibility, and is better able than other analytical technologies to keep pace with the innovative R&D developments happening within the biopharmaceutical sector today. The unprecedented level of detail provided by LC-MS-based assays can lead to improvements in biotherapeutic design and production, resulting in more stable, effective and homogeneous products and, ultimately, better patient outcomes. Already LC-MS-based assays are increasingly being adopted into the biopharmaceutical and biomanufacturing domain and are being used to set more precise specifications for critical quality attributes and to monitor production processes. Multi-attribute monitoring with LC-MS is gaining acceptance as a viable, cost-effective alternative to conventional analytical assays. We expect in the near future to see increasing use of LC-MS at all stages of the product development life cycle of antibody-based therapeutics.

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