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Structural mass spectrometry in biologics discovery: advances and future trends

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Mass spectrometry (MS) is one of the key techniques in protein characterization. In this article, the workflow for MS-based structural characterization of biologics in biopharmaceutical drug discovery is presented, including characterization of primary and higher order structures. Advances in MS techniques in protein characterization are illustrated, including electron transfer dissociation MS (ETD-MS) for primary structure analysis and hydrogen/deuterium exchange MS (HDX-MS) for probing protein higher order structures and mapping epitopes. Future trends in applications of MS to evaluate and optimize candidate molecules in biologics stability studies is also described.

Since the introduction of recombinant human insulin as a therapeutic drug approximately 30 years ago, biologics (therapeutic proteins) have become the second largest biopharmaceutical product category after vaccines. Compared with small-molecule drugs, biologics have several distinct advantages, including high specificity, high efficacy, long circulatory half-lives, fewer side effects and higher regulatory approval rates [1]. These therapeutic agents have been used in the treatment of many life-threatening diseases, such as cancer, infectious diseases, inflammation and genetic disorders [2–4]. Pharmaceutical companies are now dedicating more of their pipelines to biologics. The biologics market is estimated to reach a value of approximately US$102.4 billion in 2011, an increase of approximately 9.6% from 2010 [5]. In biologics drug discovery, there are two categories of proteins: the target protein (found in the body) and the therapeutic protein (drug candidate). Target proteins normally present as a set of molecules, and therapeutic proteins that specifically bind to the target proteins are selected either *in vivo* or *in vitro* [6]. In drug discovery, the goal is to find a drug candidate with superior biophysical, pharmacokinetic (PK) and pharmacodynamic (PD) properties to maximize its chances of making it through downstream development successfully. It can take up to 15 years to develop one new therapeutic protein from the earliest stages of drug discovery to the time it is available on market [7].

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MS in structural characterization of biologics

Biologics produced by recombinant DNA technologies are generally complex, heterogeneous, and subject to a variety of modifications. The biological efficacy, clearance, safety and immunogenicity of biologics are highly dependent on their structures. Therefore, there is a growing need for protein structural characterization, particularly during the drug discovery phase when a large number of candidates are being investigated. MS is an essential technique for characterizing biologics because of its analytical sensitivity, resolution, selectivity and specificity (Fig. 1) [8–10]. It is primarily used to support selection of host expression systems, identification of clones with the most favorable quality attributes, and evaluation of both *in vitro* and *in vivo* molecular stabilities. When coupled to online liquid chromatography (LC) separation, MS can provide detailed information about the primary structure of a protein, such as its molecular weight (MW), amino acid (AA) sequence, post-translational modifications (PTMs), and degradation products. Recently, the use of MS has been dramatically expanded to provide information on higher order structures and dynamics of proteins. In particular, hydrogen/deuterium exchange MS (HDX-MS) and ion mobility MS (IMMS) are now used to investigate protein conformations and interactions with their therapeutic targets.

Molecular weight and amino acid sequence

The first step to characterize a therapeutic protein is to determine its MW and confirm its AA sequence. Both pieces of information

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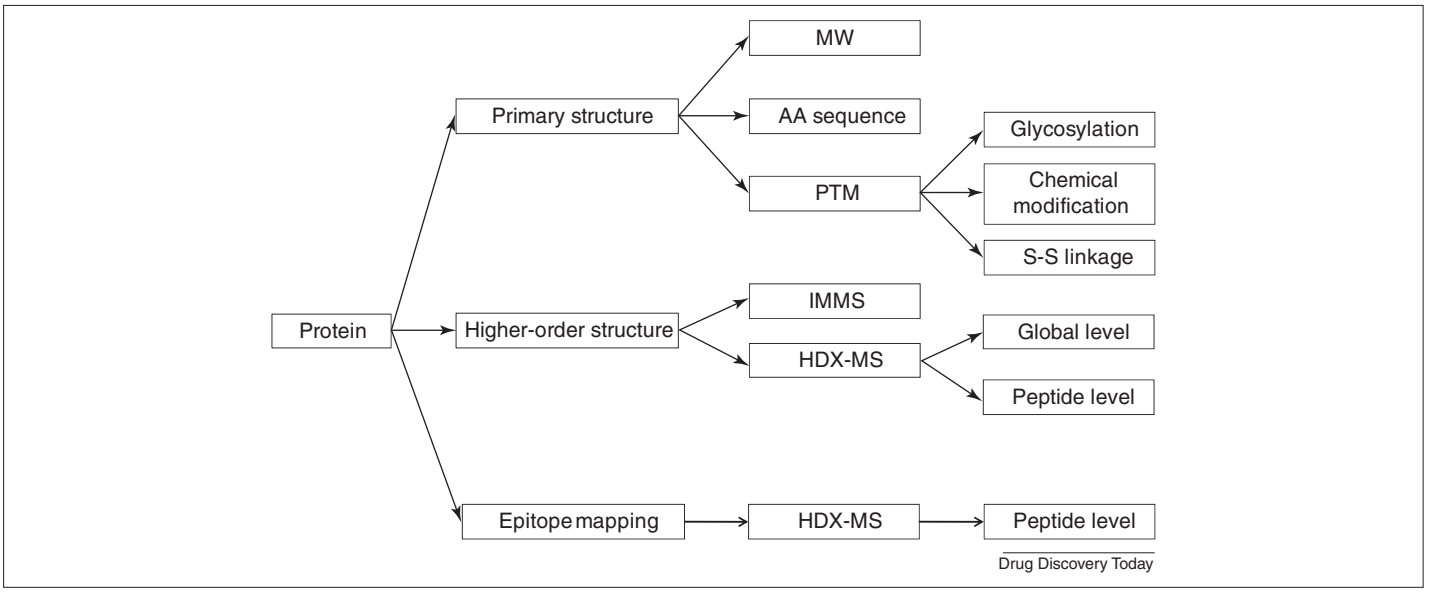

FIGURE 1

Structural characterization of biologics in drug discovery using mass spectrometry. For primary structure, MW, AA sequence and PTMs including glycosylation, chemical modifications and S–S linkages are routinely characterized by MS. IMMS and HDX-MS (global and peptide levels) are normally used for probing higher-order structures of biologics. Epitope mapping experiments can be carried out using HDX-MS. *Abbreviations*: AA: amino acid; HDX-MS: hydrogen/deuterium exchange mass spectrometry; IMMS: ion mobility mass spectrometry; PTM: post-translational modification; S–S: disulfide bond.

are crucial for establishing product identity and integrity. For example, a two-AA discrepancy between a candidate biosimilar and the innovator monoclonal antibody (mAb) can be detected by MW measurement and located by AA sequencing [11].

Due to the large size of therapeutic proteins, the MW determination requires the use of an appropriate ionization technique and a suitable mass analyzer. Electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) are the two most commonly used ionization methods for proteins, with ESI preferable when MS is coupled to LC. Time-of-flight (TOF)-type mass analyzers have been widely accepted as the standard instrumentation for measuring MWs of large molecules because of their high resolution (HR) and mass accuracy, and wide m/z detection range [12]. For a hybrid ESI quadrupole-TOF (QTOF) instrument, the mass accuracy of intact mAbs with MWs of approximately 150 kDa can approach 25 ppm [13] or even 10 ppm [14]. The Fourier-transform ion cyclotron resonance (FT-ICR) mass spectrometer provides even higher mass accuracy [15], however, it is not as common in biopharmaceutical laboratories as the TOF-type instruments owing to its high cost and maintenance requirements. If a MW measurement is performed using a mass spectrometer with modest resolving power and mass accuracy, a 'middle-up' approach, such as limited digestion or reduction of inter-chain disulfide bonds (S–S), can be used to facilitate MW analysis as the resulting fragments are usually much smaller and easier to analyze. For example, an intact mAb can be reduced by dithiothreitol (DTT) to generate separate heavy chains and light chains [16].

Protein AA sequences can be analyzed by two approaches: 'bottom-up' and 'top-down'. In the 'bottom-up' approach, which is also referred to as peptide mapping, the protein undergoes denaturation, reduction, alkylation, and digestion. The digested peptides are then separated by LC and analyzed by MS and tandem MS (MS/MS) in a data dependent manner, whereby MS detection switches between the full MS mode and the MS/MS mode to collect masses of precursor ions and fragment ions, respectively. Due to the limited duty cycle, not all ions detected in the MS mode can be selected for fragmentation in MS/MS mode. Therefore, this type of experiments is not ideal for fast analysis or detecting rapidly eluting peaks. Recently, a new form of data acquisition, called MS^E, was introduced to maximize the instrument duty cycle [17]. MS^E simultaneously collects information for both precursor and fragment ions in the same mode by utilizing parallel alternating scans acquired at either low collision energy or high collision energy. There is no need to preselect an analyte at m/z value for MS/MS experiments. Multiple peptides can be detected, fragmented and analyzed during the same scan, ensuring that MS and MS/MS data are obtained for the entire peak in the chromatogram. Algorithms correlate the changing intensities of the precursor ions (low energy MS scan) and the changing intensities of the product ions (high energy MS scan) to identify which precursor ion is matched to selected product ions. With both precursor and fragmentation data, the sequence of each peptide can be determined, and by putting all the peptides sequences together, the sequence of the entire protein can be confirmed [18].

In the 'top-down' approach, the protein is sequenced directly in the gas phase by MS/MS or multiple-stage MS (MS^n). Compared with the 'bottom-up' approach, 'top-down' methods involve less sample handling and provide more reliable analyses since they can avoid artificial modifications, such as deamidation and AA rearrangement, which can occur in 'bottom-up' analyses [19,20]. However, the 'top-down' approach has limitations when sequencing large proteins. With traditional fragmentation methods such as collision-induced dissociation (CID), complete sequence coverage can be achieved for proteins less than 5 kDa, as the fragmentation efficiency of CID is limited for larger proteins [21]. By application of 'nonergodic' fragmentation methods, such as

electron-capture dissociation (ECD), protein sizes amenable for 'top-down' sequencing can increase up to 20 kDa [22]. For even larger proteins, the 'middle-down' approach or additional dissociation before MS/MS, such as 'nozzle-skimmer' and 'pre-folding' dissociation, can be used to break the large molecules into fragments that are sufficiently small for MS/MS sequencing [23–25]. Despite the limitations of 'top-down' approaches for direct sequencing of large proteins, they have been useful for high-throughput N-terminal sequencing, as an identity test for therapeutic proteins and as a quality control assessment of the completeness of signal sequence processing [26,27].

PTM: glycosylation, chemical modifications and S–S linkage

All biologics approved or currently under development have PTMs, which can profoundly affect protein properties relevant to their therapeutic applications. Modifications of therapeutic proteins with impact on their potency, immunogenicity and PDs are critical quality attributes of therapeutic proteins and must be monitored and controlled [28]. The advent of biosimilars further highlights the significance of PTMs, because such modifications can influence product equivalence and immunogenicity [29,30]. Proteins can display a broad range of PTMs; here we discuss the modifications that are commonly monitored at the discovery stage using MS-based methods, including glycosylation, 'hot spots' for chemical modifications such as oxidation, deamidation, and isomerization, and S–S linkage formation.

Glycosylation represents the most pronounced and complex form of protein PTMs. It can significantly change protein conformation and consequently modulate the functional activity of proteins in addition to protein–ligand interactions [31]. Glycosylation is characteristically heterogeneous in that it always presents in forms of both N- and O-linked glycosylation together with the micro-heterogeneity of the glycans attached at any given site [32]. Glycosylation analysis involves three aspects: characterization of intact protein glycosylation profiles, localization of glycosylation sites, and analysis of released glycan structures. At the discovery stage, screening of the glycosylation profile is usually sufficient. Using mass detectors with high resolving power and extended mass ranges, intact MW analysis of glycoproteins enables the distribution of the major glycoforms and their relative abundance to be monitored. N-linked glycans are more predictive than O-linked glycans because they normally attach to the Asn residue in the consensus sequence of Asn-X-Ser/Thr, where X can be any AA except Pro [33], and are primarily fucosylated biantennary complex structures with a different number of terminal galactoses. For proteins with both N- and O-linked glycosylations, removing the N-linked glycans using peptide-N-glycosidase F (PNGase F) can significantly reduce the molecular heterogeneity and facilitate mass spectra interpretation [32].

'Hot spots' for common chemical modifications of proteins include Met oxidation, Asn deamidation in Asn-Gly sequences, and Asp isomerization in Asp-Gly sequences. Identification and quantification of oxidation and deamidation using peptide mapping analysis are relatively straightforward compared with isomerization, because both oxidation and deamidation introduce mass changes to the protein, typically +16 Da for oxidation and +1 Da for deamidation. By comparing MS and MS/MS spectra of the modified and unmodified peptides, mass shifts corresponding to the specific modification can be observed. For example, the precursor of an oxidized peptide is 16 Da higher than that of the corresponding non-oxidized peptide and the MS/MS fragments of the oxidized peptide that contain the modification site are also 16 Da higher in mass compared to those of the non-oxidized peptide. The modified and unmodified peptides can usually be separated chromatographically, which enables relative quantification of the modification using either UV or MS signals. MS quantification is more sensitive than UV quantification, but it can be influenced by the difference of ionization efficiencies between the modified and unmodified peptides. UV quantification is reliable only when there is no coeluting peptide for both modified and unmodified peptides.

The method described above is not applicable for detecting isomerization because iso-Asp and Asp residues are isobaric (same mass). There are approaches using isotopic labeling or statistical analysis of the intensity ratios of b:y fragment ions from CID fragmentation of the Asp-containing peptides and the corresponding iso-Asp-containing peptides to detect isomerization, however, they are either very laborious or not applicable in every case [34,35]. ECD and its analog ETD can be used to differentiate peptides containing Asp from those containing iso-Asp because they produce unique diagnostic ions, that is, side chain loss (−60 Da) for Asp-containing peptides and (c·+58 Da) and (z – 57 Da) for iso-Asp-containing peptides at the Asp or iso-Asp site [36,37]. The (−60 Da) diagnostic peak sometimes can interfere with the side chain fragment at Arg and Glu residues on low resolution instruments [38], however, the diagnostic peaks for iso-Asp-containing peptides can always be clearly defined. Figure 2 illustrates ETD fragmentation of a peptide (VVSVLTVLHQDWLNGK) and its deamidated forms using an ion trap instrument. All of the z ions, from z_3 to z_{15}, observed in the ETD mass spectra of deamidated peptides have masses that are 1 Da higher than those generated from unmodified peptides because they all contain the deamidation site, and the diagnostic ion (z_3 – 57 Da) at m/z 246 is observed only in the ETD spectrum of deamidated peptides with iso-Asp residue.

S–S formation is a crucial PTM for stabilizing protein structure and function. For example, mis-linkages of S–S can lead to antibody structural isoforms, increase structural heterogeneity and potentially change antigen binding affinities [39]. A common strategy for characterizing S–S linkages involves comparison of a protein non-reduced digest with its corresponding reduced digest using peptide mapping [40]. Peptides that appear only in the non-reduced digest indicate the presence of S–S linked peptides, and peptides that appear only in the reduced digest represent their half-cystinyl peptide constituents. Accurate mass measurement of S–S linked peptides provides initial identification of the S–S linkage, and MS and MS/MS analysis of the reduced peptides provides AA sequence confirmation. The challenge associated with this approach is S–S scrambling that can occur during sample preparation, most likely due to the presence of free sulfhydryl groups that can induce sulfhydryl-disulfide exchange under basic pH conditions [41]. Alkylation of the free sulfhydryls before enzymatic digestion can minimize S–S scrambling [40]. Alternatively, S–S linkage can be analyzed by directly identifying the S–S linked peptides produced from the non-reduced digest by using ETD-based fragmentation, because ETD preferentially breaks apart the

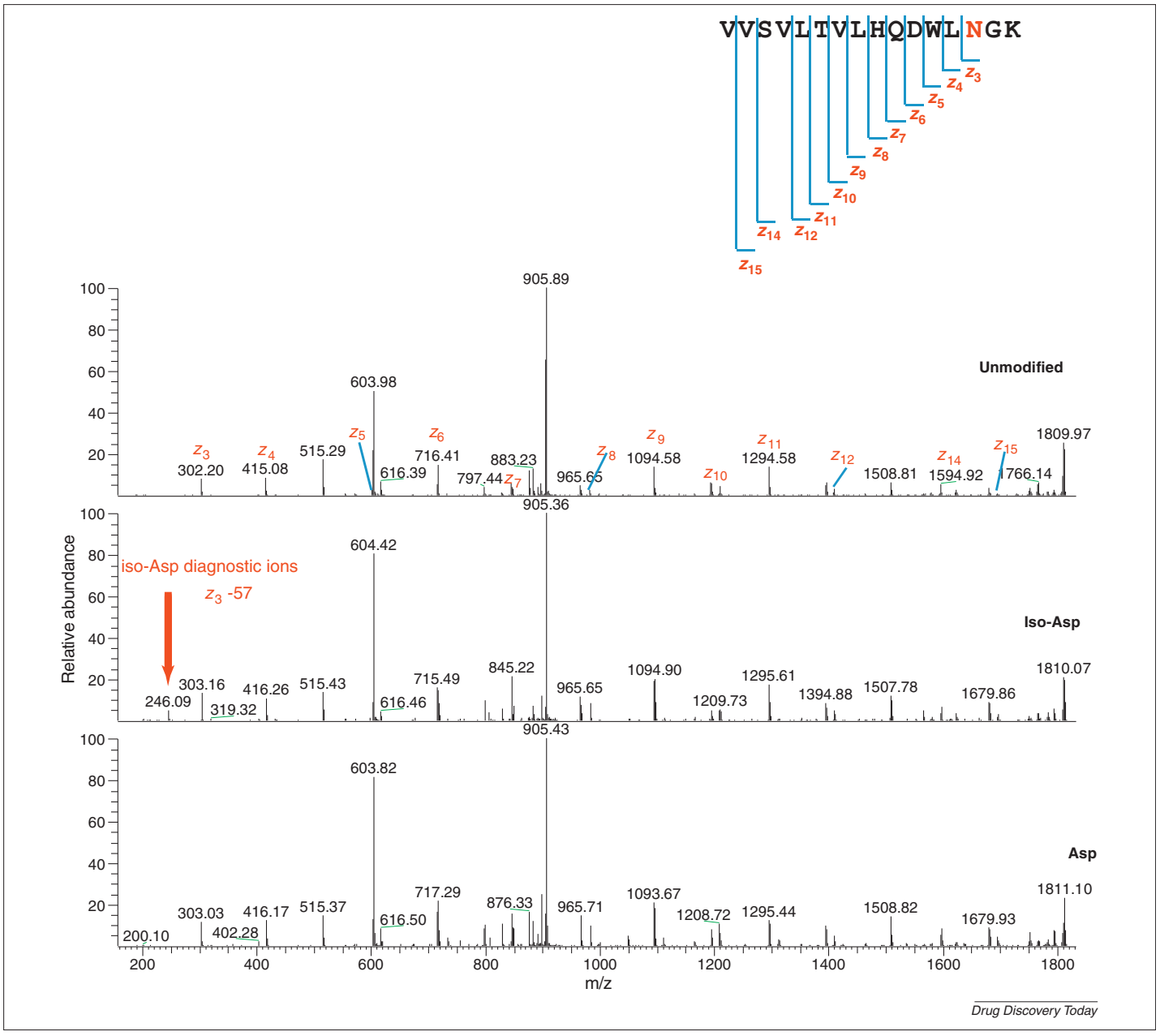

FIGURE 2

ETD mass spectra of an unmodified peptide (VVSVLTVLHQDWLNGK) (top) and its deamidated products with either iso-Asp (middle) or Asp (bottom) residue. Compared with the unmodified peptide, *z* ions from the deamidated peptides that include the deamidation sites, that is, from z_3 to z_{15}, all have mass increases of 1 Da. The ETD mass spectrum of the iso-Asp-containing peptide also has the diagnostic ion (z_3 – 57 Da), differentiating it from the Asp-containing peptide.

S–S and generates dissociated half-cystinyl peptide constituents, which can be identified with further fragmentation such as CID (ETD/CID MS^3) [42,43].

Higher-order structure

Investigation of protein structures and conformational dynamics are invaluable to fully understand how proteins drive and contribute to basic biological and biochemical events. Proteins that are not folded properly are frequently targets for various degradation pathways and are usually prone to aggregation, which could potentially trigger an immune response [44]. Several MS-based techniques are capable of characterizing protein higher-order structures. For example, ESI-MS can differentiate different folding states of the same protein by displaying different charge state distributions [45], and IMMS, with its additional separation based on protein size, shape, and conformation, has been applied to resolve S–S heterogeneity of IgG2 antibodies [46].

More recently, MS is combined with HDX to investigate protein structures in solution at both intact molecular level (global conformation) and peptide level (local conformation) [47], including biopharmaceutical comparability studies and evaluations of protein conformational dynamics upon modifications [48,49]. Compared with other techniques such as X-ray crystallography and nuclear magnetic resonance (NMR) spectroscopy, HDX-MS is more sensitive, involves simpler sample preparation, and can analyze protein mixtures that are more complex. In HDX-MS, the

exchange rates of protein amide hydrogens with deuteriums from deuterated buffer are monitored by MS. Because the HDX rate is dependent on protein exposure to the solvent and on inter-/intramolecular hydrogen bonding, the information obtained from HDX-MS can be correlated to the protein structure. With automated instrumentation becoming commercially available, HDX-MS will likely be regularly used in analytical laboratories for exploring protein higher-order structures in the near future. The primary challenge with this technique is data analysis of HDX measurements at the peptide level, including peak assignments and calculation of average deuterium uptake, which can be complicated by the non-specific enzymatic digestion and shifted and/or overlapped isotopic peak distributions. Robust and highly reproducible chromatographic separation together with HR-MS has been used to facilitate this type of data analysis [50,51].

Epitope mapping

Epitope mapping, the characterization of interactions between protein antigens (i.e. epitopes) and antibodies, is crucial in discovery and development of therapeutic antibodies. It is particularly important in the selection of lead candidates with similar epitopes. Epitope mapping involves precisely characterizing the binding site of an antibody to its corresponding target protein. Approaches such as the 'gold standard' X-ray crystallography, mutagenesis, synthetic antigen peptide screening, and MS-based methods that use limited proteolysis or chemical cross-linking have been reported for mapping epitope binding sites, however, these methods are usually labor intensive [52,53]. A recent study illustrated the use of oxidative labeling method with OH radicals for epitope mapping [54]. This approach involves complicated data analysis and is generally difficult for kinetic studies. HDX-MS has been increasingly adopted in this area, because the peptides from the antigen–antibody complex that are involved in binding would demonstrate protection from HDX (lower HDX rates) compared to the same peptides in the free form of the antigen or the antibody. However, other peptides that are located far from the binding site may also alter HDX rates as a result of allosteric conformational change (induced by binding but not at the binding site), complicating the data analysis. Another challenge with the HDX approach is the need to generate common proteolytic peptides (between the free antibody and/or antigen and the complex) that cover most of the molecular sequence to ensure reliable assessment of the binding site. The use of multiple enzymes (e.g. using pepsin and protease type XIII sequentially) has been shown in a recent study to achieve over 90% sequence coverage [55]. In that study, the epitope binding sites of two complexes identified by HDX-MS were found to be consistent with the sites identified by mutagenesis, molecular modeling, electron microscopy and synthetic antigen peptide screening. The peptides involved in binding also exhibited more significant changes in HDX rates (more protection) compared with the peptides involved in allosteric conformational change (less protection) [55]. Recent incorporation of ECD/ETD to HDX-MS for fragmenting labeled peptides enabled measurements of HDX rates at single AA residue level [56,57] because ECD/ETD possesses a low degree of intramolecular migration of peptide amide hydrogens.

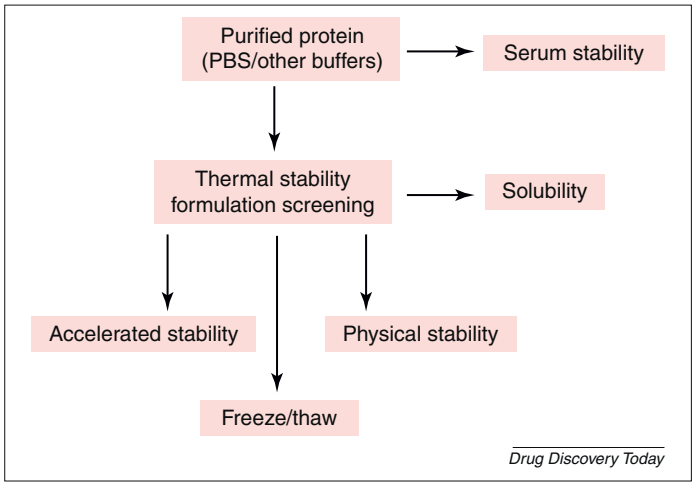

FIGURE 3

Workflows for biologics stability studies in biologics drug discovery. Starting with the purified protein, its thermal stability is evaluated and initial formulation screening is performed through accelerated stability study, freeze and/or thaw study and physical stability study. In addition, protein solubility and serum stability are also evaluated. *Abbreviation*: PBS: phosphate buffered saline.

MS in stability studies of biologics

During drug discovery, the susceptibility of protein drug candidates to degradation and modifications both *in vitro* (during production and storage) and *in vivo* (after administration in patients) needs to be assessed to fully evaluate the developability of candidate molecules from the perspective of biophysical properties. Figure 3 outlines various stability studies for candidate molecules in biologics drug discovery. MS-based methods are often used to characterize degradation products and/or fragments and chemical modifications for candidate molecules under stressed conditions.

In vitro *stability*

Fragmentation is one of the major degradation pathways of biologics in liquid formulation. Under conditions such as elevated temperatures, exposure to chemicals, light, or a combination of these, protein fragmentation often occurs at the C-terminal side of an acidic residue, or near a Ser residue, and the predominant cleavage site lies between Asp and Pro [58]. The resulting fragments usually can be resolved by ultra-high pressure liquid chromatography (UHPLC) separation and the fragmentation sites can be assigned by accurate mass measurements. The relative abundance of fragmentation and/or degradation products can be calculated from either the UV chromatographic peak abundance or MS peak abundance.

Besides fragmentation, biologics are also susceptible to modifications, which can be assessed by peptide mapping. The extent of modifications, including the 'hot spots' for oxidation, deamidation and isomerization, are calculated from either UV or MS signals of the modified and unmodified peptides. Figure 4 shows an example on relative quantification of isomerization in the peptide (FNWYVDGVEVHNAK) by extract ion chromatogram (EIC). Two peaks were obtained by extracting MS signals that match the calculated mass of the peptide, one with Asp residue and the other with iso-Asp residue, and their differentiation was achieved by ETD-MS. By integrating peak areas of these two peaks, the relative abundance of isomerization can be calculated.

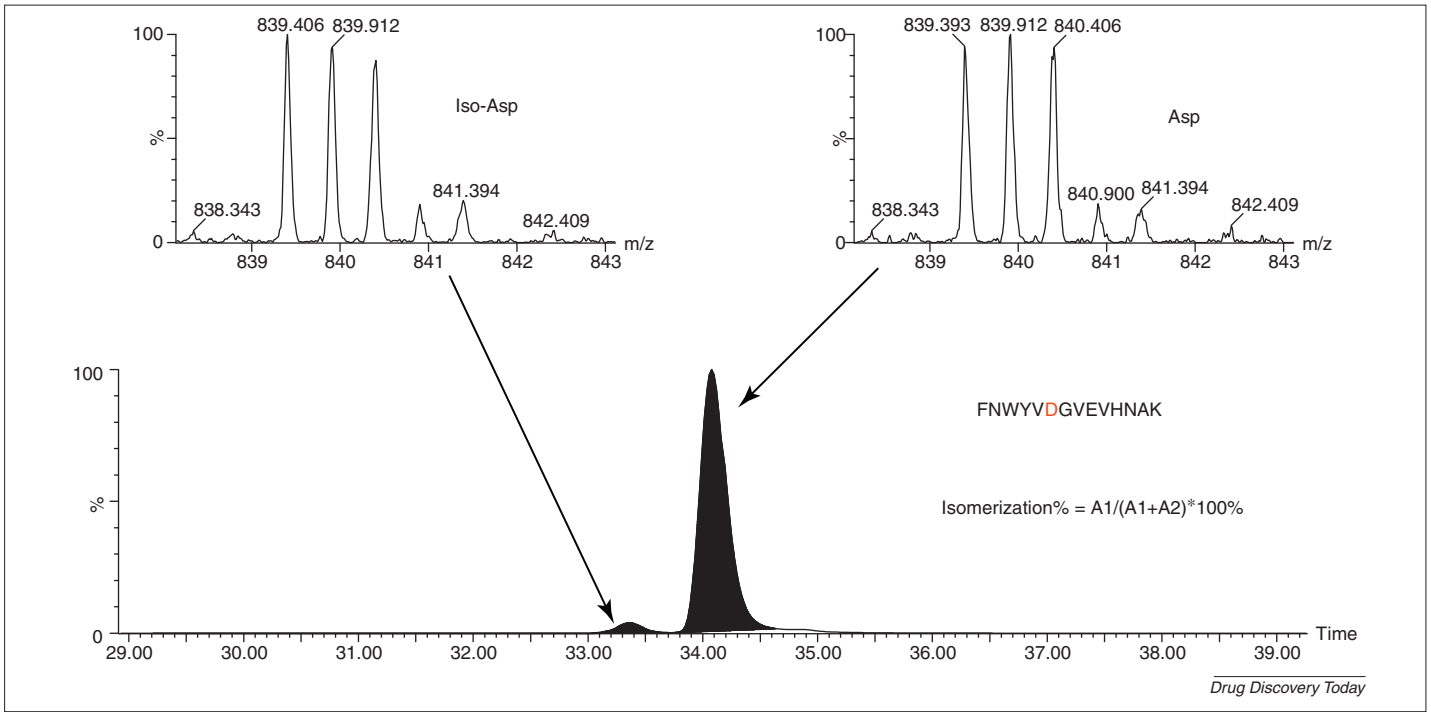

FIGURE 4

Relative quantification of isomerization by extract ion chromatogram. Mass spectrometry signals were extracted based on monoisotopic peak *m/z* values. Peak areas were integrated for calculation of isomerization percentage.

In vivo *stability*

In addition to degradation and/or modifications that occur during production and storage, *in vivo* biotransformation (i.e. proteolysis, chemical modifications) of biologics can also occur after administration to patients. For example, *in vivo* deamidation of human antibodies in the Fc region has been observed as a natural process under physiological conditions [59]. In cases where the *in vivo* modifications can impact drug efficacy, the bioavailability and serum lifetime of the drug needs to be predicted from both the PK data and the *in vivo* degradation kinetics. The *in vivo* stability data provide important information about the biophysical properties of biologics in the intended therapeutic environment.

The *in vivo* stability of biologics can be characterized using similar MS-based methods as those used for *in vitro* stability characterization after the protein is extracted and purified from the body fluids, because the sensitivity and specificity of the MS-based techniques are usually limited by the low abundance of the protein and the suppression effects of biological matrix components. One effective approach for *in vivo* analysis is to use immunoaffinity capture for purification and enrichment of the protein of interest before MS analysis to reduce sample complexity. Nowadays, with highly specific antibodies available for a wide range of biologics, immunoaffinity capture is becoming a practical way of retrieving proteins from a biological matrix and is amenable to downstream MS-based analysis.

One of the most convenient ways to perform immunoaffinity capture is to use antibody-coated magnetic beads as the affinity probe [60,61]. For example, by immobilizing magnetic beads with protein G, a specific antibody can be coupled to the beads through interaction with protein G, and then the antigen can be captured by the antibody specifically through antigen–antibody interactions. All the other non-binding components are washed away and the protein can be eluted for further MS analysis.

Immunoaffinity chromatography, which replaces the magnetic beads with an immunoaffinity column [62], can also be used to capture the protein of interest. By packing the columns with a specific antibody-coated resin, the protein of interest can be selectively captured. A scaled-down version of the immunoaffinity column is the pipette tip that contains activated silica-based resin with covalently bound antibodies [63]. Together with an automated workstation that is equipped with multiple channels, high-throughput sample preparation can be achieved.

Concluding remarks

MS is one of the most highly utilized analytical techniques in biologics discovery. Structural features of proteins, including MW, AA sequence, S–S linkages, glycosylation profile, and many other PTMs in addition to higher-order structures can be characterized using MS-based techniques. Continuous technical developments in MS instrumentation will provide newer capabilities with higher sensitivity, resolution, and mass accuracy, further improving the performance of MS in protein characterization. The use of ETD-MS and HDX-MS in biologics research will continue to evolve with increased applications. The unique role of MS in biologics stability studies will be further expanded to provide comprehensive characterization of candidate molecules during both *in vitro* and *in vivo* studies in the process of optimizing and selecting proteins with enhanced biophysical properties.

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