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Characterization of protein therapeutics by mass spectrometry: recent developments and future directions

Guodong Chen, Bethanne M. Warrack, Angela K. Goodenough, Hui Wei, David B. Wang-Iverson and Adrienne A. Tymiak

Bioanalytical and Discovery Analytical Sciences, Research and Development, Bristol-Myers Squibb Company, P.O. Box 4000, Princeton, NJ 08543, USA

Mass spectrometry (MS) has become a powerful technology in the discovery and development of protein therapeutics in the biopharmaceutical industry. This review article describes recent developments and future trends in the characterization of protein therapeutics using MS. We discuss top-down MS for the characterization of protein modifications, hydrogen/deuterium exchange MS and ion mobility MS methods for higher order protein structure studies. Quantitative analysis of protein therapeutics (*in vivo*) by MS as an orthogonal approach to immunoassay for pharmacokinetics studies will also be illustrated.

Introduction

Since the introduction of the first recombinant-DNA-derived protein insulin in the 1980s and the launch of interferons (IFNs) and interleukins in the 1990s, the protein therapeutics market has shown a healthy double-digit growth [1]. Currently, there are more than 200 companies that are actively involved in various aspects of protein therapeutics development. It is estimated that the global protein therapeutics market will reach a value of close to $90 billion this year, a big jump from $25 billion in 2001 [1]. More than 300 antibodies and 400 other recombinant proteins entered clinical trials between 1980 and 2004 [2]. The overall US approval success rate for protein therapeutics was approximately 35% between 1990 and 1997 [2]. This is in contrast to traditional small-molecule pharmaceuticals, for which the overall success rate was approximately 11% for the top ten biggest drug companies between 1991 and 2000 [3]. Major causes of small-molecule drug attrition were lack of efficacy and safety. By contrast, the protein therapeutics market is driven by innovative therapies with well-understood mechanisms. Their high efficacy, safety and ability to treat life-threatening diseases such as cancer, inflammation and genetic disorders have revolutionized modern medicine. For example, human IFN-α-2b was the first recombinant protein to be approved and marketed as a protein drug for the treatment of hairy cell leukemia in 1987. Subsequently, it has been used in the treatment of hepatitis B and C viruses by interfering with infectious agents and preventing the spread of diseased cells [4].

The growth of protein therapeutics has largely been due to advances in recombinant DNA technology that have provided the means to produce protein therapeutics in the discovery of novel protein drugs. Compared with small-molecule pharmaceuticals, protein therapeutics have distinct characteristics, as shown in Table 1. Because of their unique production processes and structural features, there are many challenges in characterizing protein therapeutics [5–7]. These protein drugs often contain microheterogeneity associated with modifications (such as glycosylation, deamidation, oxidation and disulfide bond formation) and free thiols as a result of expressions, purifications and storage. Other challenges include protein conformational changes upon modifications, noncovalent interactions between protein drugs and receptor proteins, and protein aggregation caused by misfolding. The presence of biological matrices and complex serum/plasma proteomes for *in vivo* samples often requires careful sample preparation before subsequent analysis. In addition, arrays of protein therapeutics platforms such as cytokines, enzymes, peptides, monoclonal antibodies and fragments contribute to the complexity in protein sample analysis.

As one of the most highly utilized analytical techniques in pharmaceutical research and development, mass spectrometry (MS) has been widely used in the characterization of protein therapeutics because of its analytical sensitivity, selectivity and specificity [8–15]. Advances in ionization methods, including

Corresponding author:. Chen, G. (guodong.chen@bms.com)

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TABLE 1

Comparison of small molecules and protein therapeutics

Small molecules	Protein therapeutics
Low molecular weight	High molecular weight
Chemically synthesized	Generated in living cells
Well-defined physicochemical properties	Complex biophysical properties (3D structure, modifications)
Single chemical entity	Often heterogeneous product (process dependent)
ADME studies	Limited metabolism (catabolized to endogenous amino acid)/proteolytic degradation
Often no immunogenic response	Immunogenicity

electrospray ionization (ESI) [16] and matrix-assisted laser desorption/ionization (MALDI) [17], have expanded the role of MS in protein characterization. The development of novel MS instrumentation and ion activation methods contributed significantly to the protein characterization capability of MS with enhanced accuracy, throughput and detection limit at or below femtomoles of materials. It is now possible to analyze high molecular weight proteins with a mass range over 500 kDa [18,19].

This review article is not intended to cover all aspects of protein therapeutics characterization by MS. The focus of this article is on recent developments and future trends in the characterization of protein therapeutics using MS, including top-down MS for the characterization of protein posttranslational modifications (PTMs) [20], hydrogen/deuterium (H/D) exchange MS [21] and ion mobility MS (IMS) [22–24] methods for higher order protein structure studies. Quantitative analysis of protein therapeutics (*in vivo*) by MS as an orthogonal methodology to immunoassay for pharmacokinetic (PK) studies is also discussed [25,26].

Top-down MS

The current gold standard in protein analysis is the 'bottom-up' approach because of its high-throughput format, well-developed MS instrumentation and software [27]. This method relies on enzymatic digestion of proteins and subsequent analysis of digested peptides by MS, as shown in Fig. 1. Typically, peptide mass fingerprinting (PMF) experiments are carried out on digested peptides by liquid chromatography (LC)/MS or MALDI/MS. The PMF data can be subjected to database search for protein identification. Alternatively, tandem MS (MS/MS) experiments by collision-induced dissociation (CID) can be carried out on digested peptides to obtain fragment ions for database search (sequence tag) [28]. For protein PTM analysis, the combination of PMF and MS/MS experiments is a good approach to structural elucidation of protein PTMs. The bottom-up approach has several drawbacks, however, including variation in sequence coverage (5–70%) and cleavage of labile PTMs caused by CID experiments at the peptide level.

ECD/ETD

As an emerging MS method for protein analysis, top-down MS is highly attractive [29]. It involves direct analysis of intact proteins using high-resolution MS and dissociation of ionized proteins by MS/MS experiments (Fig. 1). Potentially, 100% sequence coverage is possible and labile PTMs probably remain intact during top-down experiments. Newly developed activation methods in top-down

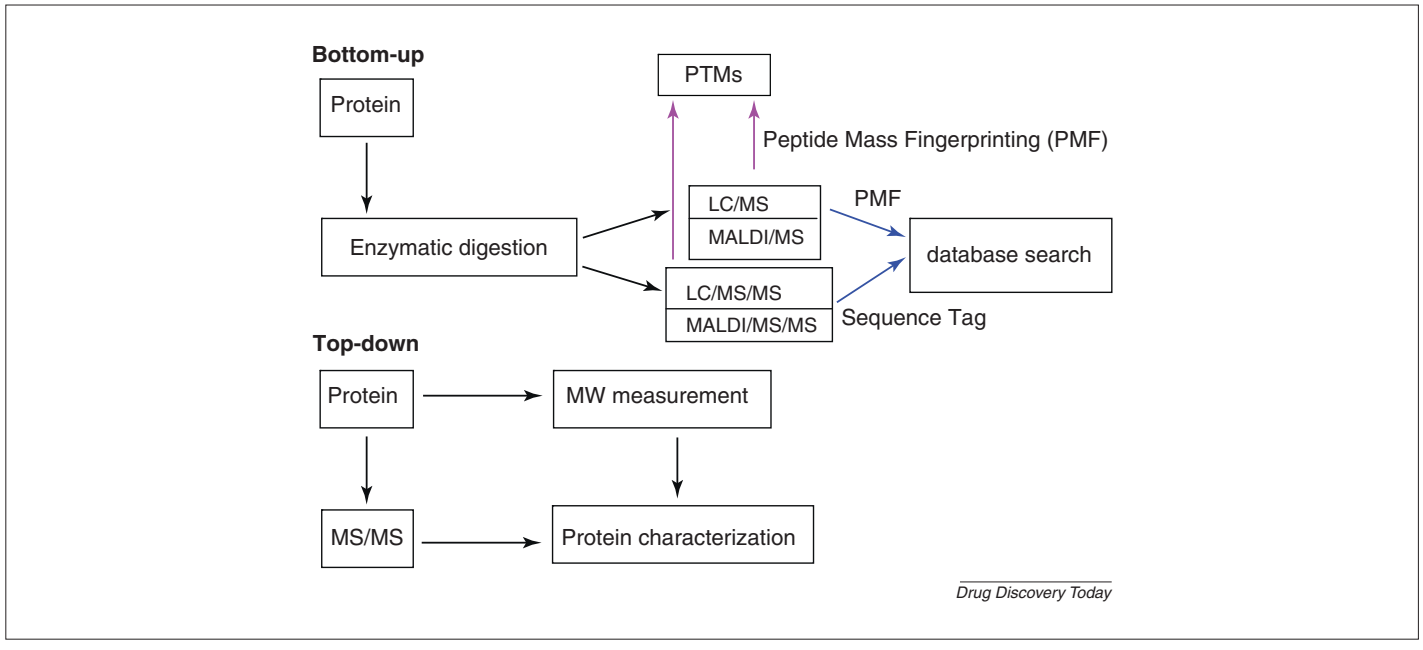

FIGURE 1

General approaches to protein characterization.

experiments include electron-capture dissociation (ECD) [30] and electron-transfer dissociation (ETD) [31], resulting in cleavages of N–C(R) bonds and the generation of c and z fragment ions. Several studies have demonstrated benefits of this technique in protein characterization.

As an illustration, we will focus on deamidation/isomerization studies using ECD/ETD. Deamidation is a chemical degradation pathway that mainly involves an asparagine residue being converted into aspartic acid (Asp) and/or isoaspartic acid (isoAsp). In addition, some Asp residues might undergo isomerization to form isoAsp residues. A mechanism for deamidation of asparagine residue and isomerization of Asp residue to isoAsp residue is displayed in Fig. 2. All of these modifications can result in the loss of activity of the protein therapeutic or even trigger immunogenicity. The formation of an isoAsp residue might have more adverse effects because the insertion of the methylene group into the protein backbone can directly impact protein stability and tertiary structure [32,33]. Because of the nature of isomers, structural differentiation of Asp and isoAsp products by MS is analytically challenging. Direct MS analysis is unable to resolve these two isomers (because they have the same molecular weight). Conventional CID experiments are not sufficient to differentiate these two isomers.

A recently introduced ECD method generates a more uniform cleavage pattern with more useful side-chain cleavages as a result of gas-phase reaction of low-energy electrons with multiply charged peptide/protein ions. Because isoAsp residue has a different structure from Asp, ECD mass spectra of Asp and isoAsp products could be substantially different. ECD experiments performed on Fourier-transform ion cyclotron resonance MS using Asp-containing peptides and isoAsp-containing peptides from synthetic peptides and proteins established the differentiation

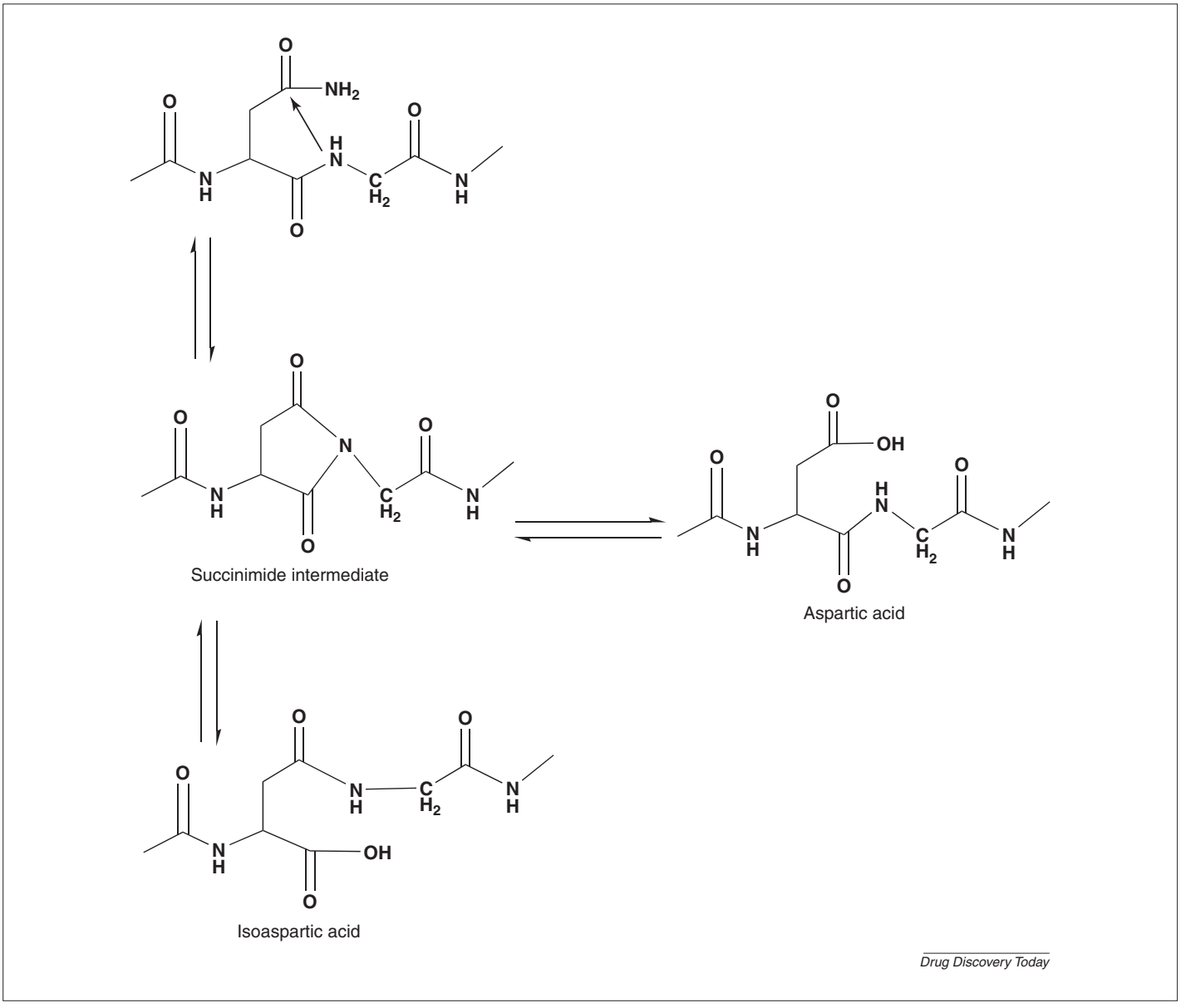

FIGURE 2

Deamidation of Asn residue and isomerization of Asp residue to isoAsp residue.

ability for these two isomers using unique diagnostic fragment ions [32,34–36]; the backbone cleavages at $c_n + 57$ (or $c_n + 58$) and $z_m - 57$ (m = peptide length $- n$) for the isoAsp form and a side-chain cleavage at M-60 Da for the Asp form. Importantly, the backbone cleavage ions enabled the detection and localization of the isoAsp form. The side-chain cleavage ion determined the presence of the Asp form (not the position of the Asp residue). Similarly, the ETD method employs transfer of an electron to multiply charged peptide/protein ions using singly charged anions, generating odd-electron fragment ions via radical-based rearrangements. ETD experiments carried out on an ion trap instrument enabled the differentiation of isoAsp and Asp residues using backbone cleavage ions ($c_n + 57$ and $z_m - 57$), although the low resolution of the ion trap instrument made detection of the Asp residue's diagnostic peak difficult because of interference of side-chain fragment ions from arginine residues [37]. Recent ETD experiments with supplemental activation of the doubly charged deamidated tryptic digested peptide ions further increased the abundance of backbone fragment ions ($c_n + 57$, $z_m - 57$) and sequence coverage from ~50% to ~85% [38]. Clearly, top-down methods (ECD/ETD) at the peptide level are applicable to the deamidation/isomerization studies as shown in this case. Further improvements in instrumentation and methodologies could provide robust characterization on intact proteins by top-down MS.

A crucial task in the characterization of protein therapeutics is disulfide bond mapping to ensure the proper formation of disulfide bonds in proteins because it is related to protein stability and function. A typical method for disulfide bond mapping includes the enzymatic digestion of nonreduced proteins by pepsin at pH 2–4 [39]; however, the nonspecific cleavages from pepsin can increase the complexity of the digested peptides. Other enzymes such as trypsin or Asp-N can provide specific cleavages, although the use of these enzymes under basic pH might lead to disulfide bond scrambling [40]. The conventional CID method is inefficient in dissociating S–S bonds. Recently, the ETD approach has been implemented to overcome this limitation, demonstrating the usefulness of S–S bond dissociation by ETD in disulfide bond mapping in recombinant protein therapeutics [41].

ISD

Another type of top-down MS method is in-source decay (ISD) in a MALDI time-of-flight (TOF) mass spectrometer [42]. ISD occurs at the nanosecond time scale inside the MALDI source region, after the laser shot. ISD MALDI mass spectra contain largely c and z ions [N–C(R) bond cleavage], probably arising from hydrogen radical transfer from matrix to analyte and inducing fragmentation at the transfer position. Crucial factors for the formation of ISD fragment ions include increased laser intensity, delayed extraction and the choice of matrix. The ISD fragmentation exhibits uniform cleavages across protein sequence with no preference to labile bonds. Therefore, labile PTMs probably remain intact during the fragmentation process, and simple fragment ion spectra from large proteins can be generated by ISD MALDI-MS. One important application of this technique in protein therapeutics research is the characterization of conjugated proteins, including the covalent attachment of nontoxic and nonantigenic polymers such as polyethylene glycol (PEG) to active proteins (PEGylation). The purpose of PEGylation is to prolong the half-life of protein therapeutics in the body for effective potency [43]. Analytical characterization of PEGylation including the determination of the PEGylation site is challenging. The traditional approach involves bottom-up methods using enzymatic digestion and PMFs [44,45]: it is a lengthy sample preparation process that could lead to potential sample loss and reduced sensitivity. As expected, the complexity of analysis rises enormously with the increasing size of protein therapeutics.

A recent report described the use of ISD MALDI-MS to determine the PEGylation site [46]. In this study, a model peptide with 31 amino acids was conjugated with 20 kDa linear PEG at the side chain of lysine[19]. ISD MALDI-MS experiments were performed on the free peptide and PEGylated peptide using 2,5-dihydroxybenzoic acid as the matrix. The ISD MALDI-MS spectrum of the free peptide was observed with abundant c, y and z fragment ions. In contrast to the free peptide, fragment ions in the PEGylated peptide were truncated at the residue (lysine[19]) where PEG was covalently attached. This observation was in line with the expectation that cyclization and large modifications (such as PEGylation) on a protein can introduce a gap or a truncation in sequencing analysis. This study demonstrated the potential analytical utility of the ISD MALDI-MS technique for the characterization of conjugated protein therapeutics with the simplicity of experiments and data interpretation, as well as improved throughput.

H/D exchange MS and IMS

One of the major challenges in characterizing protein therapeutics is to define their higher order structures and the conformational dynamics that often dictate their biological activity, stability and safety. Classical biophysical methods (circular dichroism, fluorescence, differential scanning calorimetry, isothermal titration calorimetry and analytical ultracentrifugation) often lack detailed conformational characterization. Traditional NMR approaches are limited to the size of proteins being studied and require high sample concentrations that can lead to distorted protein conformation and possible aggregation. Alternatively, the X-ray crystallography method is capable of providing detailed structural information; however, it is dependent upon the formation of appropriate crystals, and the obtained solid-state structure might not reflect the solution structure with conformational dynamics. Two emerging MS techniques including H/D exchange MS and IMS have shown great potential to address this challenge in the characterization of protein therapeutics, as reported in the literature [21–24].

H/D exchange MS

The H/D exchange MS method probes protein structures by monitoring the rate and extent of deuterium exchange with backbone amide hydrogen using MS. The level of deuterium exchange depends on the solvent accessibility of backbone amide hydrogen atoms and the conformation of the protein [47]. As a sensitive analytical tool, MS can precisely measure the increase in the mass of protein upon deuterium exchange. It has been reported that the level of deuterium exchange can vary as much as 10^8-fold as a result of protein structure [48]. Two types of H/D exchange experiments can be carried out to study protein conformation. A global

view of protein conformation can be obtained by measuring the difference between the molecular weight of the exchanged protein and that of the native protein at different time points of exchange. The exchange process is normally done at room temperature by adding 10–20-fold excess of buffered D_2O at pH 7.0 to native protein solution. After incubation for a predefined time, adding a buffer at pH 2.5 and dropping the temperature to 0°C quenches the reaction, after which the reaction solution can be analyzed by LC/MS. If localized exchange information is desirable, this process can be combined with enzymatic digestion (typically using pepsin) to extract deuterium exchange information down to the peptide level (three to ten amino acid residues). Several studies have illustrated the utility of H/D exchange MS for probing conformational dynamics of protein therapeutics and potentially for studies of protein therapeutics/receptor protein interactions [49,50].

In one study, the H/D exchange MS method was applied to the characterization of IFN-β1a upon alkylation of the only free cysteine residue in IFN [49]. IFN belongs to a family of the type I IFN with broad biological activity and is widely prescribed for the treatment of multiple sclerosis. The alkylation of cysteine residue in IFN reduces IFN antiviral activity significantly, probably because of changes in protein conformation. However, classical biophysical methods did not give conclusive results. The H/D exchange MS approach yielded unequivocal evidence that higher order structure was affected and native conformation of IFN was changed by the alkylation of the free cysteine, a single chemical modification. In another study, the H/D exchange MS technique was used to evaluate global conformation and local conformational dynamics of a recombinant monoclonal antibody, IgG1 [50]. Deuterium exchange into the intact and glycosylated IgG1 indicated that the whole molecule was highly stable with a folded structure. Upon pepsin digestion and analysis, specific regions of IgG1 were localized in terms of deuterium exchange levels. Comparative H/D exchange MS experiments on glycosylated and deglycosylated IgG1 provided clues to changes of protein conformation upon deglycosylation. Two regions of IgG1 (residues 236–253 and 292–308) were found to have altered deuterium exchange behaviors, consistent with previous observations on the role of glycosylation in the interaction of IgG1 with the Fc receptor.

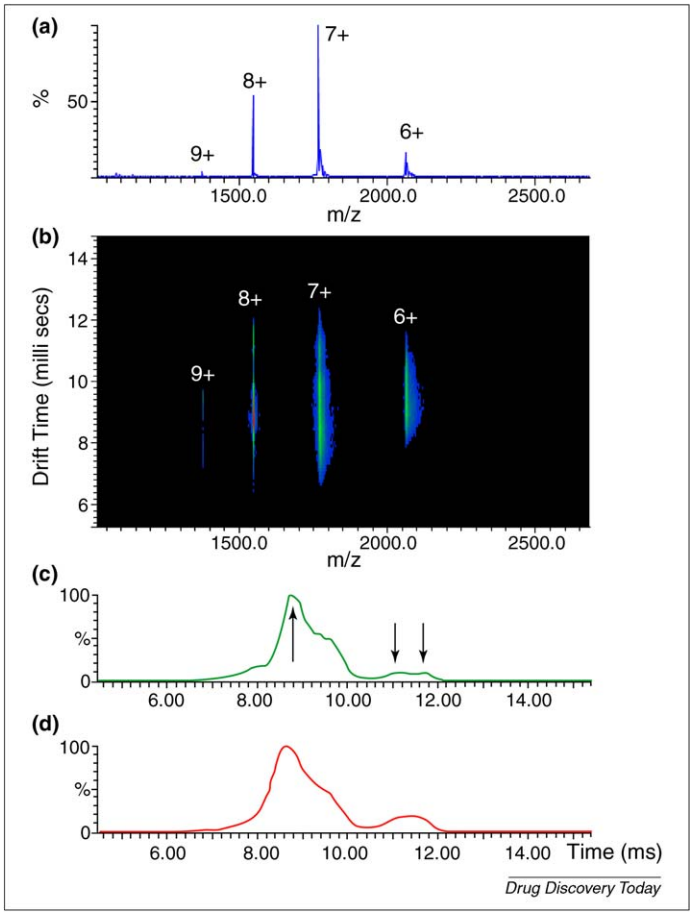

FIGURE 3

(a) Summed ESI-TOF-MS spectrum of cytochrome *c* in 10 mM ammonium acetate solution (pH 6.6) with IMS. **(b)** Driftscope displays *m/z* versus drift time for the analysis of cytochrome *c* (pH 6.6). (c) Drift-time distribution of 8+ charge state of cytochrome *c* (pH 6.6). (d) Drift-time distribution of 8+ charge state of cytochrome *c* (pH 3.3).

IMS

Another new MS technique that has received increasing attention is IMS [22–24]. It is based on gas-phase separation of protein ions with different sizes and shapes. Typically, ESI-friendly solvents are used for the generation of protein ions in IMS experiments. When a protein with different conformers (tightly folded versus less folded) travels through IMS, tightly folded conformers with smaller cross-sections move faster and can be separated from less-folded conformers of the same protein. For example, cytochrome *c* was analyzed using ESI-TOF-IMS to illustrate the utility of IMS. As shown in Fig. 3a, the ESI-TOF mass spectrum displays four major peaks with the highest peak being charge state 7+. Several minor, low-intensity peaks are also observed. When these ion species are subjected to IMS analysis, the separation of different conformers is evident, as exhibited in the plot of *m/z* versus drift time (Fig. 3b). Ion species with the same charge state travel at different drift times through IMS, indicating the existence of mixed conformers in cytochrome *c*. Furthermore, different conformers from the same charge state can be extracted from IMS data. Figure 3c plots ion mobility time versus intensity for 8+ ions with three distinct signals at drift times of 8.7, 11.3 and 11.8 ms. The 8+ ions have a high percentage of the folded conformer (shorter drift time) in comparison with the less-folded conformers (longer drift time). When cytochrome *c* was treated under denaturing conditions (pH 3.3), a higher percentage of less-folded conformers was obtained (Fig. 3d). The IMS data suggest that acidic cytochrome *c* contains more unfolded species, consistent with reports in the literature [51]. Potential benefits from IMS experiments include quantitative measurements of folding status of proteins and the assessment of protein thermal stability by monitoring mobility changes. It is important to note that IMS measures gas-phase intrinsic biophysical property of protein in the absence of solvent. Its correlation to solution structure is protein dependent. Recent studies of measurements of collision cross-sections of several protein therapeutics (human insulin, IFN-α-2a, G-CSF and human growth hormone) using IMS illustrated the potential utility of IMS as a tool to evaluate tertiary structures of protein therapeutics produced by different production/formulation processes [52]. Further

improvement of IMS performance should enable more biophysical characterization of protein therapeutics.

Quantitative analysis of protein therapeutics by MS

An important task in the discovery and development of protein therapeutics is to understand their PK behaviors, as well as the PK/pharmacodynamics relationship [25]. Currently, quantitative measurements of protein therapeutics in biological matrices are mainly accomplished using immunoassays such as enzyme-linked immunosorbent assay (ELISA). These methods have significant advantages, including high sensitivity, robustness, high throughput and the use of less sophisticated instrumentation; however, there are considerable limitations inherent to these methods. Immunoassays often require the lengthy and costly development of specific reagents (antigen/antibody) that might not be easily obtainable [26], and dynamic range and linearity might need to be addressed during method development. Immunoassays measure biochemical interactions that are subjected to interferences of biological matrices, antidrug antibodies and structural modifications of protein therapeutics. By contrast, nonimmunocapture-based MS methods directly measure protein therapeutics in biological matrices and have been shown to be viable alternatives to immunoassays [53–56].

Typical MS-based assays rely on the use of an internal standard (peptide or protein) as a reference marker for quantification. Experiments often involve protein sample preparation for the extraction/isolation of target proteins, enzymatic digestions of proteins including internal standards (peptide/protein) and LC/MS/MS analysis using multiple reaction monitoring on selected transitions of precursor ions to product ions for digested peptides. The key benefits of MS assays include rapid assay development (no special reagents required) and excellent precision, accuracy, specificity and dynamic range ($>10^3$). The presence of endogenous proteins/peptides and antidrug antibodies will not affect MS assay data. One challenge in MS assays is protein extraction/isolation from biological matrices. Various approaches have been developed to reduce the complexity of plasma/serum samples, including the depletion of high-abundance plasma proteins and the enrichment of target proteins by immunocapture. Each method has its own downsides and might not be applicable to the general analysis of protein therapeutics. In one study on the quantification of PEGylated IFN-α-2a in human serum samples, the use of monolithic C18 solid-phase extraction (SPE) was found to be effective in enriching target proteins before trypsin digestion [54]. This enrichment step not only removes more than 80% of the serum proteins for the improvement of assay sensitivity but also removes salts at high concentrations to ensure sufficient enzyme activity. The assay achieved a limit of quantification of 3.6 ng/mL with precision and accuracy well within ±20%. This approach might have general applications in the analysis of other low molecular weight proteins. In another study measuring a therapeutic monoclonal antibody in rat serum samples, a 2D-SPE cleanup method was developed for the cleanup of digested peptide mixtures to improve the assay sensitivity [55]. The procedures included spiking of the internal standard protein in serum samples, trypsin digestion, 2D-SPE cleanups using reverse-phase SPE and cation exchange SPE, and LC/MS/MS analysis. This LC/MS/MS approach was applied to rat PK studies of the therapeutic monoclonal antibody with a limit of quantification of 0.5 μg/mL. The MS assay had improved accuracy and precision in comparison with ELISA data. The results from the MS assay and ELISA were in good agreement with very similar PK parameters between these two assays, indicating that an MS-based assay is a simple and feasible approach to the *in vivo* analysis of therapeutic monoclonal antibodies.

Future directions

As illustrated in this article, MS has increasingly important roles in the characterization of protein therapeutics, including qualitative and quantitative analyses. Unique analytical challenges encountered in the discovery and development of protein therapeutics can be addressed effectively using MS techniques. Advances in the MS field will provide new capabilities for the analysis of protein therapeutics.

Built upon the initial success of ECD/ETD/ISD, top-down MS will continue to evolve into mature methodologies for the precise characterization of PTMs, including the sites of modifications and the nature and occupancy of modifications. Further instrumentation development and software integration are the keys to making top-down MS more accessible to scientists as a valuable tool in problem solving. Increasing demands for characterizing higher order structures of protein therapeutics will inevitably lead to advances in H/D exchange MS and IMS for probing protein conformational dynamics under various conditions, as well as studying therapeutic protein/receptor protein interactions for a better understanding of mechanisms of actions. Integrated MS systems and data analysis software will facilitate the quick adoption and wide application of H/D exchange MS and IMS in the biopharmaceutical industry. In addition to qualitative aspects, quantitative measurements by MS assay start to emerge as an orthogonal approach to traditional immunoassays. Initial studies have demonstrated the reliability, robustness and sensitivity of MS assays for *in vivo* analysis of protein therapeutics in biological matrices. It is expected that further refinement of MS assays will lead to broad acceptance of this approach for discovery PK studies and clinical sample analysis of protein therapeutics.

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