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Advances and challenges in analytical characterization of biotechnology products: mass spectrometry-based approaches to study properties and behavior of protein therapeutics

Igor A. Kaltashov, Cedric E. Bobst, Rinat R. Abzalimov, Guanbo Wang, Burcu Baykal, and Shunhai Wang

Department of Chemistry, University of Massachusetts-Amherst, Amherst, MA

Abstract

Biopharmaceuticals are a unique class of medicines due to their extreme structural complexity. The structure of these therapeutic proteins is critically important for their efficacy and safety, and the ability to characterize it at various levels (from sequence to conformation) is critical not only at the quality control stage, but also throughout the discovery and design stages. Biological mass spectrometry (MS) offers a variety of approaches to study structure and behavior of complex protein drugs and has already become a default tool for characterizing the covalent structure of protein therapeutics, including sequence and post-translational modifications. Recently, MS-based methods have also begun enjoying a dramatic growth in popularity as a means to provide information on higher order structure and dynamics of biotechnology products. In particular, hydrogen/deuterium exchange MS and charge state distribution analysis of protein ions in electrospray ionization (ESI)MS offer a convenient way to assess the integrity of protein conformation. Native ESI MS also allows the interactions of protein drugs with their therapeutic targets and other physiological partners to be monitored using simple model systems. MS-based methods are also applied to study pharmacokinetics of biopharmaceutical products, where they begin to rival traditional immunoassays. MS already provides valuable support to all stages of development of biopharmaceuticals, from discovery to post-approval monitoring, and its impact on the field of biopharmaceutical analysis will undoubtedly continue to grow.

1. Introduction

Biopharmaceuticals (the majority of which are protein drugs) constitute a very distinct class of medicines due to their complex nature. Unlike traditional small molecule medicines, whose masses typically do not exceed a few hundred Daltons, biopharmaceutical products are very large: the smallest biopharmaceutical (insulin) exceeds 5 kDa, while a significant number of therapeutic proteins (such as a monoclonal antibody, mAb) exceed 100 kDa. As a result, the synthetic route of production, which provides a very high level of structural fidelity to small molecule medicines is typically unavailable for protein drugs. The biotechnological mode of manufacturing protein drugs inevitably introduces at least some heterogeneity of the end-product, particularly at the level of post-translational modifications (PTM), which are very sensitive to the choice of expression systems. In addition to inherent heterogeneity, the large size of biopharmaceutical products inevitably results in increased frequency of post-production covalent modifications (the so-called non-enzymatic PTMs) that can occur as a result of storage and/or stress.

While some of the structural changes occurring in protein therapeutics as a result of stress or manufacturing process change are fairly benign, a few can lead to grave consequences, which may go well beyond loss of the drug's efficacy by eliciting an immunogenic response and raising safety concerns. This is usually a result of changes in the protein higher order structure triggered by certain key PTMs, although in most cases it is very difficult to predict if a specific PTM will affect the protein conformation. Furthermore, conformational changes may occur under certain conditions even in the absence of any PTMs (*e.g.*, due to exposure to chaotropes, air-water interfaces, lyophilization, heat stress, *etc.*). The most common conformational changes in protein drugs are those related to partial loss or destabilization of the protein higher order structure (*i.e.*, permanent or transient partial unfolding), which expose protein segments that are normally sequestered in the protein interior. Apart from complete or partial loss of function (*e.g.*, via impaired ability to interact with therapeutic targets), unfolding may also trigger an immune response by exposing protein segments recognized as foreign by the immune system, or trigger aggregation by exposing hydrophobic residues to the solvent (Figure 1).

The extreme importance of protein structure for efficacy and safety of biopharmaceutical products places a premium on the ability to characterize it at different levels, ranging from primary structure (*i.e.*, amino acid sequence and PTM) to conformation (secondary and tertiary structure) to correct assembly of multi-unit proteins (quaternary structure). Apart from the mundane tasks of quality control, structural characterization of biopharmaceutical products is also a key element throughout the drug discovery and design stages, as it enables optimization and enhancement of therapeutic properties to be carried out in a rational fashion. Finally, many analytical tools that are originally designed for structure characterization also prove useful for obtaining accurate and reliable pharmacokinetic profiles of therapeutic proteins.

2. Analytical methods for characterizing structure of protein therapeutics

The dramatic progress of experimental biophysics and biochemistry in the last several decades produced a vast array of bioanalytical methods capable of probing various aspects of biopolymer structure. However, not all of these technologies are readily applicable to characterization of biopharmaceutical products. For example, X-ray crystallography is capable of providing intimate details of protein conformation, but it can only be used to characterize native structures while the partially unfolded species would either not crystallize or be forced by the packing forces to assume a compact, native-like conformation. The dynamic character of protein structures is better captured by NMR spectroscopy; however, this technique suffers from very unforgiving molecular weight limitations, which places the majority of biopharmaceutical products out of its reach. Furthermore, the structural information produced by NMR is averaged across the entire ensemble of protein species, making it difficult to observe structurally compromised molecules that may be present in low abundance on the background of intact species.

The sensitivity issues in the analysis of biopharmaceutical products can be readily addressed by employing optical spectroscopic techniques, such as circular dichroism (CD) spectroscopy and fluorescence spectroscopy. These techniques, however, suffer from rather limited information content that can be extracted from the measurements (*e.g.*, cumulative content of secondary structure in far-UV CD and solvent exposure of aromatic residues in fluorescence spectroscopy). Several other techniques, such as size exclusion chromatography (SEC) or analytical ultracentrifugation (AUC) can provide information on the physical size of biopolymers, from which the compactness of protein conformation or the extent of oligomerization may be deduced, although they provide no detailed structural information either.

The spectacular success of biological mass spectrometry (MS) following the advent of electrospray ionization (ESI) and matrix-assisted laser desorption/ionization (MALDI) techniques two decades ago put it to the forefront of bioanalysis, especially analysis of biopolymer structure. MS has already become an indisputable technique of choice as far as the analysis of protein covalent structure, including therapeutic proteins (Srebalus-Barnes and Lim 2007; Zhang et al. 2009). It also made a tremendous impact in the field of carbohydrate analysis (Zaia 2004; Zaia 2010), and steady progress was made in recent years in developing MS-based methods for structural analysis of nucleic acids (Fabris 2010). Furthermore, the significant progress made in the past decade in developing MS-based methods to study protein higher order structure provides exciting opportunities *vis-a-vis* characterization of properties of biopharmaceutical products beyond their covalent structure.

3. Mass spectrometry for characterizing covalent structure of biopharmaceutical products

3.1. Protein sequencing

The tremendous value of ESI MS and MALDI MS for analysis of protein covalent structure (including biopharmaceutical products) was recognized immediately following introduction of these two analytical techniques; they supplanted within a very short time both chemical sequencing (Edman degradation) and gene sequencing (Fenselau 1991). Modern MS instrumentation allows masses of recombinant proteins to be measured with very high accuracy (e.g., ± 2 Da for a 150 kDa mAb, or 15 ppm (Bondarenko et al. 2009)). Since the amino acid sequence for therapeutic proteins is known, expected molecular weight can be easily calculated, and any mismatch with the experimentally determined protein masses would immediately signal deviation from the intended sequence. It has been recently shown that accurate mass measurement in some cases may provide a distinction between an innovator mAb product and its biosimilar version (Xie et al. 2010). Mass measurement also allows the heterogeneity of the protein preparation to be assessed, revealing the presence of various protein isoforms with different PTMs (Bondarenko et al. 2009) or contaminating protein species (Datola et al. 2007). In the case of multi-unit protein therapeutics (such as mAbs or antibody-based products) mass measurements following reduction of disulfide bonds and alkylation of cysteine residues can be used to confirm the structure of individual subunits (Chelius et al. 2010).

Above and beyond simple mass measurement, the great value of MS as a structural tool for characterization of protein therapeutics lies in its ability to generate sequence information. Traditionally, the sequencing task was accomplished using a scheme which is now referred to as a “bottom up” approach: the protein is digested with a suitable protease, and sequence information on each proteolytic fragment is obtained using tandem MS (or MS/MS methods). Modern methods of peptide ion fragmentation allow in many cases *de novo* sequencing to be accomplished (Standing 2003; Medzihradszky 2005; Seidler et al. 2010). Although *de novo* sequencing is rarely needed in the analysis of biopharmaceutical products, the ability to obtain structurally diagnostic fragmentation patterns is very important in the analysis of non-enzymatic PTMs (*vide infra*). A combination of proteolysis and peptide ion fragmentation in the gas phase may be used to localize the differences between the innovator product and its follow-on version (Xie et al. 2010).

In addition to the classical bottom-up approach, a new top-down strategy for protein sequencing was also introduced in the last decade. This methodology eliminates proteolysis and related solution-based protein handling steps; intact protein is transferred directly to the gas phase in the ionic form and is fragmented (McLafferty et al. 2007). This technique has recently been found to be very useful for N-terminal sequencing, which is often used both as

an identity test for therapeutic mAbs and a quality control measure to assess the completeness of signal sequence processing (Ren et al. 2009).

3.2. Characterization of enzymatic PTMs: glycosylation

Glycosylation is one of the most common (and perhaps the most complex) forms of enzymatic PTMs seen in protein therapeutics, with over two thirds of all approved biopharmaceutical products being glycoproteins (Li and d'Anjou 2009). While glycans play a variety of roles in physiology, the importance of carbohydrate chains present in protein therapeutics is largely determined by their intimate involvement in modulating stability, solubility, trafficking/biodistribution and immunogenicity/safety of biopharmaceuticals. Unlike polypeptides, biosynthesis of carbohydrate chains is controlled enzymatically, rather than genetically, which yields much greater structural diversity and heterogeneity. These two features present a major challenge from the analytical standpoint, and despite the steady progress in developing analytical technologies for structural characterization of glycoproteins, there is still a pressing need to develop new reliable, robust, and higher throughput approaches (Brooks 2009; Higgins 2010).

Biological MS is indisputably one of the most powerful analytical methods for the analysis of enzymatic PTMs (Larsen et al. 2006), and one area in particular that has greatly benefited from the dramatic progress in this field is glycomics (Marino et al. 2010; Zaia 2010). Many of these tools have been utilized in the analysis of glycosylation of biopharmaceutical products. Typically, the analysis begins with a glycoform profiling, where ESI MS or MALDI MS is used to obtain mass distribution of the glycoprotein. If the carbohydrate weight fraction is modest (<5% of the total protein weight), mass distributions can be clearly resolved and provide a useful signature of the glycan size distribution (*e.g.*, black trace in Figure 2). Mass distributions for more complex glycoproteins become more convoluted and generally less useful. In the case of extensive glycosylation it may become difficult to obtain reliable estimates of the protein mass based on ESI MS data alone; a combination of ESI MS and gas phase chemistry may be needed in order to reduce the complexity of the protein ion ensemble as a pre-requisite for reliable mass measurements (Abzalimov and Kaltashov 2010).

Glycan release from glycoproteins can be carried out using peptide N-glycosidase F (PNGase F) and/or PNGase A for N-glycans and chemical methods (*e.g.*, hydrozinolysis) for O-glycans (Marino et al. 2010). Methods of tandem MS/MS, especially recently introduced electron-based ion fragmentation methods (such as electron capture dissociation, ECD, and electron transfer dissociation, ETD), can be used in combination with exoglycosidases to obtain detailed structural information on released glycans (Zaia 2004; Marino et al. 2010; Zaia 2010). However, complete determination of structure (especially with novel glycans) may require the use of orthogonal methods, such as NMR and X-ray crystallography in addition to MS (Marino et al. 2010).

Finally, glycosylation site analysis is typically carried out by identifying glycopeptides among proteolytic fragments (*e.g.*, by comparing peptide maps for intact and de-glycosylated protein). If peptide mapping of de-glycosylated protein is not feasible (*e.g.*, due to poor solubility of the carbohydrate-free form of the protein), glycopeptides can be identified in the digest of intact glycoprotein by observing characteristic losses (*e.g.*, 162 Da for hexose residues) in survey MS/MS spectra obtained with collision-activated dissociation (CAD) of peptide ions, since the labile nature of glycosidic bonds in the gas phase leads to their facile dissociation (see Figure 3). While this feature is certainly useful as far as identifying glycopeptides in complex mixtures, it may prevent more precise localization of glycosylation sites. In these cases other fragmentation techniques (ECD and ETD) can be

effectively employed, as they preferentially cleave peptide backbone, leaving the carbohydrate chains mostly intact (Mirgorodskaya et al. 1999).

In addition to becoming a primary analytical tool for characterizing glycosylation of biopharmaceuticals (Huhn et al. 2009), MS-based glycosylation analysis is also emerging as an attractive means of comparing follow-on protein therapeutics with innovator drugs (Jiang et al. 2010; Xie et al. 2010) and other comparability studies (Reid et al. 2010; Liu et al. 2011).

3.3. Characterization of “designer” PTMs

Glycosylation remains the most common form of PTM found in many therapeutic proteins, and efforts continue to be made to enhance these products by re-engineering the glycosylation patterns using enzymatic means (Rich and Withers 2009). At the same time, covalent structure is also modified chemically in a significant proportion of protein drugs. One of the most common types of such modification is chemical conjugation of a protein with a synthetic polymer chain, such as polyethylene glycol, PEG (Greenwald et al. 2003; Gaberc-Porekar et al. 2008; Veronese and Mero 2008). Characterization of PEGylated biopharmaceuticals is usually focused on two questions: the extent of PEGylation (*i.e.*, average number of polymer chains attached to a single protein molecule) and the location of the conjugation sites. The extent of PEGylation can be evaluated by measuring the mass of the conjugated protein by MALDI MS (Seyfried et al. 2010), although pre-MS separation of conjugate forms provides more reliable results (Foser et al. 2003; Lee et al. 2008).

ESI MS is rarely used to measure masses in intact PEGylated proteins due to extreme spectral congestion typically observed in ESI mass spectra of these polydisperse species. However, charge stripping of ESI-generated multiply charged ions of PEGylated proteins may reduce the complexity of MS data and allow meaningful information on mass distributions to be obtained (Bagal et al. 2008). Identification of conjugation sites can be accomplished by comparing the peptide map of a particular form of the conjugated protein to that of the unmodified protein (Monkarsh et al. 1997; Foser et al. 2003; Arduini et al. 2004). Recently, feasibility of the top-down approach to localization of PEGylation sites has also been demonstrated (Yoo et al. 2009; Lu et al. 2010).

Another class of protein therapeutics with designer PTMs is represented by protein-drug conjugates (where “drug” could be another protein, peptide, or a small molecule medicine). As is the case with PEGylated proteins, analytical characterization is usually focused on the extent of conjugation (Lazar et al. 2005) and localization of the conjugation site(s) within the carrier protein (Wang et al. 2005).

3.4. Characterization of non-enzymatic PTMs

Chemical degradation of protein therapeutics may proceed through a variety of routes, where the most common ones are oxidation (affecting primarily methionine residues and free cysteines), de-amidation (affecting side chains of asparagines and glutamine), and amide bond hydrolysis leading to cleavage of the backbone. The latter process is readily detected by MS, as it results in a significant mass change (unless the two backbone dissociation products are still connected by a disulfide bridge, in which case the cleavage will result in a protein mass increase of 18 Da). Oxidation is also readily detected by MS, since each oxidation event results in a mass increase of 16 Da, which is usually noticeable even in the context of large proteins, such as mAbs (*e.g.*, compare red and black traces on Figure 2). De-amidation, on the other hand, produces a more subtle mass change (loss of 1 Da per de-amidation event), and usually is more difficult to detect (Figure 2, blue trace).

A more reliable detection of non-enzymatic PTMs related to protein degradation usually requires careful examination of the protein covalent structure, which also allows the modified residues to be localized within the protein sequence (Srebalus-Barnes and Lim 2007; Zhang et al. 2009). Typically, peptide mapping helps identify protein segments that undergo commonly observed chemical modifications; in some instances, however, it also yields information on more exotic degradation products (Kroon et al. 1992). Careful quantitative measurement of the extent and spatial distribution of common non-enzymatic PTMs in recombinant proteins may in certain cases provide a unique signature of the biopharmaceutical product reflecting unique manufacturing, formulation, and storage conditions, and allowing a distinction to be made between the follow-on, counterfeit, and the original innovator products (Jiang et al. 2009).

3.5. Disulfide bond mapping, disulfide scrambling problem and disulfide variants detection

Re-arrangement of disulfide bonds (commonly referred to as disulfide scrambling) is a non-enzymatic PTM, which is unique in that it does not change the protein mass. At the same time, its occurrence almost always leads to changes in the higher order structure, frequently resulting to changes in efficacy and safety profiles of biopharmaceutical products. Although the protein mass remains unchanged as a result of disulfide scrambling, a combination of liquid chromatography (LC) and MS may allow the alteration of cysteine linkage patterns to be detected by observing multiple LC peaks each corresponding to the same protein mass (Dillon et al. 2006). Rearrangement of disulfide bonds in a protein may also result in significant changes of its conformation in the gas phase, which in some cases can be detected using ion mobility mass spectrometry (Bagal et al. 2010).

Localization of scrambled disulfides usually requires a significant effort, particularly in cysteine-rich proteins, and peptide mapping must be typically complemented by tandem MS analysis. Since CAD in the positive ion mode leaves thiol-thiol linkages in peptides intact, other ion activation methods are frequently employed. ECD and negative ion CAD (Chrisman and McLuckey 2002; Bilusich et al. 2005; Bilusich and Bowie 2007) are particularly useful, since they both have the ability to cleave disulfide bonds. The latter technique is particularly appealing, since it results in very inefficient backbone cleavages, thereby allowing easy identification of disulfide-linked peptide dimers in complex mixtures (Zhang and Kaltashov 2006).

In addition to disulfide scrambling, there is another, less frequently occurring PTM that involves cysteine-cysteine bonds. The so-called trisulfides are formed by insertion of a sulfur atom into a disulfide bond. Trisulfide formation appears to be a common modification in many natural and recombinant antibodies, and occurs only in inter-chain linkages (Gu et al. 2010). Detection of these modifications is relatively straightforward, as trisulfide formation results in a noticeable mass increase not only at the peptide level (Gu et al. 2010), but also at the protein level (Pristatsky et al. 2009).

4. Mass spectrometry for characterization of conformation of biopharmaceuticals

Until recently, most applications of MS in the biopharmaceutical industry have been focused on characterizing the covalent structure of protein therapeutics (Srebalus-Barnes and Lim 2007; Zhang et al. 2009), even though the role of this technique in characterizing biopolymer structure extends well beyond sequencing and PTM mapping tasks (Kaltashov and Eyles 2002). While in some cases occurrence of non-enzymatic PTMs clearly signals that the protein is likely to be misfolded (*e.g.*, disulfide scrambling), in general there is no unequivocal correlation between the presence of certain PTMs and protein misfolding.

Indeed, some non-enzymatic PTMs are benign and lack the capacity to alter protein conformation. At the same time, protein misfolding may (and often does) occur even if the covalent structure is not compromised. Therefore, focusing characterization of a biopharmaceutical product exclusively on PTMs may generate both false-positive and false-negative outcomes *vis-a-vis* conformational integrity of therapeutic proteins. In addition, structural complexity and heterogeneity exhibited by the majority of protein drugs makes precise mapping of all PTMs in those cases a truly gargantuan task. Therefore, extending the reach of MS-based techniques to probing conformation and dynamics of protein drugs without the need for exhaustive cataloging of all PTMs would certainly benefit the field of analytical characterization of biopharmaceutical products in a very significant way.

Among the large number of MS-based techniques developed specifically to study protein non-covalent structure, two have been used successfully in the past several years to characterize conformation and dynamics of protein therapeutics, as well as their interactions with physiological targets. These are hydrogen/deuterium exchange (HDX) with MS detection and a suite of methods involving direct ESI MS analysis of biopolymers and their complexes in solution.

4.1. Direct ESI MS analysis of biopolymers and their complexes in solution: native ESI MS and protein ion charge state distribution analysis

ESI is a unique ionization technique in that it allows the protein ions to be extracted directly from solution, whose composition may be reasonably close to the “native” environment in terms of pH and ionic strength. ESI-generated biopolymer ions are typically produced as multiply charged ions, and the extent of multiple charging is determined by the physical dimensions of the biopolymer molecule in solution (Kaltashov and Abzalimov 2008). This feature of ESI MS allows it to be used as a means to probe protein higher order structure and detecting large-scale conformational transitions in solution. Indeed, natively folded proteins undergo ESI to produce ions carrying a relatively small number of charges, because the compact shape of a tightly folded polypeptide chain in solution does not allow the accommodation of a significant number of protons on its surface upon transition from solution to the gas phase. As a result, ion peaks in ESI mass spectra of proteins in aqueous solutions at neutral pH typically dominate the high m/z regions of the mass spectra and are almost always characterized by having narrow distribution of charge states (Kaltashov and Abzalimov 2008). However, once the protein molecule loses its compactness upon unfolding, a significantly larger number of charges can be accommodated on its surface. Therefore, partially or fully unfolded proteins give rise to ions carrying a significantly larger number of charges and their charge-state distributions are significantly broader than those of natively folded proteins. If native and non-native protein states coexist at equilibrium, protein ion charge state distributions are bimodal, reflecting the presence of both species in solution.

Despite the simplicity of this technique, its acceptance in the biopharmaceutical industry has been somewhat limited due to the need to carry out all measurements in the so-called “ESI-friendly” solvent systems. Non-volatile electrolytes (which are present in all biopharmaceutical formulations) have a detrimental effect on the quality of ESI MS data even at relatively low concentrations and must be removed by either dialysis or ultra-filtration before the direct ESI MS measurements are carried out. Typically, the protein is transferred to a volatile electrolyte solution (*e.g.*, ammonium acetate or ammonium bicarbonate), which frequently raises concerns that the observed protein behavior may be at least partially attributed to the influence of the new solvent system, rather than the intrinsic features of the biopharmaceutical product itself. Nevertheless, there is growing evidence, at least in the case of stressed protein therapeutics, that detection of unfolding events by direct ESI MS is not affected by the solvent exchange step (Bobst et al. 2008; Bobst et al. 2010).

Charge state distribution analysis of mAb ions produced by ESI MS has also been used in comparability studies and allowed molecules produced by the two production systems to be distinguished (Zamani et al. 2009).

4.2. Analysis of protein conformation in solution: hydrogen/deuterium exchange

HDX MS is another experimental tool that has been used in recent years to probe conformation and dynamics of biopharmaceutical products (Bobst et al. 2008; Houde et al. 2009; Burkitt et al. 2010b; Kaltashov et al. 2010). This experimental technique is reliable, robust and sensitive, and is capable not only of detecting the presence of (partially) unfolded species in solution, but also localizing protein segments with anomalous protection levels. Unlike direct ESI MS measurements, HDX MS does not place restrictive requirements on solvent composition, and the measurements can be carried out in the formulation buffer or any other relevant solvent system.

Conformational properties of proteins are probed by HDX by measuring the rates of labile hydrogen atoms exchange with the solvent, and the presence of higher order structure can alter the exchange rates in a very significant way. Protons that are involved in hydrogen bonding or sequestered from solvent in the protein interior are not able to exchange with the solvent unless they become exposed to it through a local conformational fluctuation or a more global (complete or partial) protein unfolding event (Krishna et al. 2004). Substitution of H₂O with D₂O provides a straightforward means to monitor the progress of the exchange reactions, where each individual H/D exchange event results in a mass increase of 1.01 Da, a difference that can be easily detected by MS even in the context of large proteins. Therefore, monitoring the mass change of an initially unlabeled protein placed in D₂O-based solvent allows the extent of hydrogen bonding network within the protein and its stability to be evaluated.

Although HDX MS can provide information on solvent protection of all labile hydrogen atoms, polar and charged side chains are frequently exposed to the solvent, and exchange very fast. The labile hydrogen atoms residing on backbone amides are typically better reporters of higher order structure due to their intimate involvement in H-bonding networks that maintain the integrity of secondary structural elements. Contribution of the labile hydrogen atoms on the side chains to the overall exchange can be easily eliminated prior to the mass measurement by bringing the solution pH to the 2.5–3.0 interval and lowering the temperature to 0–4 °C. These conditions result in facile unfolding of most of the proteins, thereby exposing all labile hydrogen atoms to the solvent. However, the intrinsic (chemical) exchange rate of backbone amide protons under these conditions is very slow (Bai et al. 1993), causing them to be mostly preserved, while labile hydrogen and deuterium atoms on the side chains exchange quickly (Dempsey 2001).

The ability to decelerate selectively the exchange of backbone amide hydrogen atoms also allows local information on protein higher order structure and dynamics to be obtained by carrying out proteolysis under the slow exchange conditions prior to MS analysis. Pepsin is one of the very few proteases that are active within the pH range 2.5–3.0, and can be used to probe backbone amide protection following the quench of the exchange reactions (Englander 2006; Konermann et al. 2010). Since the slow exchange of backbone amide hydrogen atoms continues even under quench conditions, protein processing and analysis must be relatively fast in order to preserve the local exchange details. Following the quench of the exchange reactions, all sample handling steps can be carried out on-line, including proteolysis, reduction of disulfide bonds, LC separation of peptic fragments and MS analysis of their deuterium content (Bobst et al. 2009).

4.3. Higher order structure of protein drugs: conformational changes triggered by stress and designer PTM

Several recent reports highlight the power of native ESI MS and HDX MS as a means to detect changes in conformation and dynamics of recombinant protein therapeutics caused by either non-enzymatic PTMs under stress conditions (Bobst et al. 2008; Bobst et al. 2010; Burkitt et al. 2010a) or engineered PTMs (Houde et al. 2010b). An example is shown in Figure 4, where extensive oxidation of a recombinant form of acid- β -glucocerebrosidase (GCase, a recombinant glycoprotein used for treatment of Gaucher's disease by enzyme replacement therapy (Grabowski 2008)) results in a noticeable change of the protein ion charge state distribution in ESI MS, revealing the presence of a less compact conformation in solution (represented by charge states +17 through +28 in Figure 4).

Further insight into the structure of this partially unfolded state of GCase is provided by HDX MS (Figure 5), where HDX MS is used to probe local conformational dynamics of intact and oxidized GCase (Bobst et al. 2010). The uneven distribution of conformational flexibility across the backbone of intact GCase highlights the importance of the delicate balance between the protein structure and dynamics. Oxidation clearly shifts this balance by adversely affecting stability of several protein segments. Surprisingly, the segments containing the oxidation sites (*e.g.*, 447–456 in Figure 5) are not affected by the covalent modifications, while several segments that are remote from the modified residues (*e.g.*, 185–209) exhibit noticeable decrease in backbone protection. A detailed analysis of HDX MS data for the intact and oxidized forms of GCase (Bobst et al. 2010) indicates that the segments whose protection is affected by the oxidation tend to form a contiguous cluster on the protein surface (Figure 5B). This suggests that the greatly diminished activity of GCase caused by extensive oxidation results from the decrease of its conformational stability, while the catalytic function is not affected directly (Bobst et al. 2010).

The ability of HDX MS to detect changes in conformation of protein drugs resulting from stress or engineered PTMs has led to suggestions that this technique be included in the arsenal of comparability studies of biopharmaceutical products (Houde et al. 2010a; Kaltashov et al. 2010).

4.4. Aggregation of protein drugs

Aggregation propensity is an inherent feature of most proteins, including biopharmaceutical products, and presents one of the most significant barriers to developing efficient therapies in terms of both drug safety and the economics of the manufacturing process (Wang 2005). The centrality of aggregation to success or failure of a specific biopharmaceutical product places a premium on our ability to understand the general mechanism of protein aggregation, as well as to detect and monitor this process under specific conditions (Weiss et al. 2009; Wang et al. 2010) or make relevant predictions based on sequence information (Wang et al. 2009). Despite significant recent advances in understanding this phenomenon, tracking and detailed description of the aggregation processes using the existing experimental tools remains a very difficult task. One of the reasons is the extreme complexity of the populations of aggregating proteins, which typically exhibit a high degree of structural heterogeneity (both in terms of oligomerization state of individual species and conformation of their constituents). Furthermore, the highly transient nature of such systems makes it nearly impossible to track their behavior in real time.

In recent years, MS was applied to study aggregation of protein drugs, although such studies are typically limited to the analysis of various species that are populated at the end-point of the aggregation process (Tobler and Fernandez 2002). The presence of soluble aggregates and their heterogeneity in biopharmaceutical products is frequently assessed by size

exclusion chromatography (SEC). Although the size of soluble aggregates is typically evaluated using light scattering techniques, the ability of ESI MS to maintain the integrity of non-covalent complexes in the gas phase can also be used to measure the extent of protein oligomerization in various SEC fractions (Kükler et al. 2010). However, the size information on soluble oligomers provided in such measurements could be biased due to several factors. First, protein oligomers are very dynamic species; collecting, storing and processing the SEC fractions prior to mass analysis may easily change the size distribution as shown in the inset in Figure 6 for SEC-isolated oligomers of recombinant anti-thrombin III (AT). Second, if several oligomeric species are present in the sample, the ionic abundance distribution is likely to be biased towards lower molecular weight components (*e.g.*, the majority of the ionic species in the “oligomers” SEC fraction of AT in Figure 6 correspond to trimers and tetramers with little contribution from larger oligomers). Some of these concerns (*e.g.*, alteration of the oligomer size distribution during fraction collection) may be addressed by using the on-line SEC-ESI MS scheme (Brady et al. 2008), but it has not been applied to study aggregation behavior of biopharmaceutical products as yet.

A different approach to study aggregation of protein drugs utilizes a temperature controlled ESI source, where the protein is heated at a fixed temperature for a certain period of time, immediately followed by MS analysis (Wang et al. 2011). This approach allows one to observe simultaneously soluble oligomers formation and conformational changes within monomeric species by monitoring both changes in ionic mass and charge state distributions (Figure 7). The ability to carry out measurement under both equilibrium and non-equilibrium conditions using this approach is likely to make this technique an attractive tool in heat-stress stability studies.

5. Interaction of biopharmaceuticals with therapeutic targets and physiological partners

The impact of MS-based techniques discussed in preceding sections extends well beyond quality control of protein drug formulations. In fact, many of them show tremendous promise for the discovery and development stages as well. Several examples of using MS-based experimental strategies to study interaction of protein drugs or drug candidates with their therapeutic targets and physiological partners presented below illustrate how this technique may catalyze design of new biopharmaceutical products.

5.1. Molecular mechanism of drug/target interaction

The unique ability of native ESI MS to preserve non-covalent associations in the gas phase (Loo 1997; Yin and Loo 2009) has been useful not only in characterizing protein quaternary structure, but also in monitoring protein interaction under near-physiological conditions with a variety of ligands, ranging from metal ions and small organic molecules to large biopolymers. This unique feature of ESI MS allows it to be used as a tool to identify species capable of binding to a specific protein from libraries of small organic ligands (Bruce et al. 1995) and even mixtures of complex biopolymers (Abzalimov et al. 2007). This same feature can be used to monitor binding of protein drugs to their therapeutic targets (*e.g.*, antibody-antigen interaction (Tito et al. 2001)) and physiological partners (*e.g.*, protein-cognate receptor interaction (Kaltashov et al. 2010)). ESI MS readily provides information not only on the ability of a specific therapeutic protein to bind to its intended target, but also reveals the binding stoichiometry, as shown in Figure 8 for the interaction between transferrin receptor (TfR) and a fusion protein designed to contain transferrin (Tf) and human growth hormone (Amet et al. 2009). In some cases it is possible to obtain binding affinities for protein/receptor systems based on ESI MS data (Leverence et al. 2010).

More detailed characterization of a protein drug/target interaction can be provided by HDX MS. It was pointed out a decade ago that this technique has a great potential for aiding optimization of a small molecule drug candidates by providing information on structure and dynamics of the binding site at the therapeutic target (Woods and Hamuro 2001). The idea of incorporation of MS in the workflow of the discovery and development of protein therapeutics is also beginning to enjoy attention following recent reports that it may aid epitope mapping (Coales et al. 2009; Obungu et al. 2009) and, therefore, facilitate rational design of biopharmaceutical products with enhanced binding to therapeutic targets.

An example of HDX MS-assisted characterization of protein/receptor binding is shown in Figure 9, where this technique is used to probe the conformation of Tf alone and in the receptor-bound form. The ability of Tf (or any Tf-based biopharmaceutical product) to enter cells critically depends on its recognition by TfR; therefore, detailed information on Tf/TfR interaction at the molecular level is extremely important for the drug development process. Unfortunately, the large size and the presence of paramagnetic ferric ion (Fe^{3+}) places the Tf/TfR complex out of reach of NMR, while its dynamic character and high carbohydrate content make acquisition of high-resolution X-ray diffraction (and even low resolution cryo-electron microscopy (Cheng et al. 2005)) data highly problematic. However, HDX MS readily provides information on involvement of various protein segments in Tf/TfR interaction. For example, two similarly sized peptic fragments of Tf (71–81 and 612–621) whose deuterium content evolution is shown in Figure 9 represent protein regions with increased backbone mobility in the absence of the receptor. The first fragment (which maps to a loop region in the crystal structure of the protein, see red-colored segment in Figure 9) has very low backbone protection, and the other peptide (which maps to a helix-loop-strand segment, multi-colored in Figure 9) exhibits intermediate level of protection. The ability of the latter region of Tf to exchange with solvent is completely eliminated upon binding to the receptor, and the peptide becomes highly protected (see blue traces in Figure 9), suggesting strong involvement of this flexible loop region in forming the protein/receptor interface. In contrast, the flexible loop discussed earlier is not affected at all by the Tf/TfR interaction.

This type of analysis, particularly in the absence of a reliable crystal structure of a protein drug/therapeutic target complex will become a truly invaluable tool for designing new and enhancing existing biopharmaceutical products (*e.g.*, in optimizing conjugation of a payload to a carrier protein). Furthermore, in addition to structural information on protein drug/target binding, HDX MS methodology can also be used to determine binding affinity (Tu et al. 2010).

5.2. MS-based methods to study pharmacokinetics and pharmacodynamics of protein therapeutics

Clinical success of any drug (both small molecule medicines and biotechnology products) critically depends not only on its ability to interact with a desired therapeutic target in a highly specific manner, but also on its pharmacokinetics. Biopharmaceuticals frequently exhibit unique and unpredictable pharmacokinetic profiles which reflect their differential susceptibility to proteolysis, renal clearance, interaction with the host's immune system and many other factors (Lin 2009). Analytical methods have always been critically important for obtaining valid pharmacokinetics data, but until recently immunoassays and bioactivity assays were the two mainstays of the analytical support of pharmacokinetics of protein drugs, while MS-based methods were used only for a limited range of niche applications (Baumann 2006). However, the explosive growth of proteomics in recent years, and the central role played by MS in this field (Fenselau 2007) have resulted in a dramatic expansion of the scope of MS-based protein quantitation methods in pharmacokinetic studies of protein drugs (Ezan et al. 2009).

Since a protein drug in circulation typically represents a very small fraction of the total plasma proteome, quantitation of a therapeutic protein in biological fluids with high sensitivity usually requires protein enrichment as a first step. Immunocapture offers perhaps the most straightforward way to increase the sensitivity of quantitation by increasing concentration of the protein analyte and dramatically reducing matrix interference and signal suppression from other proteins in the sample (Ezan et al. 2009), although some other means of protein pre-concentration prior to the MS analysis are also possible (Figure 10).

Accurate and reliable quantitation of proteins using MS-based approaches is impossible without proper internal standards (IS), which can be prepared in a variety of ways. Although IS preparation can be achieved using a protein that is structurally similar to the biopharmaceutical product (Yang et al. 2009), or by chemical modification of the protein drug or its fragments (Dai et al. 2005; Ji et al. 2009), isotopically labeled IS are usually more desirable. Since most biopharmaceuticals cannot be expressed in simple bacterial systems, generation of an isotopically labeled version of the therapeutic proteins as an internal standard may be very challenging. In some cases it may be possible to introduce a partially labeled IS by feeding at least one isotopically labeled amino acid to the expression system (Heudi et al. 2008); however, peptide labeling at the proteolytic step (Fenselau and Yao 2009) is usually a more attractive alternative due to the relative ease and universality of labeling that is achieved using this scheme (Figure 10).

Combination of immunocapture with MS detection allows quantitation of protein drugs in biological fluids to be carried at concentrations as low as 20 ng/mL, which compares favorably with most ELISA-based quantitation protocols; at the same time MS typically offers far better precision (Ezan and Bitsch 2009). Presently, a significant drawback of MS-based quantitation (particularly when IS are utilized) is lower analysis throughput, although continuing improvements in automation are likely to address this shortcoming in the near future.

6. Conclusions/outlook

Biological MS offers a great variety of approaches to study structure and behavior of complex protein drugs both *in vitro* and *in vivo*. MS has already become a default tool for characterizing covalent structure of protein therapeutics, including sequencing information, as well as distribution and nature of both enzymatic and non-enzymatic PTMs. Recently, MS-based methods have also been evaluated as a means to provide information on higher order structure and dynamics of biotechnology products. In particular, HDX MS and charge state distribution analysis of protein ions in ESI MS offer a convenient way to assess the integrity of the protein conformation. Native ESI MS also allows the interactions of protein drugs and with their therapeutic targets and other physiological partners to be monitored using simplified model systems. MS-based methods are also beginning to enjoy popularity as a means to study pharmacokinetics of biopharmaceutical products. The overall impact of MS-based techniques in the field of biopharmaceutical analysis is already very significant and is expected to continue to grow, providing valuable support to all stages of development of biopharmaceuticals, from discovery to post-approval monitoring.

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References

- Abzalimov RR, et al. Glycosaminoglycans as naturally occurring combinatorial libraries: Developing a mass spectrometry-based strategy for characterization of anti-thrombin interaction with low molecular weight heparin and heparin oligomers. *Anal Chem.* 2007; 79:6055–63. [PubMed: 17658885]
- Abzalimov RR, Kaltashov IA. Electrospray ionization mass spectrometry of highly heterogeneous protein systems: Protein ion charge state assignment via incomplete charge reduction. *Anal Chem.* 2010; 82:7523–6. [PubMed: 20731408]
- Amet N, et al. Insertion of the designed helical linker led to increased expression of Tf-based fusion proteins. *Pharm Res.* 2009; 26:523–8. [PubMed: 19002568]
- Arduini RM, et al. Expression, purification, and characterization of rat interferon- β , and preparation of an N-terminally PEGylated form with improved pharmacokinetic parameters. *Prot Exp Purif.* 2004; 34:229–42.
- Bagal D, et al. Resolving disulfide structural isoforms of IgG2 monoclonal antibodies by ion mobility mass spectrometry. *Anal Chem.* 2010; 82:6751–5. [PubMed: 20704363]
- Bagal D, et al. Gas-phase proton-transfer chemistry coupled with TOF mass spectrometry and ion mobility-MS for the facile analysis of poly(ethylene glycols) and PEGylated polypeptide conjugates. *Anal Chem.* 2008; 80:2408–18. [PubMed: 18324791]
- Bai Y, et al. Primary structure effects on peptide group hydrogen exchange. *Proteins.* 1993; 17:75–86. [PubMed: 8234246]
- Baumann A. Early development of therapeutic biologics - pharmacokinetics. *Curr Drug Metab.* 2006; 7:15–21. [PubMed: 16454690]
- Bilusich D, Bowie JH. Identification of intermolecular disulfide linkages in underivatized peptides using negative ion electrospray mass spectrometry. A joint experimental and theoretical study. *Rapid Commun Mass Spectrom.* 2007; 21:619–28. [PubMed: 17279483]
- Bilusich D, et al. Direct identification of intramolecular disulfide links in peptides using negative ion electrospray mass spectra of underivatized peptides. A joint experimental and theoretical study. *Rapid Commun Mass Spectrom.* 2005; 19:3063–74. [PubMed: 16200662]
- Bobst CE, et al. Detection and characterization of altered conformations of protein pharmaceuticals using complementary mass spectrometry-based approaches. *Anal Chem.* 2008; 80:7473–81. [PubMed: 18729476]
- Bobst CE, et al. Impact of oxidation on protein therapeutics: Conformational dynamics of intact and oxidized acid- β -glucocerebrosidase at near-physiological pH. *Protein Sci.* 2010; 19:2366–78. [PubMed: 20945356]
- Bobst CE, et al. Existence of a noncanonical state of iron-bound transferrin at endosomal pH revealed by hydrogen exchange and mass spectrometry. *J Mol Biol.* 2009; 388:954–67. [PubMed: 19324057]
- Bondarenko PV, et al. Mass measurement and top-down HPLC/MS analysis of intact monoclonal antibodies on a hybrid linear quadrupole ion trap-orbitrap mass spectrometer. *J Am Soc Mass Spectrom.* 2009; 20:1415–24. [PubMed: 19409810]
- Brady LJ, et al. Molecular mass analysis of antibodies by on-line SEC-MS. *J Am Soc Mass Spectrom.* 2008; 19:502–9. [PubMed: 18258452]
- Brooks S. Strategies for analysis of the glycosylation of proteins: Current status and future perspectives. *Mol Biotechnol.* 2009; 43:76–88. [PubMed: 19507069]
- Bruce JE, et al. Bio-affinity characterization mass spectrometry. *Rapid Commun Mass Spectrom.* 1995; 9:644–50. [PubMed: 7647362]
- Burkitt W, et al. Conformational changes in oxidatively stressed monoclonal antibodies studied by hydrogen exchange mass spectrometry. *Protein Sci.* 2010a; 19:826–35. [PubMed: 20162626]
- Burkitt W, et al. Conformational changes in oxidatively stressed monoclonal antibodies studied by hydrogen exchange mass spectrometry. *Protein Science.* 2010b; 19:826–35. [PubMed: 20162626]
- Chelius D, et al. Structural and functional characterization of the trifunctional antibody catumaxomab. *Mabs.* 2010; 2:309–19. [PubMed: 20418662]

- Cheng Y, et al. Structure of the human transferrin receptor-transferrin complex. *Cell*. 2004; 116:565–76. [PubMed: 14980223]
- Cheng Y, et al. Single particle reconstruction of the human apo-transferrin-transferrin receptor complex. *J Struct Biol*. 2005; 152:204–10. [PubMed: 16343946]
- Chrisman PA, McLuckey SA. Dissociations of disulfide-linked gaseous polypeptide/protein anions: ion chemistry with implications for protein identification and characterization. *J Proteome Res*. 2002; 1:549–57. [PubMed: 12645623]
- Coales SJ, et al. Epitope mapping by amide hydrogen/deuterium exchange coupled with immobilization of antibody, on-line proteolysis, liquid chromatography and mass spectrometry. *Rapid Commun Mass Spectrom*. 2009; 23:639–47. [PubMed: 19170039]
- Dai S, et al. Quantification of sifuvirtide in monkey plasma by an on-line solid-phase extraction procedure combined with liquid chromatography/electrospray ionization tandem mass spectrometry. *Rapid Commun Mass Spectrom*. 2005; 19:1273–82. [PubMed: 15834846]
- Datola A, et al. Characterisation of a novel growth hormone variant comprising a thioether link between Cys182 and Cys189. *ChemMedChem*. 2007; 2:1181–9. [PubMed: 17576647]
- Dempsey CE. Hydrogen exchange in peptides and proteins using NMR-spectroscopy. *Progr Nucl Magn Res Spectrosc*. 2001; 39:135–70.
- Dillon TM, et al. Optimization of a reversed-phase high-performance liquid chromatography/mass spectrometry method for characterizing recombinant antibody heterogeneity and stability. *Journal of Chromatography A*. 2006; 1120:112–20. [PubMed: 16448656]
- Englander SW. Hydrogen exchange and mass spectrometry: A historical perspective. *J Am Soc Mass Spectrom*. 2006; 17:1481–9. [PubMed: 16876429]
- Ezan E, Bitsch F. Critical comparison of MS and immunoassays for the bioanalysis of therapeutic antibodies. *Bioanalysis*. 2009; 1:1375–88. [PubMed: 21083085]
- Ezan E, et al. Bioanalysis of recombinant proteins and antibodies by mass spectrometry. *Analyst*. 2009; 134:825–34. [PubMed: 19381370]
- Fabris D. A role for the MS analysis of nucleic acids in the post-genomics age. *J Am Soc Mass Spectrom*. 2010; 21:1–13. [PubMed: 19897384]
- Fenselau C. Beyond gene sequencing: Analysis of protein structure with mass spectrometry. *Annu Rev Biophys Biophys Chem*. 1991; 20:205–20. [PubMed: 1867716]
- Fenselau C. A review of quantitative methods for proteomic studies. *J Chromatogr B*. 2007; 855:14–20.
- Fenselau C, Yao X. ¹⁸O₂-Labeling in quantitative proteomic strategies: A status report. *J Proteome Res*. 2009; 8:2140–3. [PubMed: 19338309]
- Foser S, et al. Isolation, structural characterization, and antiviral activity of positional isomers of monopegylated interferon α -2a (PEGASYS). *Prot Exp Purif*. 2003; 30:78–87.
- Gaberc-Porekar V, et al. Obstacles and pitfalls in the PEGylation of therapeutic proteins. *Curr Opin Drug Discov Dev*. 2008; 11:242–50.
- Grabowski GA. Phenotype, diagnosis, and treatment of Gaucher's disease. *Lancet*. 2008; 372:1263–71. [PubMed: 19094956]
- Greenwald RB, et al. Effective drug delivery by PEGylated drug conjugates. *Adv Drug Del Rev*. 2003; 55:217–50.
- Gu S, et al. Characterization of trisulfide modification in antibodies. *Anal Biochem*. 2010; 400:89–98. [PubMed: 20085742]
- Heudi O, et al. Towards absolute quantification of therapeutic monoclonal antibody in serum by LC-MS/MS using isotope-labeled antibody standard and protein cleavage isotope dilution mass spectrometry. *Anal Chem*. 2008; 80:4200–7. [PubMed: 18465883]
- Higgins E. Carbohydrate analysis throughout the development of a protein therapeutic. *Glyco conj J*. 2010; 27:211–25.
- Houde D, et al. Characterization of IgG1 conformation and conformational dynamics by hydrogen/deuterium exchange mass spectrometry. *Anal Chem*. 2009; 81:2644–51. [PubMed: 19265386]
- Houde D, et al. The utility of hydrogen/deuterium exchange mass spectrometry in biopharmaceutical comparability studies. *J Pharm Sci*. 2010a e-pub: n/a-n/a.

- Houde D, et al. Post-translational modifications differentially affect IgG1 conformation and receptor binding. *Mol Cell Proteom.* 2010b; 9:1716–28.
- Huhn C, et al. IgG glycosylation analysis. *PROTEOMICS.* 2009; 9:882–913. [PubMed: 19212958]
- Ji C, et al. A Universal Strategy for Development of a Method for Absolute Quantification of Therapeutic Monoclonal Antibodies in Biological Matrices Using Differential Dimethyl Labeling Coupled with Ultra Performance Liquid Chromatography-Tandem Mass Spectrometry. *Analytical Chemistry.* 2009; 81:9321–8. [PubMed: 19842637]
- Jiang H, et al. Characterization of the glycosylation occupancy and the active site in the follow-on protein therapeutic: TNK-tissue plasminogen activator. *Anal Chem.* 2010; 82:6154–62. [PubMed: 20552988]
- Jiang HT, et al. Mass spectrometric analysis of innovator, counterfeit, and follow-on recombinant human growth hormone. *Biotechnol Prog.* 2009; 25:207–18. [PubMed: 19224592]
- Kaltashov IA, Abzalimov RR. Do ionic charges in ESI MS provide useful information on macromolecular structure? *J Am Soc Mass Spectrom.* 2008; 19:1239–46. [PubMed: 18602274]
- Kaltashov IA, et al. H/D exchange and mass spectrometry in the studies of protein conformation and dynamics: Is there a need for a top-down approach? *Anal Chem.* 2009; 81:7892–9. [PubMed: 19694441]
- Kaltashov IA, et al. Conformation and dynamics of biopharmaceuticals: transition of mass spectrometry-based tools from academe to industry. *J Am Soc Mass Spectrom.* 2010; 21:323–37. [PubMed: 19963397]
- Kaltashov IA, Eyles SJ. Studies of biomolecular conformations and conformational dynamics by mass spectrometry. *Mass Spectrom Rev.* 2002; 21:37–71. [PubMed: 12210613]
- Konermann L, et al. Mass spectrometry combined with oxidative labeling for exploring protein structure and folding. *Mass Spectrom Rev.* 2010; 29:651–67. [PubMed: 19672951]
- Krishna MMG, et al. Hydrogen exchange methods to study protein folding. *Methods.* 2004; 34:51–64. [PubMed: 15283915]
- Kroon DJ, et al. Identification of sites of degradation in a therapeutic monoclonal-antibody by peptide-mapping. *Pharm Res.* 1992; 9:1386–93. [PubMed: 1475223]
- Kükrer B, et al. Mass spectrometric analysis of intact human monoclonal antibody aggregates fractionated by size-exclusion chromatography. *Pharm Res.* 2010; 27:2197–204. [PubMed: 20680668]
- Larsen MR, et al. Analysis of posttranslational modifications of proteins by tandem mass spectrometry. *Biotechniques.* 2006; 40:790–8. [PubMed: 16774123]
- Lazar AC, et al. Analysis of the composition of immunoconjugates using size-exclusion chromatography coupled to mass spectrometry. *Rapid Commun Mass Spectrom.* 2005; 19:1806–14. [PubMed: 15945030]
- Lee DL, et al. Preparation and characterization of monopegylated human granulocyte-macrophage colony-stimulating factor. *J Interf Cyt Res.* 2008; 28:101–12.
- Leverence R, et al. Noncanonical interactions between serum transferrin and transferrin receptor evaluated with electrospray ionization mass spectrometry. *Proc Natl Acad Sci USA.* 2010; 107:8123–8. [PubMed: 20404192]
- Li H, d'Anjou M. Pharmacological significance of glycosylation in therapeutic proteins. *Curr Opin Biotechnol.* 2009; 20:678–84. [PubMed: 19892545]
- Lin JH. Pharmacokinetics of biotech drugs: Peptides, proteins and monoclonal antibodies. *Curr Drug Metab.* 2009; 10:661–91. [PubMed: 19702530]
- Liu C, et al. Assessment of the quality and structural integrity of a complex glycoprotein mixture following extraction from the formulated biopharmaceutical drug product. *J PharmBiomed Anal.* 2011; 54:27–36.
- Loo JA. Studying noncovalent protein complexes by electrospray ionization mass spectrometry. *Mass Spectrom Rev.* 1997; 16:1–23. [PubMed: 9414489]
- Lu XJ, et al. Elucidation of PEGylation site with a combined approach of in-source fragmentation and CID MS/MS. *J Am Soc Mass Spectrom.* 2010; 21:810–8. [PubMed: 20189826]

- Marino K, et al. A systematic approach to protein glycosylation analysis: a path through the maze. *Nat Chem Biol.* 2010; 6:713–23. [PubMed: 20852609]
- McLafferty FW, et al. Top-down MS, a powerful complement to the high capabilities of proteolysis proteomics. *FEBS J.* 2007; 274:6256–68. [PubMed: 18021240]
- Medzihradszky, KF. *Biological Mass Spectrometry*. Vol. 402. San Diego: Elsevier Academic Press Inc; 2005. Peptide sequence analysis; p. 209–44.
- Mirgorodskaya E, et al. Localization of O-glycosylation sites in peptides by electron capture dissociation in a fourier transform mass spectrometer. *Anal Chem.* 1999; 71:4431–6. [PubMed: 10546526]
- Monkarsh SP, et al. Positional isomers of monopegylated interferon α -2a: Isolation, characterization, and biological activity. *Anal Biochem.* 1997; 247:434–40. [PubMed: 9177709]
- Niles R, et al. Acid-catalyzed oxygen-18 labeling of peptides. *Anal Chem.* 2009; 81:2804–9. [PubMed: 19243188]
- Obungu VH, et al. Determination of the mechanism of action of anti-FasL antibody by epitope mapping and homology modeling. *Biochemistry.* 2009; 48:7251–60. [PubMed: 19588926]
- Pristatsky P, et al. Evidence for trisulfide bonds in a recombinant variant of a human IgG2 monoclonal antibody. *Anal Chem.* 2009; 81:6148–55. [PubMed: 19591437]
- Reid CQ, et al. Rapid whole monoclonal antibody analysis by mass spectrometry: An ultra scale-down study of the effect of harvesting by centrifugation on the post-translational modification profile. *Biotechnol Bioeng.* 2010; 107:85–95. [PubMed: 20506289]
- Ren D, et al. Top-down N-terminal sequencing of Immunoglobulin subunits with electrospray ionization time of flight mass spectrometry. *Anal Biochem.* 2009; 384:42–8. [PubMed: 18834850]
- Rich JR, Withers SG. Emerging methods for the production of homogeneous human glycoproteins. *Nat Chem Biol.* 2009; 5:206–15. [PubMed: 19295526]
- Seidler J, et al. De novo sequencing of peptides by MS/MS. *Proteomics.* 2010; 10:634–49. [PubMed: 19953542]
- Seyfried BK, et al. MALDI linear TOF mass spectrometry of PEGylated (glyco)proteins. *J Mass Spectrom.* 2010; 45:612–7. [PubMed: 20527029]
- Srebalus-Barnes CA, Lim A. Applications of mass spectrometry for the structural characterization of recombinant protein pharmaceuticals. *Mass Spectrom Rev.* 2007; 26:370–88. [PubMed: 17410555]
- Standing KG. Peptide and protein *de novo* sequencing by mass spectrometry. *Curr Opin Struct Biol.* 2003; 13:595–601. [PubMed: 14568614]
- Tito MA, et al. Probing molecular interactions in intact antibody: antigen complexes, an electrospray time-of-flight mass spectrometry approach. *Biophys J.* 2001; 81:3503–9. [PubMed: 11721011]
- Tobler SA, Fernandez EJ. Structural features of interferon-gamma aggregation revealed by hydrogen exchange. *Protein science: a publication of the Protein Society.* 2002; 11:1340–52. [PubMed: 12021433]
- Tu TT, et al. Protein-peptide affinity determination using an H/D exchange dilution strategy: Application to antigen-antibody interactions. *J Am Soc Mass Spectrom.* 2010; 21:1660–7. [PubMed: 20444623]
- Veronese FM, Mero A. The impact of PEGylation on biological therapies. *BioDrugs.* 2008; 22:315. [PubMed: 18778113]
- Wang G, et al. Direct Monitoring of Heat-Stressed Biopolymers with Temperature-Controlled Electrospray Ionization Mass Spectrometry. *Anal Chem.* 2011;83. in press. [PubMed: 22122543]
- Wang L, et al. Structural characterization of the maytansinoid monoclonal antibody immunoconjugate, huN901 DM1, by mass spectrometry. *Protein Sci.* 2005; 14:2436–46. [PubMed: 16081651]
- Wang W. Protein aggregation and its inhibition in biopharmaceuticals. *Int J Pharm.* 2005; 289:1–30. [PubMed: 15652195]
- Wang W, et al. Protein aggregation-Pathways and influencing factors. *Int J Pharm.* 2010; 390:89–99. [PubMed: 20188160]
- Wang XL, et al. Potential aggregation prone regions in biotherapeutics: A survey of commercial monoclonal antibodies. *mAbs.* 2009; 1:254–67. [PubMed: 20065649]

- Weiss WF, et al. Principles, approaches, and challenges for predicting protein aggregation rates and shelf life. *J Pharm Sci.* 2009; 98:1246–77. [PubMed: 18683878]
- Woods VL Jr, Hamuro Y. High resolution, high-throughput amide deuterium exchange-mass spectrometry (DXMS) determination of protein binding site structure and dynamics: utility in pharmaceutical design. *J Cell Biochem.* 2001; 37S:89–98.
- Xie HW, et al. Rapid comparison of a candidate biosimilar to an innovator monoclonal antibody with advanced liquid chromatography and mass spectrometry technologies. *Mabs.* 2010; 2:379–94.
- Yang ZP, et al. A sensitive and high-throughput LC-MS/MS method for the quantification of pegylated-interferon-alpha(2a) in human serum using monolithic C-18 solid phase extraction for enrichment. *J Chromatogr B.* 2009; 877:1737–42.
- Yin S, Loo JA. Mass spectrometry detection and characterization of noncovalent protein complexes. *Methods Mol Biol.* 2009; 492:273–82. [PubMed: 19241039]
- Yoo C, et al. Toward top-down determination of PEGylation site using MALDI in-source decay MS analysis. *J Am Soc Mass Spectrom.* 2009; 20:326–33.
- Zaia J. Mass spectrometry of oligosaccharides. *Mass Spectrom Rev.* 2004; 23:161–227. [PubMed: 14966796]
- Zaia J. Mass spectrometry and glycomics. *OMICS.* 2010; 14:401–18. [PubMed: 20443730]
- Zamani L, et al. Discrimination among IgG1- κ monoclonal antibodies produced by two cell lines using charge state distributions in nanoESI-TOF mass spectra. *J Am Soc Mass Spectrom.* 2009; 20:1030–6. [PubMed: 19251439]
- Zhang M, Kaltashov IA. Mapping of protein disulfide bonds using negative ion fragmentation with a broadband precursor selection. *Anal Chem.* 2006; 78:4820–9. [PubMed: 16841900]
- Zhang Z, et al. Mass spectrometry for structural characterization of therapeutic antibodies. *Mass Spectrom Rev.* 2009; 28:147–76. [PubMed: 18720354]

Figure 1. A schematic representation of the potential effects of stress or process change on the properties of a biopharmaceutical product. The space-filled parts of the protein structure correspond to structural elements that are frequent targets of non-enzymatic PTMs: methionine oxidation (red), acidic residues deamidation (blue), disulfide scrambling (orange), change in glycosylation pattern (green).

Figure 2.

Mass distribution of mAb obtained by deconvolution of ESI MS data reveals the presence of several glycoforms in the intact protein. Forced oxidation of the protein with 0.06% H_2O_2 for 4 hours at room temperature (red-filled spectrum) results in a noticeable mass increase, as each oxidation event increases the protein mass by 16 Da. The mass change caused by mild deamidation at pH 9.5, 37 °C for one week (blue-filled spectrum) is barely detectable, as each deamidation event increases the protein mass by only 1 Da.

Figure 3.

Fragment ion (CAD) mass spectrum of a proteolytic peptide fragment of mAb readily reveals the presence of a carbohydrate chain (whose facile fragmentation gives rise to a series of abundant fragment ions at $m/z > 1,300$), but is not informative *vis-à-vis* the amino acid sequence (the sequence information can be obtained by using electron-based ion fragmentation techniques, such as ECD or ETD).

Figure 4. Nano-ESI mass spectra (inset contains the deconvoluted spectra) of 6 μ M GCcase (blue) and GCcase-ox (red) solutions in 50mM ammonium acetate, pH 4.5. The gray trace shows a reference mass spectrum of GCcase under denaturing conditions (50% acetonitrile, 5% acetic acid). Reproduced with permission from (Bobst et al. 2010).

Figure 5.

Isotopic distributions of peptide ions representing peptic fragments (185–209, 220–227, 384–396, and 447–456) of GCase (blue) and GCase-ox (red) following 1 and 100 min of HDX in solution. The gray traces represent isotopic distributions within the same peptide for denatured GCase, which allowed for complete exchange (*i.e.*, the maximum level of amide exchange for the peptide) and was analyzed under the same conditions as native GCase. Location of these segments within the crystal structure of GCase is shown in structure **A**. Local backbone amide protection deduced from HDX MS measurements mapped to GCase structure are colored based on ΔHDX (difference between oxidized and intact forms of GCase, exchange time 100 min) is shown in structure **B**. Stars in the lower right panel indicate ionic signal of covalently modified peptide carrying one of the oxidation targets (Met-450). Adapted with permission from (Bobst et al. 2010).

Figure 6.

ESI MS analysis of monomer (black trace) and oligomer (gray trace) SEC fractions of human AT-III, which was in storage for 1 month at 4°C. The SEC chromatograms of the entire sample (black) and re-injected oligomer fraction (gray) are shown in the inset and highlight the dynamic nature of the oligomer fraction. The oligomer fraction was kept at 4°C for 2 weeks prior to re-injection and ESI MS analysis.

Figure 7. Temperature-controlled ESI MS of heat-stressed recombinant AT-III (1 μ M solution, pH 8) reveals the onset of protein unfolding (via appearance of high charge-density monomer ions at $m/z < 3,000$) and aggregation (appearance of abundant oligomer ions at $m/z > 6,000$) above 50°C.

Figure 8.

ESI MS of a mixture of a recombinant transferrin-growth hormone protein (GHT) and the soluble part of transferrin receptor (TfR) shows facile formation of a 1:1 complex under near-physiological conditions (130 mM ammonium acetate 20mM ammonium bicarbonate, pH 7.4). Signal corresponding to unbound TfR reflects the excessive amount of the receptor in solution, while no signal of unbound GHT could be detected (the gray trace represents the reference mass spectrum of free GHT in solution in the absence of TfR).

Figure 9.

HDX MS within two segments of Tf in the presence (blue) and the absence (red) of the cognate receptor. The exchange was carried out by diluting the protein stock solution 1:10 in exchange solution (50 mM sodium phosphate buffer 100mM sodium chloride in D₂O, pH adjusted to 7.4) and incubating for a certain period of time as indicated on each diagram followed by rapid quenching (lowering pH to 2.6 and temperature to near 0°C). The black trace at the bottom of each diagram shows unlabeled protein and the dotted lines represent the endpoint of the exchange reaction. Location of these segments in Tf is shown within the context of the low-resolution structure of Tf/TfR (Cheng et al. 2004). Adapted with permission from (Kaltashov et al. 2009).

Figure 10.

The schematic diagram showing generation of O^{18} -labeled internal standard to assist protein quantitation in complex biological matrices: affinity purification is used to extract the protein of interest from the biological sample (*e.g.*, immunocapture, or other affinity extraction methods, such as heparin column for AT-III, metal affinity column if the protein has a built-in His-tag, *etc.*), followed by proteolysis and LC/MS or LC/MS/MS detection. Proteolysis of the internal standard is carried out in H_2O^{18} , which introduces an isotopic tag on each fragment peptide (by placing O^{18} atoms at the C-terminal of each peptide) without changing its LC elution profile (Fenselau and Yao 2009). The diagram on the right shows a total ion chromatogram of tryptic digest of exogenous human transferrin extracted from bovine serum (black trace); the blue and red traces correspond to extracted ion chromatograms for ions representing tryptic fragment peptides KPVEEYANCHAR derived from Tf in serum (blue) and the internal standard (red). The mass spectrum at the bottom of the diagram shows isotopic clusters of these two peptides (charge state +3). To avoid any signal interference, the spacing between the two isotopic clusters was increased by acid-catalyzed O^{18} -labeling of IS tryptic fragments (which places O^{18} atoms on acidic side chains (Niles et al. 2009)) prior to LC/MS analysis.