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Mass Spectrometry for the Biophysical Characterization of Therapeutic Monoclonal Antibodies

Hao Zhang, **Weidong Cui**, and **Michael L. Gross***
Department of Chemistry, Washington University in St. Louis St. Louis, MO 63130, USA

Abstract

Monoclonal antibodies (mAbs) are powerful therapeutics, and their characterization has drawn considerable attention and urgency. Unlike small-molecular drugs (150-600 Da) that have rigid structures, mAbs (~150 kDa) are engineered proteins that undergo complicated folding and can exist in a number of low-energy structures, posing a challenge for traditional methods in structural biology. Mass spectrometry (MS)-based biophysical characterization approaches can provide structural information, bringing high sensitivity, fast turnaround, and small sample consumption. This review outlines various MS-based strategies for protein biophysical characterization and then reviews how these strategies provide structural information of mAbs at the protein level (intact or top-down approaches), peptide, and residue level (bottom-up approaches), affording information on higher order structure, aggregation, and the nature of antibody complexes.

Keywords

Monoclonal Antibody; Mass Spectrometry; Native ESI; Top-down and Bottom-up; Protein Footprinting; hydrogen/deuterium exchange; FPOP; ion mobility

1. Therapeutic monoclonal antibodies (mAbs)

Therapeutic mAbs may have become the most popular drug candidates following their introduction into the clinic in the late 1980s [1]. Their high specificity and low side effects make mAbs powerful human therapeutics for oncology, autoimmunity/inflammation, infectious diseases, and metabolic disorders [2]. At present, approximately 30 therapeutic mAbs are being marketed. The sales contributed approximately $18.5 billion to the US economy in 2010 [1]. The high demands for new therapeutic mAbs have trigged a burst of mAb-based drug development. For example, 16 human mAbs entered the clinic during 1985-1996, whereas during 1997-2008, 131 human mAbs became available [3]. In 2011, more than 300 mAb-based thereapeutics were in clinical trials [2]. As older mAbs come off patent and go into production as generic drugs, the need for characterizing their higher order structure in quality control becomes even more important, motivating this review.

*corresponding author: CB 1134, One Brookings Dr., St. Louis, MO 63130, USA Fax: 314-935-7484 mgross@wustl.edu.

1.1 Introduction to mAbs

Therapeutic mAbs are glycoproteins that belong to the immunoglobulin (Ig) family. Ig's are used by the immune system to identify and neutralize foreign organisms or antigens [4,5]. Ig's are classified in five groups, IgA, IgD, IgE, IgG and IgM (as α, δ, ε, and μ), based on the structure of their constant regions [6]. At present, most approved mAbs are from IgG's (γ-immunoglobulin). IgG's have the typical "Y"-shaped structure comprised of two identical heavy and light chains (H and L chains) (Figure 1). All heavy and light chains are covalently linked by disulfide bonds. IgGs can be further classified into four groups, IgG1, 2, 3, and IgG4 (as γ-1, γ-2, γ-3 and γ-4) on the basis of different patterns of inter-chain disulfide bonds and heavy-chain sequences. IgG1, 2 and 4 are widely used in therapeutics, whereas IgG3, which has a shorter serum half-life, is rarely used.

Each heavy chain contains one variable (VH) and three constant domains (CH1, CH2 and CH3), whereas each light chain contains one variable (VL) and one constant domain (CL). In the heavy chain, CH1 and CH2 are linked by a hinge region that contains inter-heavy chain disulfide bonds (IgG1 and IgG4 have two disulfide bounds in hinge region, whereas IgG2 has four). Antigen binding is mediated by the variable region, mainly by three loops connecting individual β-strands, which are called the complementarity determining regions (CDRs), from both heavy and light chains. Upstream of the heavy chain (VH and CH1) is the disulfide-bond-linked light chain (VL and CL), known as the fragment antigen-binding (Fab) region. The downstream constant regions (CH2 and CH3) of the heavy chain are called the fragment crystallizable (Fc) region, which is responsible for effector function during recycling [7].

A milestone in the development of therapeutic engineered mAbs was the introduction of murine mAbs from hybridomas [8]. Clinical applications of murine mAbs (suffix: -omab) began in the late 1980s [9]. Dissimilarities between murine and human immune systems led to clinical failure of those antibodies. Murine antibodies are engineered to generate chimeric mouse-human mAbs (~65% human in molecules, suffix: -ximab) by fusing the murine-variable regions onto human-constant regions [10]. The humanized mAbs (~95% human in molecules, suffix: -zumab) are produced by grafting murine hyper-variable regions on amino acid domains of human antibodies [11]. Both chimeric mouse-human and humanized mAbs have reduced immunogenicity and increased serum half-life [12]. With the development of phage-display technology and various transgenic mouse strains expressing human variable domains [13,14], fully human mAbs (suffx: -umab) with significantly reduced immunogenic potential and high similarity to human endogenous IgGs, have become rich sources of new therapeutics [1,3,15].

1.2 The challenge of verifying higher order structure of therapeutic mAbs—

Unlike traditional small molecular drugs (150-600 Da), mAbs are large macromolecules (~150 KDa) with four polypeptide chains held in place by tens of inter- or intra-disulfide bonds as well as by non-covalent interactions [6]. For example, one approved therapeutic mAb, trastuzumab, has 6560 carbon atoms, 10132 hydrogens, 2090 oxygens, 1728 nitrogens and 44 sulfur atoms [16]. The functional form of the protein depends on its higher order structure (HOS), referring to the tertiary 3-D architecture determined by the secondary alpha-helices and beta-sheets, building upon the primary structure, and the quaternary complex formed by interacting/binding with other entities. Sources affecting the HOS of mAbs are not limited to primary structures. Variations in PTMs (post-translational modifications), mutations and modifications can trigger changes in HOS to affect binding to an antigen or to Fc-gamma and Fc-Rn receptors. Production and storage of therapeutic mAbs can introduce significant changes of HOS. From the view of patient health, HOS variations of these proteins can pose serious safety issues [17,18]. HOS can be fleeting for proteins; HOS is certainly more dynamic than primary structure. Although strategies to

determine the primary structure of mAbs, including mutations, PTMs, and other modifications, have been available for decades, approaches to verify HOS are still needed. Although circular dichroism (CD), fluorescence and related optical spectroscopic methods are used for rapid HOS characterization [19], many regional but important structural changes are missed [20]. In the recently published draft guidelines for quality control of biosimilars (copies of therapeutic mAbs that are coming off patent), the US Food and Drug Administration (FDA) acknowledged that "a protein's three-dimensional conformation can often be difficult to define precisely using current physicochemical analytical technology". New approaches are under development to meet this challenge [16,20]. In this review, we focus on the new and promising MS-based biophysical approaches as means for characterization of mAb HOS.

2. Mass spectrometry based protein biophysics

2.1 Overview—The advantages of MS in biology are attracting the attention of structural biologists who address the biophysical properties of proteins [21]. Modern MS instrumentation and proteomics methods offer two major approaches to interrogate protein biophysics. One is an intact or top-down approach that employs native electrospray ionization (ESI), ion-mobility measurements, and fragmentation, usually by interaction with electrons, to provide a global view of the protein of interest [22]. The other is a bottom-up approach combining either protein footprinting [23] or cross-linking [24] that provide detailed peptide and even amino-acid-residue information. These terms "top down" and "bottom-up" first appeared in the MS-based proteomics literature [25]. Compared to bottom-up, top-down is less mature, requiring the invention of electron capture dissociation (ECD) [26] in 1998. Both top-down and bottom-up MS approaches have the advantages of small sample consumption, nearly no limit to protein size, and ability to determine in the gas phase the native or near-native protein properties. Furthermore, MS can be combined with protein footprinting to give an approach that is tolerant to solution media containing MS-unfriendly small molecules. Native ESI and top-down sequencing offer high throughput and unique specificity for oligomeric states and stoichiometry of native protein samples. By combining these complementary MS methods, important structural information can be discovered with intermediate structural resolution. We will review the principles of both approaches in the following subsections.

2.2 Intact and top-down based approach—At present, proteins and protein complexes with MW even at the mega-Dalton range can be directly analyzed by MS [27]. Top-down protocols provide information without requiring proteolytic digestion of protein samples prior to MS analysis [28]. Removing the digestion step should significantly reduce analysis time. Intact proteins and protein complexes are then interrogated close to their functional forms, even as protein assemblies [29]. Species existing in different oligomeric states or with PTMs can be analyzed separately. Although targeted analysis (e.g., oligomer specific analysis) can also be accomplished by bottom-up approaches by adding a pre-separation step, top-down approaches are more efficient. Using an approach targeting intact proteins, we can directly monitor the charge-state distribution and obtain stoichiometry or measure ion mobility of intact proteins or of protein complexes and capture some information about shape and changes in shape by keeping the protein in near-native states in the gas phase. Tandem MS capabilities available on most commercial MS instruments can also be employed to elucidate the conformations by fragmenting portions of a protein or protein complex and interpreting the decomposition reactions. An overview of top-down MS strategies is summarized in Figure 2A where native ESI in the upper half preserves the protein structure; while normal ESI in the lower half deals with proteins that are denatured, and such proteins can be studied further by limited or specific proteolysis to generate large

peptide fragments (middle-out) to improve the sequence coverage by MSMS in a way of middle-down/up that is similar to bottom-up approach but for large peptides.

2.2.1 Native MS (or Native ESI): Native MS has proved to be an alternative strategy to investigate structure in the near-native state of intact proteins and their complexes by using MS platforms that work for gas-phase species [22]. Prior to conducting an MS experiment, the original buffer solution that maintains the protein native structure is exchanged with a volatile ammonium acetate solution that mimics the native buffer but favors solvent (and salt) evaporation during ESI to release protein ions that bear a memory of the structure in solution [30]. In this way, the ionized proteins carry less charge (are less protonated on the surface) than those generated by normal ESI, which utilizes denaturing solutions comprised of water and organic solvents at low pH. As a result, the ions seen in the mass spectrum produced by a native ESI experiment appear at higher *m/z*.

The non-covalent interactions within a protein and between subunits of protein assemblies can also be preserved in the gas phase [29]. It is sometimes relatively straightforward to determine the stoichiometry of a complex by using native MS. More importantly, variations within proteins or protein complexes can be monitored based on their charge-state distribution in native MS [31]. Highly ordered or compact proteins and protein complexes have smaller surface areas than disordered or unfolded ones. This difference can be directly read out from the charge-state distribution [32]. Following the introduction of protein ions into the gas phase, they can be interrogated by ion mobility or tandem MS to obtain structural information, all in a top-down manner [33,34].

2.2.2 Ion mobility measurements: Gas-phase ion mobility provides a collisional cross-section (CCS) of a particular ion drifting through in inert buffer gas in a low electrical field [35]. In the drift region, an ion experiences many collisions with inert buffer gas molecules. The upshot of this ion drift is a two-dimension projection (or CCS) of the three-dimension shape of the ion in free rotation. The readout is the time for the ion to pass through the drift region (called an arrival time distribution (ATD) or drift time). Ions with a large cross section drift more slowly, affording an outcome that is similar to that of a native-gel experiment. Thus, proteins and protein complexes can be separated based on the difference of their native states. An important application of ion mobility is to provide experimental evidence on native protein conformation, which appears to be preserved, at least in part, in the gas phase when the protein is introduced by native MS [36]. Mobility can be applied to differentiate the structure of two ions of the same *m/z*, thus providing information on shape and size in the gas phase [37]. Robinson and coworkers [38,39] have used native ESI and ion mobility to establish a calibration curve for the CCS of proteins and protein complexes. Furthermore, these approaches have also facilitated the investigation of large membrane-embedded protein complexes [40].

2.2.3 Top-down fragmentation: MS/MS-based fragmentation of protein ions affords information on primary structure. A number of approaches can be used to activate ions: collisionally activated dissociation (CAD), sometimes called collision-induced dissociation (CID) [41], electron-capture dissociation (ECD) [26], electron-transfer dissociation (ETD) [42], surface-induced [43] dissociation, and photon-induced dissociation [44] . Top-down fragmentation in this context provides structural connectivity and compositions of the protein or protein assembly [34]. As exemplified by its applications in proteomics [45], top-down sequencing overcomes a disadvantage of bottom-up in which the digestion of the starting proteins leads to a loss of information about protein isoforms, especially for PTMs.

Thus far, CAD/CID in a quadruple/time-of-flight (TOF) instrument has played the dominant role for in characterization of native protein complexes; it reveals the stoichiometry and

topology of a protein assembly [46]. Electron-capture dissociation (ECD) in a Fourier transform ion cyclotron resonance (FTICR) mass spectrometer can identify flexible regions of a protein [47] to reveal isomerization of small proteins from solution to the gas phase [48]. Surface-induced dissociation (SID) tends to release and to distribute charges symmetrically among the fragmented subcomplexes [49].

2.3 Bottom-up approaches

In bottom-up approaches, proteins are digested into peptides before MS analysis. Conformational information of a protein *must be encoded into peptides beforehand*. This encoding can be achieved by labeling solvent-accessible amino acid side chains or backbones. The labeling reagent can involve one reactive group that attaches the reagent to various amino acids (protein footprinting), or have two reactive groups that form a linkage between two amino acids of a protein (protein crosslinking).

2.3.1 Protein footprinting—Footprinting examines ligand binding and conformational changes by determining the solvent accessibility of macromolecules through their sensitive responses to chemical or enzymatic modification and cleavage reactions [50]. Protein footprinting as a complementary approach to probing protein conformation has rapidly developed during the last decade [51]. Footprinting strategies are labeling approaches that include amide hydrogen deuterium exchange (HDX) and hydroxyl-radical based labeling; they are becoming sufficiently characterized that they can have wide usage. MS-based protein footprinting approaches can be classified into two groups: reversible and irreversible, as is summarized in Figure 2B. Protein footprinting can also be combined with top-down MS, as was shown recently [52,53].

In this review, we focus on the original bottom-up approach for protein footprinting.

A. Reversible HDX footprints therapeutic proteins: The hydrogens of solvent-accessible amino-acid backbones and side chains can exchange with deuterium when a protein in normal water is diluted into a buffer containing D_2O, initiating deuterium uptake by the amides and other active sites of the protein. The mass shift induced by deuterium uptake can be monitored by MS to reveal some structural features of a protein [54]. The resolution of the HDX MS platform can be further improved by adding proteolytic digestion accompanied by LC separation [55]. At present, even super protein complexes, like viral capsids and *E coli* ribosome, have been analyzed by HDX MS [56,57], well beyond the capabilities of NMR, which was the dominant tool in the early development of HDX.

There are three types of hydrogens in proteins: those on side-chain carbons, on heteroatoms, and on the backbone amide linkages. There is no measureable exchange for the carbon hydrogens, whereas the exchange rate for most hydrogens on heteroatoms is too fast to be followed during normal HDX LCMS experiments (except for those on histidine [58]). Those on the backbone amides of all amino acids (except proline) exchange at measurable rates. The amide hydrogen exchange can be significantly slowed (effectively quenched) such that the rate constant decreases by 10000 times when the conditions (pH 7 at 25 °C) are changed (pH 2.5 at 0 °C) prior to MS analysis [59]. The quenching condition (pH 2.5) is compatible with denaturing positive-ion electrospray ionization (ESI), making MS a good detector for the outcome of HDX. More importantly, mass shifts (induced by deuterium uptake) instead of peak intensities are measured in MS based HDX, which avoids the problems of changes in ionization efficiency for the various constituent peptides. Nevertheless, sample preparation and analysis of the peptides to give “regional information” still must be done quickly (e.g., < 10 min), limiting chromatographic resolution and restricting the use of complex matrices or samples for study.

B. Irreversible footprinting approaches: For irreversible approaches, the labeled protein sample can survive extensive separation and purification after the labeling experiment, whereas reversible HDX suffers back exchange. The labeling approaches can be relatively general or site-nonspecific [60] (e.g., hydroxyl radical labeling), or site-specific (such as carboxyl group labeling).

Hydroxyl radicals footprint therapeutic proteins: Radicals are usually highly reactive and have a short life time, making them good labeling reagents in protein footprinting. The generation and control of radicals, however, are not easy. Although development of other radical besides •OH as protein footprinting reagents have been reported [53,61], the most popular radical in protein footprinting remains the hydroxyl radical, which is similar in size as water molecules and is highly reactive toward approximately two thirds of the amino-acid side chains.

Hydroxyl radicals can be generated by electron-pulse radiolysis, synchrotron radiolysis of water, laser photolysis of hydrogen peroxide, Fenton and Fenton-like reactions, and high-voltage electrical discharges [62]. Although Fenton and Fenton-like reactions were used early on for protein footprinting [63], the speed of Fenton and Fenton-like reactions is relatively slow (minutes). To speed up the process and avoid label-induced unfolding, synchrotron radiolysis of water [64] and the laser photolysis of hydrogen peroxide to make radicals [65,66] are the most promising. The photolysis of water in the kilovolt X-ray range causes water to ionize and lose a proton to give hydroxyl radicals [67]. No reagent besides the solvent water is required in this experiment. The reaction time can be controlled by irradiation time. Chance's group developed a systematic approach that uses synchrotron light source, found in national labs, for protein footprinting studies [68]. Recently, that group investigated the water distribution in the membrane-embedded channel complexes [69]. The access to synchrotron light sources, however, limits the general application of this method. Laser photolysis of hydrogen peroxide is an alternative that can be set up in most chemistry laboratories.

We developed a laser photolysis approach, which we call Fast Photochemical Oxidation of Protein (FPOP), to form •OH, in a few-nanosecond, 248 nm laser pulse that photolyzes low concentrations of hydrogen peroxide (0.04%, 15 mM) in a flowing solution containing the protein of interest. FPOP limits the radical lifetime by using a scavenger (free amino acids, like glutamine or histidine) to ensure the labeling reaction takes place within approximately 1 μs [70-72]. We took advantage of the fast labeling of FPOP to study fast protein folding by a "pump-probe" method whereby we use two lasers, one to supply a temperature jump, and the second to generate hydroxyl radicals that footprint the protein as a function of its folding time (hundreds of microsec) [73].

Glycl ethyl ester and other reagents label proteins in a site-specific manner: A variety of chemical reagents can be used to modify specifically certain amino acids [23]; an example is the carbodiimide-mediated coupling reaction between glycine ethyl ester and the carboxyl groups of a protein [74]. Another example of a reactive amino acid group is the thiol of cysteine, which can be modified by several reagents (e.g., iodoacetamide, NEM [75]). Major drawbacks of site-specific labeling are that the labeling is slow relative to FPOP, and less conformational information can be obtained because the target residues on the surface are limited in number (i.e., the method has limited structural resolution). Site-specific labeling is recommended for studies of very complicated systems that have hundreds of target residues. An advantage is the data analysis, identifying and quantifying labeled products, can be simplified because the target list can be narrowed. We reported several examples that utilized site-specific labeling for the study of complicated systems like membrane-embedded protein complexes implicated in photosynthesis and cancer [76,77].

2.3.2 Protein cross-linking—The chemical-labeling attribute of protein footprinting can be extended to chemical cross linking by using bi-functional labeling reagents (e.g., *N*-hydroxysuccinimide esters). Chemical cross-linkers can modify two amino-acid side chains within a designed distance and form a covalent linker between the two residues, provided the residues are with the distance constraint [24,78,79]. The cross-linked peptides can be identified by LC-MS/MS after proteolytic digestion. Information about the distance between the two residues, defined by the length of cross-linker reagent, can be used to determine adjoining proteins in a protein-protein interaction or to locate within a protein two interacting domains. With the development of new isotope-encoded linkers, cleavable linker, and tagged linkers [80], protein crosslinking has become a more effective tool to determine sites of protein-protein interaction in complicated biological systems.

3. MS-based characterization of antibodies

The higher order structural variations of mAbs and their dynamics must be addressed during discovery and development as therapeutics. Many approaches taken from structural biology can be applied [16,19,20]. Traditional biophysical techniques, like X-ray crystallography (X-ray), nuclear magnetic resonance (NMR) and cryo-electron microscopy, are hampered by the size of the protein, the amount available, and the need to determine dynamics of mAbs. MS has become an essential analytical tool for the therapeutic mAb development owing to its superior resolution and speed, allowing it to monitor primary structure and locate post-translational modifications (PTMs) [6,81]. Variations of primary structure, including those of disulfide-linkage location, amino-acid sequence, PTM location, and other in-storage modifications can be determined by MS in all phases of mAb production. Although MS-based structural approaches are still limited by the lower resolution compared to NMR and X-ray, they are more efficient because they have high sensitivity, can be applied to proteins in various environments, and have high throughput.

Applications by MS are rapidly growing [21,82]. Currently, many MS-based biophysical approaches focus on differentiation of mAb isomers [83,84]. Those isomers could arise as a consequence of primary structural variations or variables in production and storage. In this section, we review MS strategies that can address three important and challenging issues: quality control of high order structure (HOS), assessment of aggregation, and mapping of antibody-antigen interfaces. To provide future perspective, we include several new developments in the MS-based biophysical studies to demonstrate their potential for mAb characterization.

3.1 Higher order structure (HOS)

3.1.1 Intact and top-down approaches—The observation of intact mAbs in native MS is the most direct measurement of an antibody. We now can introduce mAbs into the gas phase with minimal perturbation of their native conformation. As a simple first approach, we can follow the lead of Kaltashov et al. [85]. Who reported that protein conformational variations can be directly observed by observing their charge-state variations when introduced to the mass spectrometer by native MS. This approach is recommended as an early one to apply for quality control of mAb HOS [86].

Combining native MS and ion mobility provides a simple and direct shape/size measurement of mAbs. Structural information obtained from ion mobility refers to the global conformations of species even with the same MW. The first demonstration of ion mobility for antibodies was by Bagal et al [87] to differentiate, using native ESI, the conformations of IgG2 isomers caused by disulfide linkage variations. They used IgG1 as a control because it does not have S-S isoforms, and found that IgG2 has a longer drift time than IgG1 and shows two distinct peaks in the ATD of each charge state. They concluded

that these two peaks correspond to IgG2-A (shorter time) and IgG2-B forms, respectively. The double peaks were not caused by glycosylation, which was confirmed by redox-enrichment of A and B after refolding in the presence and absence of GuHCl. Heck and coworkers [88] used native MS to monitor the CH3 domain swapping between human IgG4 molecules, a process related to spontaneous Fab-arm exchange to form bi-specific antibodies [89]. Beck and coworkers [90] monitored the dynamics of this process by using native MS combined with time-resolved ion mobility; their results demonstrate the high potential of IM-MS for characterizing biopharmaceutical protein products.

We in collaboration with colleagues at Pfizer [91] used a strategy combining native ESI, ion mobility, ECD top-down, and hydroxyl-radical footprinting (FPOP) to characterize five IgG2 disulfide isomers including the wild type. Ion mobility showed two major drift time peaks for the WT isoform, consistent with Bagal's observation [87]. The shorter drift time peak of the WT is approximately the same as those of the other four mutants. ECD in a top-down mode sequenced a flexible portion of structure, providing a result that is consistent with the FPOP results (Figure 3).

3.1.2 Bottom-up approaches—At present, the most used bottom-up approach for characterizing mAb HOS is HDX. Most HDX studies differentiate mAb structural variations and provide evidence that the HOS of an unknown protein is that of a reference (i.e., both give nearly identical HDX kinetics and extents (footprints) at the peptide level). Differences in HDX pinpoint regions that have changed their HOS. Summaries of protocols for HDX MS applications can be found in recent publications [92,93]. Here, we describe several examples that demonstrate how HDX MS provides HOS information of mAbs.

Glycosylation changes antibody behavior, increases solubility, imparts longer shelf-life, increases resistance to unfolding and proteolysis, and lowers aggregation rates [94]. Little is known about the relevant structural changes caused by glycosylation. Houde et al. [95] utilized HDX MS to probe the conformational changes for IgG1 with and without glycans. HDX MS can also be used to probe structural variations and receptor binding induced by other PTMs (e.g., methionine oxidation and fucosylation) [96,97]. One major mAb isoform is a charge variant (or charge heterogeneity) [98] designed to maintain optimized electrostatic interactions of the favored structure and have the desired reactivity. Modification, degradation, and covalent adduction can result in net changes of the positive or negative charge (pI value) of a mAb, and ultimately change its structure and stability. Tang et al. [99] demonstrated how HDX and an extended approach to give affinity (i.e., SUPREX) can characterize conformation and stability of charge variants of human IgG1.

Variations of HOS may also result from storage of mAbs. Furthermore, effects of excipients used in the therapeutic preparation also need to be evaluated as to their effect on the stability of mAbs. Manikwar et al. [100] reported the use of HDX MS to measure local dynamics of mAbs stored in different environments. This approach can be extended to examine the effects of salts on stability, aggregation propensity, and local flexibility of mAbs [101].

Hydroxyl-radical-based protein footprinting methods are a more recent development than HDX, but they can also be applied to protein therapeutics to afford information similar to that of HDX. Watson et al. [102] demonstrated the use of FPOP in structural studies of the protein therapeutic, granulocyte colony-stimulating factor (GCSF). Recently, we reported the application of FPOP for the structural characterization of conformational differences of mAb IgG2 isomers. We found that the FPOP results are consistent with top-down ECD data and point to flexible regions of the protein. Furthermore, FPOP identifies local conformational changes and reveals solvent accessibility in the CDR [91]. Thus, FPOP complements HDX but may be more reliable and versatile because it imparts an irreversible

change to the protein on the microsecond timescale. Moreover, analysis of the outcome of free-radical footprinting can take advantage of advances in analytical proteomics. Radical-labeling outcomes are a measure of solvent accessibility, and the speed of FPOP can be used to locate dynamic or flexible regions of a protein. Other slower labeling approaches including HDX give a time-averaged view.

Site-specific labeling can also measure solvent accessibility of certain residues. Zhang et al. [103] applied the specific labeling reagents, sulfosuccinimidyl acetate, for lysine and *p*-hydroxylphenylglyoxal for arginine, to reveal a positive-charge patch on an antibody. Free mAbs and a resin-bound antibody were labeled and analyzed by LC-MS/MS. The charge distributions as well as the antibody-resin binding interface were successfully located.

3.2 Protein Aggregation

Antibody aggregation is a common problem occurring in protein manufacturing and storage [104]. The importance arises because the functional form of a protein is often the monomer, whereas higher oligomers not only reduce the dose efficiency but also pose toxicological problems. This is a serious issue facing the biotechnology and pharmaceutical industries [105]. Understanding the mechanism of aggregation is important, therefore, not only for manufacturing and storage of proteins but also for shedding light on protein aggregation in general. It is not surprising that protein aggregation is attracting a considerable attention given that serious problems in human health can be caused by protein aggregation. The analytical tools used for analysis of protein aggregations in neurodegenerative disease were recently reviewed [106], and they may also be applicable to the problem of antibody aggregation.

3.2.1 Intact and top-down approaches—Native ESI can be used to analyze monomeric antibodies and their associated glycosylated forms. Given the narrower charge-state distribution produced by native ESI, ion signals are more concentrated than in normal ESI. Thus, the sensitivity for the low-abundant modified forms becomes higher. The Heck group in the Netherlands has observed IgG4 oligomers up to tetramers by using this approach and even monitored the dynamics of IgG4. When size-exclusion chromatography (SEC) is integrated with native ESI, and combined with fractionation, the antibody aggregates can be separated and analyzed in more detail [107].

3.2.2 Bottom-up approaches for aggregation—Understanding the mechanism of aggregation and locating the aggregation interface are vital to develop preventive measures. We [108] developed a pulsed HD exchange labeling strategy that can monitor the conformational changes during aggregation processes of Abeta-42 peptide, adding a new approach to the “toolbox” for protein aggregation studies. The approach should have applicability for study of aggregation of other proteins, possibly including antibodies. The advantages of this new platform are that no modification (e.g., addition of a fluorophore) of the protein is needed, information can be achieved at the peptide level, and factors that affect aggregation can be readily evaluated for following the HDX patterns.

Cross-linking can also be applied to proteins in different oligomeric states collected after SEC separation. Identification of resulting linked peptides by bottom-up MS pinpoint the interface and interaction regions.

Freeze-thaw stresses induce protein aggregation. Zhang et al. [109] reported the use of MS-based HDX to assess the impact of freeze-thaw cycling on protein structure, demonstrating that the aggregation mechanism of mAbs under thermal and freeze-thaw stresses can be revealed by HDX MS [110]. Engen’s group [111] used H/DX and other biophysical measurements to compare the monomeric and dimeric forms of two mAbs, respectively, to

connect aggregation with the structural changes in antibodies, They found that the dimerization has no effect on the deuterium uptake between monomer and dimer forms of one of the mAbs. However, the other mAb monomer showed subtle changes in HDX in the CH 2 domain and the hinge region between CH 1 and CH 2 domains, as compared with its dimer form.

Cold storage of proteins, particularly with traces of hydrogen peroxide carried over from FPOP, for example, can lead to protein oxidation [112]. Usually removal by various solid-phase desalting methods, catalase treatment, or freeze-drying after protein footprinting is critical to insure no uncontrolled oxidation, which does occur even at −80 °C. Taking a positive view of this phenomenon, we see possibilities to use cold chemical oxidation to bring insights in a manner similar to FPOP or Fenton chemistry to protein aggregation occurring in cold storage.

The combination of size-exclusion chromatography and hydroxyl radical labeling should be considered for the characterization of mAb dimers [113]. The small amount of dimer in the mAb product can be separated by SEC and labeled by hydroxyl radicals generated via synchrotron irradiation (ionization of water). The dimer interface as well as the dimer orientation can be elucidated by this footprinting and site-specific digestion[113].

Gu and coworkers [114] used cross-linking to probe the interaction of the Fc moiety of a mAb expressed in Chinese hamster Ovary cells. They formed aggregates by incubation at 40 °C for 6 h, followed by reaction with the bifunctional crosslinker BS3. Their results suggest the interaction occurs at three Fc molecules in the CH2 and CH3 domains, indicating not only the Fab region but also the Fc region can aggregate.

3.3 mAb complexes

3.3.1 Intact and top-down approaches—Antibody aggregates can be treated as homogeneous complexes whereas antibody/antigen complexes are heterogeneous. Because of the fast turnaround of native MS, it can be used to screen small–molecules for drug candidates. For mixtures of small molecules, the stoichiometry, relative intensity, and affinity of mab/Ag complexes can be obtained in one experiment. The relative intensity should reflect the relative abundance of a complex, which can be used for relative quantification, assuming there is no discrimination in the native spray. Klassen and coworkers [115] have extended their program on protein/carbohydrate complexes to assess antigen-binding fragments in an assay of carbohydrates. Combining this approach with ECD top-down sequencing should locate the binding pocket in such systems.

3.3.2 Bottom-up approaches—Antibody-antigen interactions are the core function of mAb therapeutics. Understanding this interaction and locating the interface (epitopes) are crucial in mAb design. Epitope mapping is also involved in the patentability and protection of intellectual property [93]. Footprinting by HDX and MS and comparing the outcomes of free antigen and antibody-antigen complex can locate the epitope binding region. A number of examples of this approach were reported [109,116-119]. Similar approaches should be possible with FPOP, and an example is illustrative, underscoring the advantage of irreversible labeling for epitope mapping [120]. One should be cautious in the interpretation of epitope mapping data by FPOP or HDX. The changes induced by antigen binding may be away from the epitope binding, resulting from remote or allosteric effects [121], and these changes will also be picked up. Furthermore, the binding imparts thermodynamic stability to the protein that may cause overall protection. Complementary information from other techniques, like modeling, site-directed mutagenesis, and x-ray [119], should help resolve these issues.

4. Conclusions

Recently, mass spectrometry has seen dramatic evolution in instrumentation and method. In the meantime, the biotechnology and pharmaceutical industries are in a stage of expansion that requires new analytical means to characterize their protein products at each step from preparation to storage and shipping. Mass spectrometry is poised to address higher order structure, aggregation, and binding with antigens, providing a powerful physicochemical approach that spins off its development for protein biophysics and structural biology. MS information can serve as guideline for production of the next generation of engineered therapeutic proteins. No single method, however, can provide complete information of a complex protein system. Rather, utilization of combined methods (Figure 4) including native MS, top-down and bottom-up sequencing, footprinting, and modeling should be effective in generating a full picture of a protein.

For the future, the technological advances of mass spectrometry will continue, driven in part by the complex problems in biological sciences. For example, the recently modified Orbitrap Q-Exactive instrument has demonstrated high sensitivity and resolving power for large proteins and protein complexes [122] as demonstrated by Heck and coworkers [123], who employed this instrument to analyze intact antibodies. The implementation ETD, SID, or UV photodissociation [124] for MSMS may provide a new dimension of information for mAbs. UV photodissociation, owing to its fast pulsing and high fragmentation efficiency, may drive top-down sequencing to a new level. These combinations coupled with SEC in the front end would increase the power of native ESI and top-down MS/MS. Trapped ion mobility spectrometry (TIMS) [125] has become a new member of the ion mobility family, showing high resolution in just several centimeters drift length and suggesting that improvement in IM are also forthcoming. All these new developments would add to the collection of tools for the characterization of protein therapeutics.

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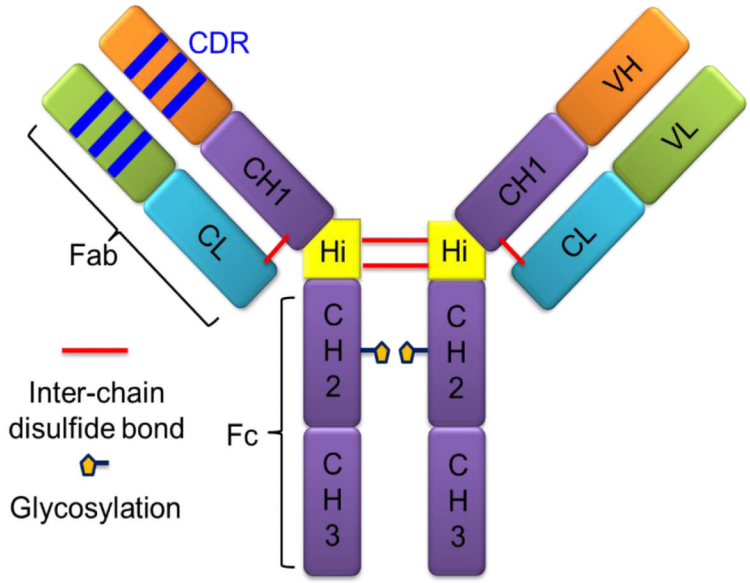

Figure 1.

IgG structure (IgG1). The global structure of IgG1 has two identical heavy chains and light chains. Four chains are attached covalently with inter-chain disulfide bonds and also by non-covalent interactions. The two constant regions from heavy chains (CH2 and CH3, Fc regions) respond to the binding to Fc gamma and FcRn receptors. The variable region from both light and heavy chains contains antigen-binding regions (CDRs). Variable regions with the close constant region together are called the Fab region. The Fab and Fc region are linked by the hinge region in the heavy chain. In the IgG1, there is a glycosylation site on the second constant region (CH2).

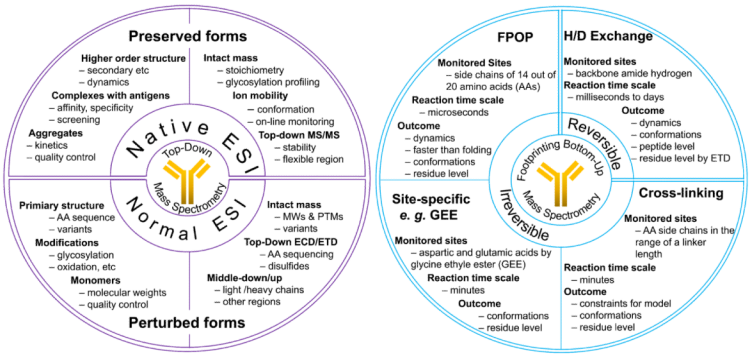

Figure 2.

Overview of top-down and bottom-up MS based protein biophysical studies. The left circle is the summary of top-down approaches. The right circle is the summary of bottom-up approaches.

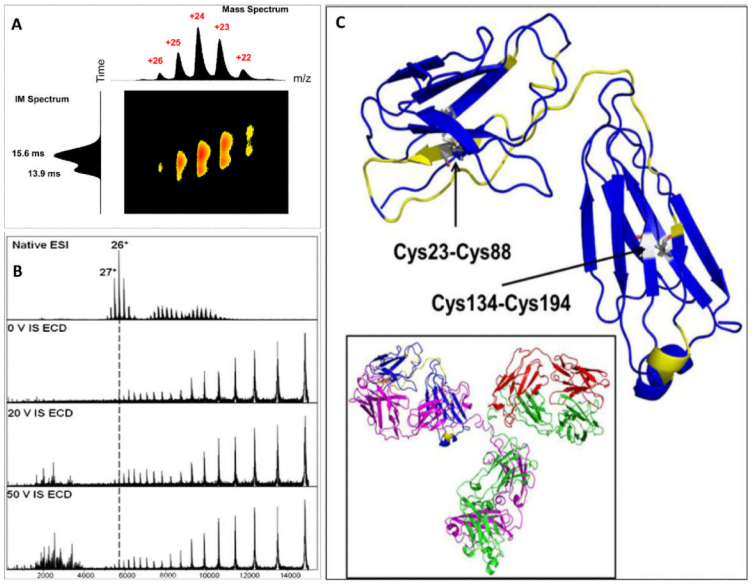

Figure 3.

Native ESI, IM and ECD mass spectra of WT IgG2, (a) ion mobility separation, (b) ECD top-down with in-source activation, and (c) highlighted region in yellow of light chain in CDR showing ECD cleavage sites. (Copied with permission from JASMS)

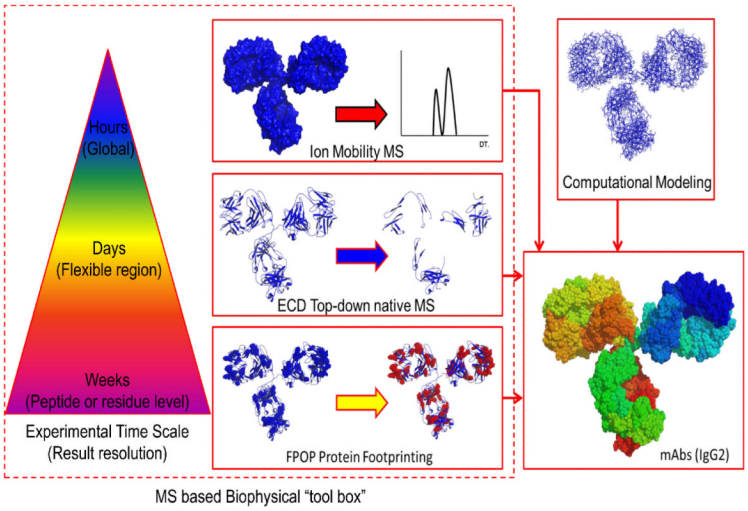

Figure 4.

The MS based biophysical "tool box" in characterizations of IgG2 with different S-S bond networks. This is an example of combining multiple approaches, not limited to MS, to provide complementary structural information of mAbs. The experimental time scale and structural resolution are labeled.