


REVIEW

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Advances in mass spectrometry-based approaches for characterizing monoclonal antibodies: resolving structural complexity and analytical challenges

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Abstract

Mass spectrometry (MS)-based intact mass analysis and structural characterization of biotherapeutic proteins such as monoclonal antibodies (mAbs) are a crucial characterization approach from upstream drug development to downstream product analysis. Due to various endogenous modifications leading to the structural heterogeneity and several *N*-linked glycan species resulting in macro-heterogeneity, it is challenging to characterize the mAbs. Hence, it is essential to understand the micro-heterogeneity of such proteins with high level of complexity which may vary in charge, size, or hydrophobicity. The development of high-throughput native separation techniques hyphenated with MS with high sensitivity and excellent mass accuracy has improved the top/middle down analysis, intact mass detection, subunit analysis, enhanced sequence coverage, and accurate localization of site-specific modifications. In this review, we have focused on the critical inroads taken for the improvement in MS-based techniques to resolve the challenges related to analysis of mAbs. Various MS-based techniques and their role in high-order structural analysis and the progress in software development have been explained, and further, the challenges remaining have been discussed.

Keywords Monoclonal antibody, Native, Top down, High throughput, Mass spectrometry

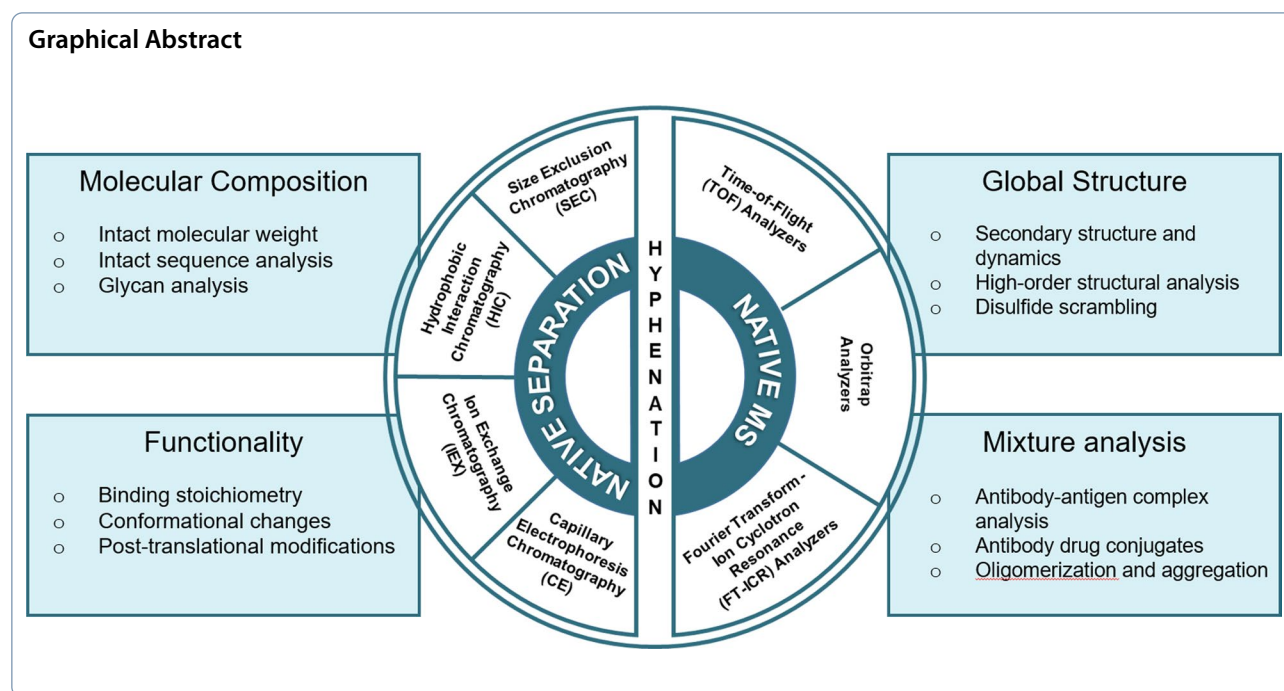
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Introduction

Although mass spectrometry (MS) was born in the field of physics during the hunt for electrons by Joseph John Thomson, since then it has come a long way and today it has become a practically ubiquitous research instrument in the domain of analytical chemistry (Griffiths 2008). With several scientific breakthroughs such as accurate measurement of atomic weights, identification of isotopes, and characterization of elements and molecular structures, it has proved to be one of the most essential complex instruments in the field of science and technology. Throughout the twentieth century, the developments in the MS showed its practicality to the world. By the 1940s, commercial MS was available; however, the first groundwork for modern MS was led by three chemists, Fred McLafferty, Klaus Biemann, and Carl Djerassi. They revealed the distinct mechanism for fragmentation of organic compounds which in turn led to the discovery of unknown structures of organic molecules by MS (Reinhardt 2006).

In 1946, the first concept of time of flight (TOF) was proposed by William E. Stephens (Wolff and Stephens 1953), and gradually in the mid-1950s, Wolfgang Paul reported the first quadrupole mass filter (Wolfgang and Helmut 1960). In 1974, Alan Marshall and Melvin Comisarow revolutionized ion cyclotron resonance (ICR) MS by applying Fourier transform (FT) to it and the first commercial FT-ICR MS was developed which is one of the valuable ultrahigh-resolution MS today. Although several ionization techniques such as electron ionization,

fast atom bombardment (FAB), thermospray ionization, and plasma desorption were developed, however, none of them was of much use for analysis of large biomolecules and required a high concentration of small biomolecules. Eventually in the 1980s, with the development of soft ionization techniques such as electrospray ionization (ESI) and matrix-assisted laser desorption ionization (MALDI), a revolution was brought in the field of biological MS-based characterization of biomolecules (Karas et al. 1985).

Recent developments in ionization techniques and mass analyzers have led to the improved potential for resolution, sensitivity, accuracy, and reliability, and hence, MS has become a significant integral part of comprehensive protein studies. In the field of structural biology, MS has emerged as a complementary tool to other biophysical techniques such as EM, NMR, and X-ray crystallography. By coupling MS with diverse separation techniques, complex mixtures of proteins can be easily separated, identified, and analyzed which can be additionally utilized to study protein interactions, post-translational modifications (PTMs), etc. MS-based proteomics approaches can be classified into various types based on their workflow. In a typical bottom-up approach, protein samples are enzymatically digested to produce smaller peptides to be characterized by MS. On the contrary, with advancements in MS techniques, intact proteins can also be characterized by the top-down approach.

In the past few decades in the field of biotechnological and pharmaceutical research, therapeutic monoclonal

antibodies (mAbs) have emerged as an enticing treatment for immunological diseases, neurodegenerative diseases, cancers, and various other diseases due to their high specificity and reduced side effects. Muromonab-CD3 was a murine mAb against T-cell CD3 and was the first therapeutic mAb to be approved by US FDA in 1986. Initially, murine antibodies were used for therapeutic mAb development until chimeric and humanized mAbs provided a better immunogenic profile decreasing the immunogenicity of murine mAbs (Lu et al. 2020). The development of humanized antibodies using the complementary-determining region (CDR) grafting approach was a remarkable development that hastened the approval of therapeutic mAbs. The success of humanized mAbs led to the new technologies to develop fully human mAbs (Jones et al. 1986). As a result, currently, most of the research focuses on fully human mAbs.

One of the significant breakthroughs in MS approaches is native MS, enabling the characterization of biomolecules in their native folded state. Notably, in recent years, native MS has gained considerable traction as an advanced and efficient technique for analyzing mAbs (Thompson et al. 2014), their derivatives (Valliere-Douglass et al. 2012; Hengel et al. 2014), and Ag–Ab complexes (Atmanene et al. 2009; Fo et al. 2013). It is instrumental in studying mAb interactions with ligands, receptors, or other molecules. It enables the detection of intact mAb complexes, providing insights into binding stoichiometry and affinity, which are crucial for understanding therapeutic efficacy. It provides information on the intact mass, glycosylation patterns, and higher-order structure without the need for extensive sample preparation or enzymatic digestion. In this review, we aim to delve into the recent developments and advances in native protein separation techniques, focusing specifically on the MS-based analysis of intact mAbs.

Chemical structure of antibodies

MABs are therapeutic antibodies that have monovalent affinity and can bind to only single epitopes. They are made of four polypeptide chains, including two identical light and two identical heavy chains bounded by disulfide bonds into a “Y”-shaped tetramer. The C-terminal and the N-terminal regions of the antibody chain are further subdivided into constant and variable regions. The antigen-binding sites are present in the variable regions as the amino acid sequence in this region varies largely, whereas the constant region of the heavy chain determines the isotype and effector functions of the antibodies. Based on the variation in amino acid sequences of these constant regions, immunoglobulins (Ig) are classified into five classes, namely IgA, IgD, IgE, IgG, and IgM, and their heavy chains are denoted as α , δ , ϵ , γ , and

μ , respectively. On the other hand, light chains are categorized into kappa (κ) and lambda (λ) types; however, there are no known functional differences (Moorthy et al. 2015). There are 3 functional components of an intact antibody molecule, i.e., two F_{ab} or fragment binding domains and the F_c or the fragment crystallizable. The hinge region connects the two F_{ab} and the F_c domains which provide the F_{ab} regions with better conformational flexibility as compared to the F_c domain (Hayashi et al. 2000).

IgG is the most abundant class of antibodies found in the human body; hence, to understand the chemical structure of antibodies, we will exclusively focus on IgG structure, although other classes of antibodies differ from IgG by the number of structural domains or in the length of the heavy chain. In the case of IgG, the heavy chain consists of one variable (V_H) and three constant (C_{H1} , and C_{H3}) domains, whereas the light chain consists of only one variable (V_L) and one constant (C_L) domains as shown in Fig. 1. Each variable domain contains 3 regions known as “hypervariable loops,” also known as complementarity-determining regions (CDRs), that identify the antigen. IgG is further subdivided into IgG1, IgG2, IgG3, and IgG4 subclasses based on the length of the hinge region (Chiu et al. 2019). Understanding these structural intricacies illuminates the biological function and therapeutic potential of antibodies and ADCs.

Native MS instrumentation

Performance metrics in high-resolution MS

Essential metrics in the performance of native MS analysis need to enhance continuously with the complexity and ever-increasing size of therapeutic molecules. The urge to investigate macromolecules with molecular weights (MW) in the mega Dalton (Da) mass range has continuously pushed the mass range bounds of native MS (Snijder et al. 2013). Sensitivity and high mass accuracy are also critical parameters for quality control analysis of large therapeutic macromolecules (Rathore et al. 2018). Further demands for distinguishing minute mass differences between species are leading to the development of new-age high-resolution native MS technologies.

Mass resolution is typically defined as the difference in mass between two equally abundant peaks and is expressed as $m/\Delta m$, where m represents the m/z ratio of a singly charged ion and Δm is obtained at a peak height of 50% also known as the full width at half maximum (FWHM) (Tsybin et al. 2019). Often mass resolution is confused with a mass resolving power which is the ability of the equipment to distinguish small m/z value differences between two peaks and is defined as the peak width, Δm (Murray et al. 2013). With respect to small molecules and peptides, high-resolution MS is used to

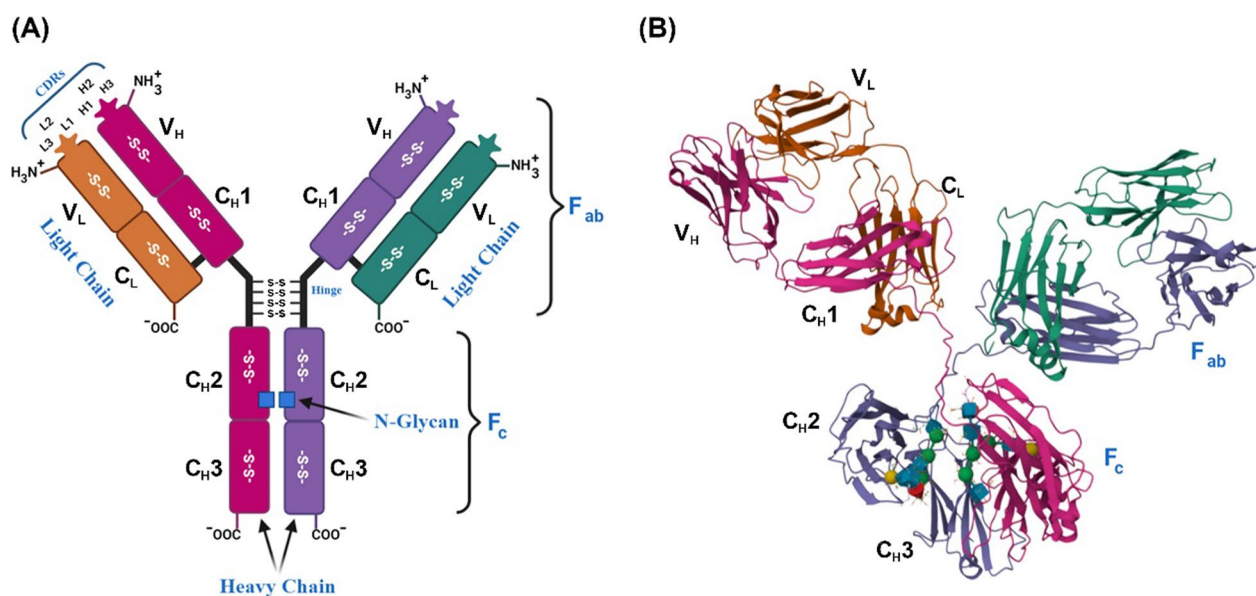


Fig. 1 **A** Schematic representation of typical IgG structure **B** ribbon representation of intact IgG1 isotype (PDB: 1IGY) (Harris et al. 1998)

determine accurate masses to distinguish isotope patterns. Contemporary FT MS instruments can achieve the highest resolution in the range of millions and sub-ppm mass accuracies (Bowman et al. 2020). FT-ICR MS has been reported to achieve the absolute highest resolution beyond 10^6 at $m/z=200$ Th followed by Orbitrap analyzers. However, for larger molecules (MW ~ 150 kDa), the isotopic distribution sometimes overlaps due to the presence of salt adducts or other solvent compounds detected along with the target samples (Fig. 2A). As a result, signals acquired are sometimes distorted. Due to this effect, the measured peaks of globular proteins are much wider than predicted and this is more evident for bigger molecules (Fig. 2B).

Moreover, for the analytes in the MDa range, the methods used for desalting and complete desolvation of ions are partly effective (Tamara et al. 2021). PTM-related microheterogeneity occurs in large multiply charged macromolecules with a minute difference in m/z ratio (Snijder et al. 2013). The large multiply charged protein assemblies are also difficult to analyze in the native condition since they are more prone to decay due to field imperfections. Nonetheless, intermediate instrumental resolutions can prevent the decay of large ion signals due to shorter acquisition time in FT-based native MS systems (Kafader et al. 2020).

Typically, mass accuracy is described as the mass error as a fraction of the hypothetical mass, which is frequently expressed in ppm or ppb. The achievable mass accuracy is often determined by the resolving power of the equipment. Often in native MS, the actual peak width

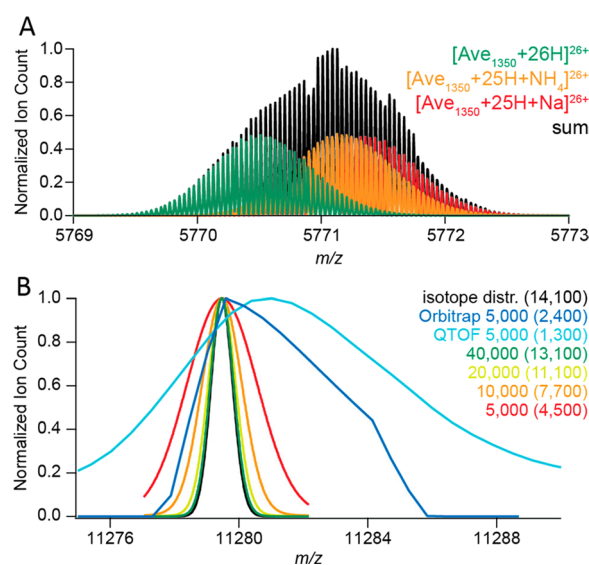


Fig. 2 **A** Effects of adduct ions on mass resolving power. Individual simulations of the isotope distributions of unmodified (green), ammonium-bound (orange), and sodium-bound (red) averagine protein ions were combined to create the final mass spectrum (black); **B** mass spectra of GroEL (+71 charged) ions on a QTOF (cyan) and Orbitrap (blue) simulated with MassLynx ver 4.1 at maximum mass resolution of 40,000 (green), 20,000 (yellow), 10,000 (orange), and 5,000 (red). The natural isotope envelope of GroEL is denoted with black. Reprinted with permission from Ref (Lössl et al. 2014). Copyright 2014 American Chemical Society

transcends the theoretical peak width specified by the instrumental resolution (Lössl et al. 2014). It is essential to obtain monoisotopic masses either directly or by

presuming from the isotope distribution during peptide sequencing or analysis of small proteins. However, in the case of pure macromolecules, it is difficult to obtain isotopically resolved spectra (Senko et al. 1995). Technically, a fine mass calibration either internally or externally is very essential to obtain high mass accuracy. However, internal calibration of the mass analyzers is quite challenging; hence, external calibration is more popular.

Analyzer-specific systemic mass measurement errors (MMEs) vary considerably since various analyzers follow a distinct method of mass analysis. For instance, systemic MME can occur due to a change in temperature during LC separation (Loboda et al. 2000); popular analyzers such as Orbitrap or FT-ICR used in native MS can also suffer electric field imperfections (Makarov et al. 2019) or space charge effects leading to MME (Gorshkov et al. 2010), or even, outdated calibration coefficient may also contribute to MME. However, MME should be eliminated in order to equal the mass accuracy to the mass measurement precision.

Features of mass analyzers

Mass analyzers with broader m/z ranges are essential since under native conditions large protein assemblies generate significantly fewer charges by ESI compared to denaturing conditions. Initially, TOF analyzers were extensively relied upon for native MS due to their theoretically unlimited m/z range (Boesl 2017). However, significant advancements in native MS platforms over the past few decades have prioritized the development of technologies like Orbitrap and FT-ICR mass analyzers. These advancements focus on achieving higher resolution, enhanced mass accuracy, and an extended m/z

range, thereby enhancing the applicability and versatility of native MS beyond its earlier limitations.

TOF mass analyzers

Although TOF analyzers were developed in the 1940s by the name “Velocitron” (Cameron and Eggers 1948), however, main advancements that were crucial for native MS came considerably late in the 1970s, when TOF-reflectron was developed by Mamyrin et al. (Mamyrin et al. 1973). With the development of orthogonal acceleration TOF (oa-TOF) in the early 1990s, the resolution of TOF analyzers was further improved by combining the velocity and position of the accumulated ions before acceleration (Dawson and Guilhaus 1989). Soon Q-TOF mass analyzers were developed by hyphenating ion selecting quadrupole (Q) compartment with TOF (Fig. 3). In the late 2000s, first-generation Q-TOFs were modified by introducing low-frequency quadrupole, high transmission grid ion optics, high-pressure collision cell, and a low-repetition pusher which led to the pioneering development for quantification and analysis of large macromolecular assemblies.

Over the past few years, the primary focus of the development of TOF analyzers was on the improvement in high-mass ion transmission and enhancement of flight distance of ions in order to achieve high resolution. Methods such as multi-pass and multi-flection TOF (MR-TOF) were developed (Verenichikov and Yavor 2021). These innovations have led to significantly improved resolution of $>10,000$, approx. $\sim 5\text{--}10$ ppm mass accuracy, enhanced spectral quality, and high-mass ion transmission in native TOF-MS. Enhanced MR-TOF can demonstrate a resolving power of 500,000 (Yavor et al. 2018). Bruker TimsTOF Pro is a commercial ion

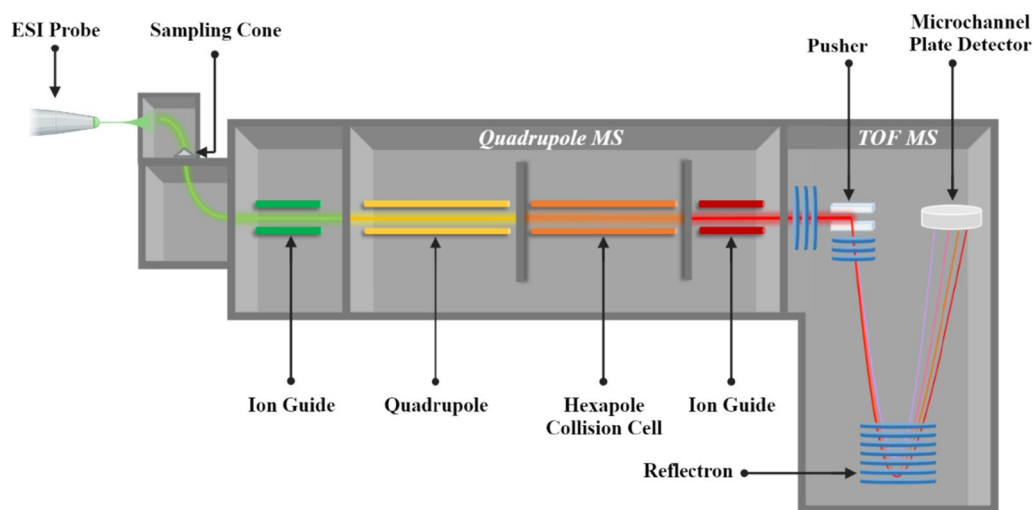


Fig. 3 Schematic representation of a typical Q-TOF MS

mobility-Q-TOF MS that separates ions on a 4D platform by parallel accumulation serial fragmentation (PASEF[®]) technology to provide enhanced specificity and high sensitivity. Despite such advancements, the capability of TOF analyzers to quantify accurate MWs of high mass protein assemblies remains a limitation maybe due to buffer or salt adducts (Lössl et al. 2014).

TOF analyzers have a distinctive feature as compared to FT-based mass analyzers. The resolution ($m/\Delta m$) of TOF analyzers remains nearly constant over the whole m/z range as the resolution is equivalent to $t/2\Delta t$, and also the m/z ratio of TOF analyzers is proportional to the square of flight time of the ions.

FT-ICR

Fourier transform ion cyclotron resonance MS (FT-ICR MS) is an ultrahigh-resolution ($>10^6$ at $m/z=200$ Th) mass analyzer with ultrahigh mass accuracy with a range in the ppb scale (Savory et al. 2011); hence, it is used extensively in the field of proteomics, metabolomics, etc. (Tucholski and Ge 2022). Moreover, due to its flexibility to couple with various fragmentation methods including EID, ETD, IRMPD, ECD, and CAD, it has long been acknowledged for top-down capabilities for straight-forward protein sequencing, and protein–ligand interaction and PTM characterization (Tamara et al. 2021; Li et al. 2018).

FT-ICR MS consists of an ion trap essentially a Penning trap (Guan and Marshall 1995) which has a homogenous axial magnetic field with electric trapping plates to trap the ions (Fig. 4). The trapped ions in the magnetic field rotate at their resonant cyclotron frequencies. An oscillating electric field (RF voltage) orthogonal to the magnetic field is applied in order to excite the synchronous cyclotron motion of the trapped ions. As a result, the ions rotating at the cyclotron frequency ($f_c = B/[2\pi(m/z)]$)

(Tamara et al. 2021; Marshall and Chen 2015) induce an image current which changes the potential of the trap electrodes generating an ICR signal. It is essential to note that detection is feasible exclusively when ions are reasonably excited at their cyclotron frequency. The ICR signal is Fourier transformed in order to analyze the frequency, and mass spectra are generated (Nikolaev et al. 2016). The superiority of the FT-ICR analyzers became obvious when the length of ion flight paths was compared to TOF analyzers. In a typical TOF analyzer, the flight path length of ions is 2–5 m, whereas ions with $m/z=1000$ Th undergo 144,346 rotations in 1 s when analyzed with 9.4 T FT-ICR, and hence, the flight path length is equivalent to 9 km (Marshall and Hendrickson 2008). FT-ICR is a pulsed technique as its mass detection procedure is repeated several times in order to obtain a spectrum with an appropriate S/N ratio. Since the mass accuracy, resolving power, and dynamic and mass range of the FT-ICR are strongly dependent on the magnetic field induction, hence, to generate a high strong magnetic field, superconducting magnets are used. Although modern FT-ICR analyzers can be equipped with >15 T magnets, for improvement in resolving power and reduction in electric and magnetic field imperfections, designs with multiple ICR cells have been proposed (Tamara et al. 2021). The compatibility of FT-ICR equipment with conventional CID fragmentation methods is low due to the requirement of ultrahigh vacuum conditions, and therefore, ExD methods are used for the analysis of large protein assemblies (Li et al. 2018; Lippens et al. 2017).

Orbitrap analyzers

Orbitrap mass analyzers are the newest member of the mass analyzers specifically in the FTMS family. It is one of the most prominent technologies to analyze the intact mass of heterogenous and large biomolecules (Fort et al.

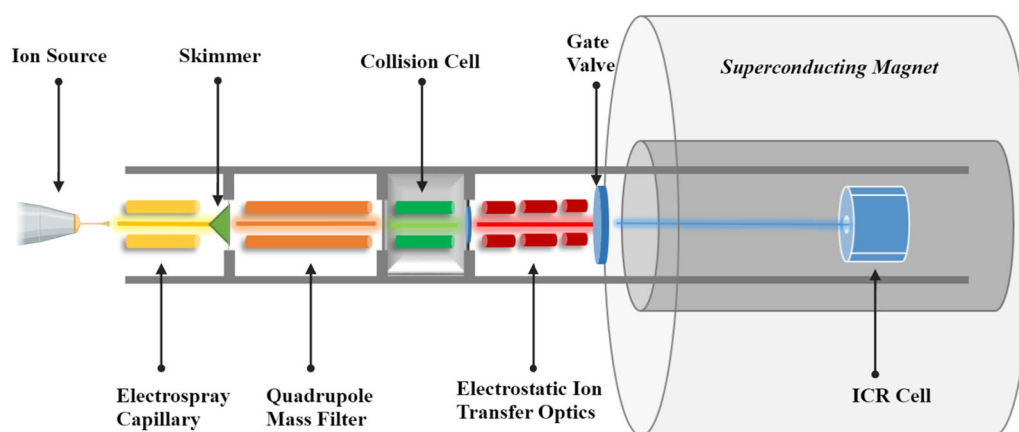


Fig. 4 Schematic representation of FT-ICR MS

2018; Wörner et al. 2020). Many characteristics from previously developed mass analyzers have been incorporated into orbitraps such as trapping principle from RF ion traps, pulsed ion injection from oa-TOF, and signal detection and processing from FT-ICR (Makarov et al. 2019). Orbitrap analyzers essentially consist of outer cup-shaped electrodes electrically separated by a very thin gap and a coaxial spindle-shaped central electrode. C-trap is a curved liner ion trap next to the Orbitrap which holds and confines the ions prior to mass detection. Subsequently, the ion packets from the C-trap are injected tangentially between the outer and the central electrodes and trapped through electrodynamic squeezing. The ions are pushed toward the broadest portion of the trap by the axial electric field generated by the conical electrodes resulting in a harmonic axial oscillation of the ions. The ions separate as they oscillate at different frequencies; however, the axial ion oscillation frequency is independent of the ion energy. The oscillating ions induce current on the outer electrodes simultaneously, and the differential amplifier detects the image current. The digitized image current is Fourier transformed, and a mass spectrum is generated (Makarov et al. 2019; Zubarev and Makarov 2013; Perry et al. 2008). The resolving power of Orbitraps is directly proportional to the number of harmonic oscillations of the ions and acquisition time, hence inversely proportional to the square root of m/z (Denisov et al. 2012).

From an experimental standpoint, native MS analyses are efficient and accurate, since data can be obtained within a minute after starting with a purified protein sample and sample preparation which involves just

desalting or buffer exchange (Rosati et al. 2012). Until recent times, ions with $m/z > 4000$ could not be isolated using commercial equipment such as the Orbitrap Exactive EMR MS (Fig. 5). However, Belov et al. reported a modified Orbitrap Exactive EMR instrument with a high mass quadrupole mass analyzer which was able to isolate intact protein complexes with MW over few hundred kDa (Belov et al. 2013). The high resolution with high sensitivity and improved desolvation are the major advantages of using this technique for analysis of slight mass changes caused by sequence variation, glycosylation, or PTMs.

High-throughput native separation

A common method for mAb characterization is RPLC combined with ESI-MS, which uses a low pH organic mobile phase as such acetonitrile and water blend (Bondarenko et al. 2009; Tsybin et al. 2011). As an alternate approach for protein characterization, native separation techniques utilizing 100% aqueous mobile phase and low concentrations of volatile salt buffer have been developed in the last decade. Native MS analysis protects the protein higher-order structure and non-covalent interactions better than the conventional denatured RPLC MS analysis as well as lowers the spectrum interference from the reduced surface charge at the same mass resolution with high charge states and low m/z ranges (Rosati et al. 2014; Schachner et al. 2016). Native separation techniques such as ion-exchange chromatography (IEX) (Bailey et al. 2018), size exclusion chromatography (SEC) (Ren et al. 2018), capillary electrophoresis (CE) (Shen et al. 2021; Le-Minh et al. 2019), and hydrophobic interaction

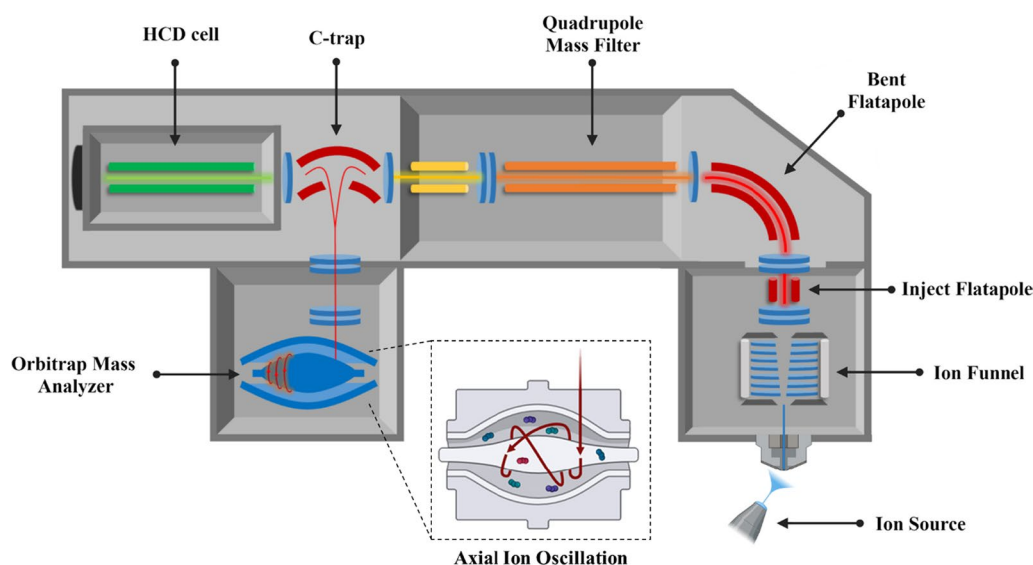


Fig. 5 Schematic diagram of the Thermo Scientific Q Exactive Orbitrap MS

chromatography (HIC) (Chen et al. 2018a) have been effectively hyphenated with MS for intact mass analysis of mAbs under native conditions (Fig. 6).

HIC separates proteins according to their surface hydrophobicity while maintaining their intact native structure. It has been utilized in the past to describe conformational changes and PTMs (Fekete et al. 2016). For the first time, Chen et al. reported the use of HIC-MS and HIC-tandem MS with ECD for top-down proteomics and utilized it for analysis of intact mass, glycosylation profiling of mAbs (Fig. 7), and structural characterization of the CDRs (Fig. 8). They used HIC column with enhanced hydrophobicity due to longer surface carbon chain and acetate buffers for retention (Chen et al. 2018a) and further demonstrated the enhanced selectivity of the mAb separation by isocratic HIC. Native RPLC-MS method was developed for the analysis of antibody–drug conjugates (ADCs) which utilize mild elution conditions, and in order to enhance the mAb elution, no or very low concentrations of organic modifiers are used (Chen et al. 2019). However, even with weak organic modifiers like isopropyl alcohol, a risk for protein denaturation remains with concentration over 15% (Bobaly et al. 2016).

High-performance SEC (HP-SEC) is commonly used for protein separation on the basis of size (Andersen et al. 2010; Arakawa et al. 2010). However, for the identification and characterization of mAb species, molecular weight determination is a general method. Thus,

HP-SEC is directly coupled with ESI-TOF MS for analysis of mAb species (Brady et al. 2008). K ukrer et al. (2010) fractionated intact IgG oligomers comprised of dimer, trimer, and tetramers by HP-SEC and characterized by native ESI-TOF MS (K ukrer et al. 2010).

CE is widely used for the separation and characterization of therapeutic compounds due to the high resolving power, selectivity, and fast separation speed of CE (Jorgenson and Lukacs 1981; Righetti 2001). For analysis of mAbs, several robust methods have been developed using CE such as for CE-SDS to evaluate product-related impurity, capillary isoelectric focusing (CIEF) was developed for the analysis of charge variants and analysis of release glycan, and capillary zone electrophoresis (CZE) is used with UV or LIF detectors (Salas-Solano et al. 2006, 2012). Further, CE can be coupled with online ESI-MS for accurate mass identification and additional sensitivity (Liu et al. 2005; Haselberg et al. 2013). CE-MS provides an interesting alternative to LC-MS separation and determination of the intact mass of therapeutic mAbs. Mei Han et al. 2016 described the coupling of CE to a TOF analyzer using an electrokinetic flow-driven nanospray sheath liquid interface for analysis of intact mass analysis of fully human mAbs and their fragments under native as well as denaturing conditions (Han et al. 2016). R omer et al. developed a method to identify mAb fragments and impurities by 2D CE-CZE-MS (R omer et al. 2019).

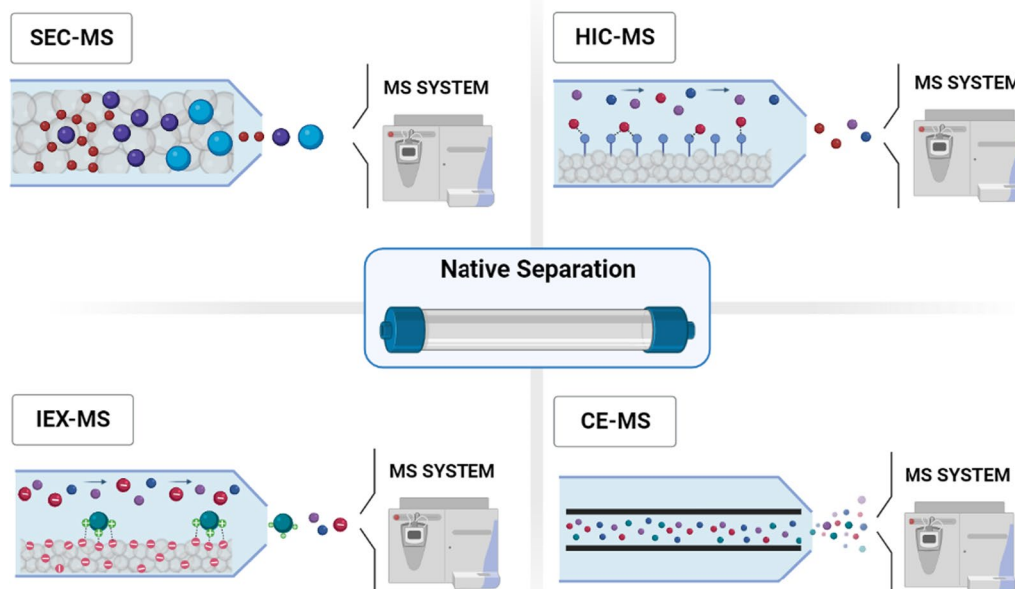


Fig. 6 Schematic representation of native separation-MS techniques

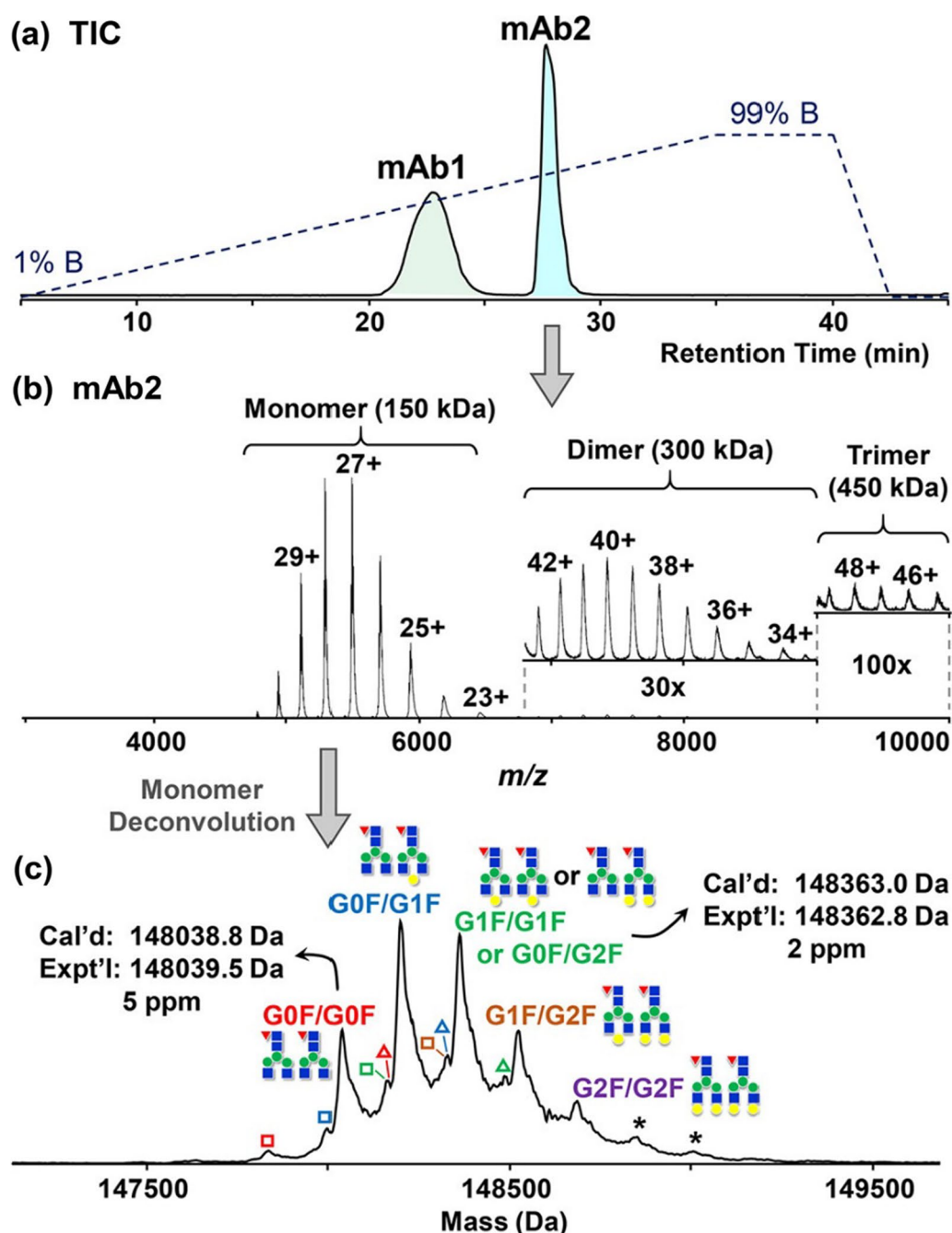


Fig. 7 Online HIC-MS of mAb mixtures on a maXis II Q-TOF mass spectrometer. **a** Total ion chromatogram demonstrating the separation of mAb1 and mAb2. The dashed line indicates the gradient changes of mobile phase B. **b** Mass spectrum of mAb2 showing the detection of monomers, dimers (30 \times zoom-in), and trimers (100 \times zoom-in). **c** Deconvoluted mass spectrum of mAb2 monomer with annotated glycosylation forms (red triangle, fucose; blue square, GlcNAc; green circle, mannose; yellow circle, galactose); hollow square represents the loss of one GlcNAc (-203 Da), hollow triangle represents the preservation of C-terminal Lys on heavy chain ($+128$ Da), and an asterisk represents the addition of a hexose ($+162$ Da). G x F indicates Fc-oligosaccharides terminated by x number of galactoses. Reprinted with permission from Ref (Chen et al. 2018a). Copyright 2017 American Chemical Society

Software and analysis platform

The richness of the information that is accessible is substantially increased by combining native MS with

top-down MS, but simultaneously data analysis is also made more difficult. Two or three layers of mass information are used in the native top-down MS analysis,

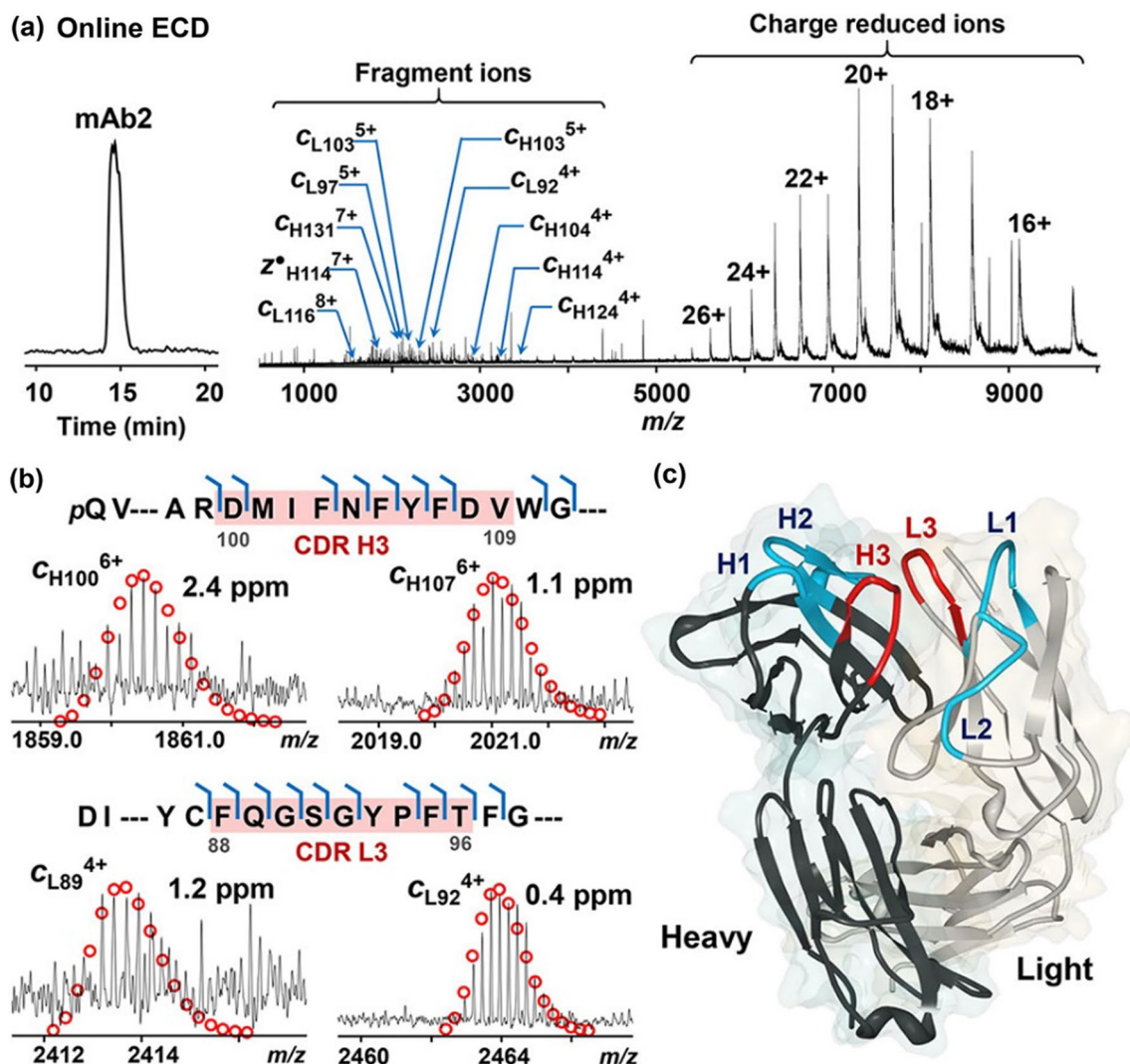


Fig. 8 Online ECD HIC-MS/MS analysis of deglycosylated mAb2 on a 12 T solarix XR FT-ICR MS. **a** HIC chromatogram and spectrum of mAb2 fragment ions and charge reduced ions. **b** CDR H3 and L3 (red highlighted regions) **c** Crystal structure of the mAb2 Fab region (PDB: 5K8A). The CDRs fragmented by ECD (H3 and L3) are highlighted in red, and the other CDRs are highlighted in blue (H1, H2, L1, and L2). Reprinted with permission from Ref (Chen et al. 2018a). Copyright 2017 American Chemical Society

according to the tandem fragmentation techniques selected. The standard procedure for CID or SID is to first break down a complex (MS1) into subunits (MS2), which were subsequently broken into sequence ions (MS3). A schematic representation of tandem MS is depicted in Fig. 9. In contrast, the complete complex (MS1) for ExD or UVPD may be instantly broken up into sequence ions (MS2). Picking an isotopic peak, deconvolution, and identification using database search, validation, and visualization are typically the stages in a basic study of native top-down MS data (Schachner et al. 2021; Chen et al. 2018b).

Deconvolution is an important step for data processing native MS and proteomics. Deconvolution is a crucial step in the processing of proteomics or native MS data, as it aggregates all charge states and establishes monoisotopic masses that can reduce the complexity of complete protein or subunits' (MS1 and MS2) spectra (Chen et al. 2017). THRASH was one of the first algorithms created for the deconvolution of high-resolution mass spectra (Horn et al. 2000) which was followed by UniDec for rapid deconvolution of ion mobility MS data (Reid et al. 2018; Marty et al. 2015) and then comes pParseTD which has great accuracy in precursor detection (Sun et al.

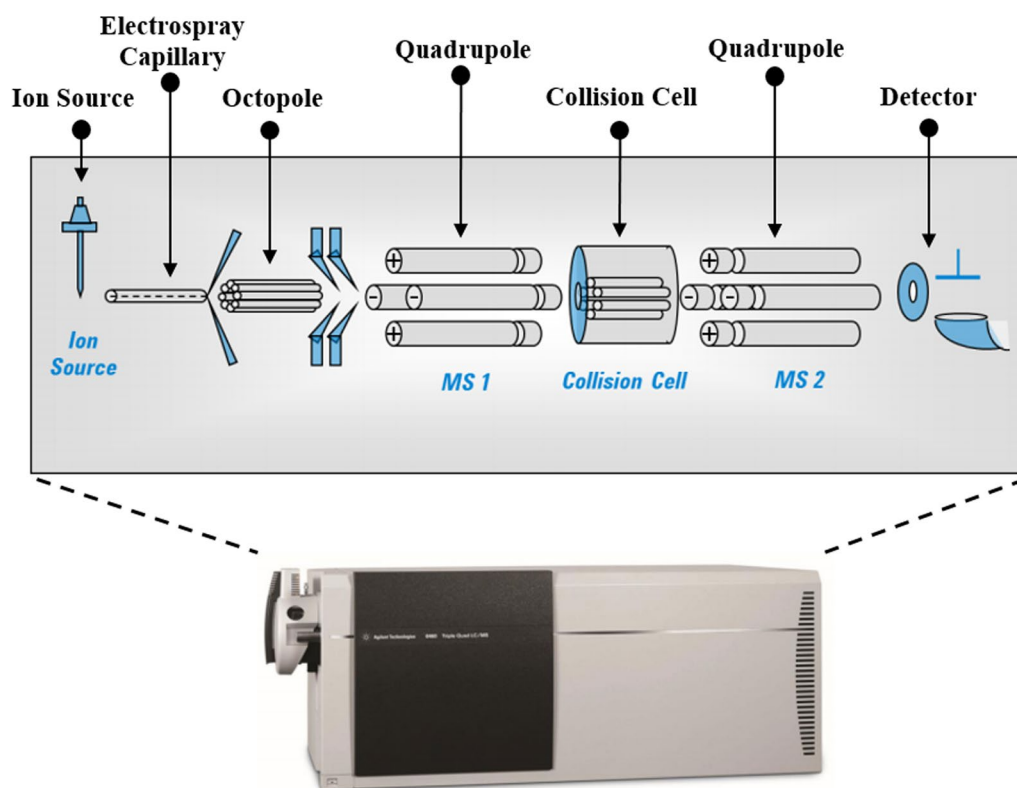


Fig. 9 Schematic representation of Agilent 6460 Triple Quad MS

2016); for more modern deconvolution of LC–MS data, there is ProMex (Park et al. 2017). Strategies for database searches are frequently employed to identify protein and characterize proteoforms. Automatically the unknown proteins in complexes can be detected by reusing top-down MS search engines (such ProSight PC/PD, BigMascot, TopPIC, and TDPortal) (Kou et al. 2016; Toby et al. 2019), and hence, these programs will display sequence coverage, draw fragment maps, and list protein candidates, whereas for protein identification ProSight Lite or MASH Explorer may be applied to observe the fragment ions on the sequence (Fellers et al. 2015; Wu et al. 2020). These tools also enable the user to assess the support for user-defined sequences comprising PTMs, cofactors, or sequence variations (such as mutations or proteolytic truncations) at various places (Schachner et al. 2021). Screening for multiple internal segments (ax, ay, az, bx, by, bz, cx, cy, and cz) can rapidly expand search times and have a negative influence on the majority of score measures due to large false discovery rates (Zenaidé et al. 2021). TDValidator and the new tool ClipsMS (Lantz et al. 2021), on the other hand, may allocate both terminal and internal fragments for top-down MS (Fornelli et al. 2018). For a more focused approach, ProSight Annotator (Greer et al. 2022) helps modify databases

to incorporate candidate proteoforms produced when signal peptides are removed, known sequence variants, PTMs, and cofactors accessible in Uniprot or Unimod (Schachner et al. 2021). All alterations are taken into account to be covalent and not transitory in typical top-down MS software.

Native MS characterization of antibodies Intact antibody sequence elucidation

The basic structure of therapeutic mAbs can be evaluated using the well-established methodologies of the “bottom-up” protein sequencing approach which involves reduction, alkylation, and proteolytic digestion followed by LC–MS/MS quantification (Ren et al. 2009; Fodor and Zhang 2006). However, quantification of PTMs of degraded states of a therapeutic mAbs using such techniques can be difficult. To overcome such challenges, top-down approaches can be used by directly injecting the intact mAbs for sequence analysis and PTM evaluation (Catherman et al. 2014). Due to highly structured and disulfide bond-protected complex regions of antibodies, it is still difficult to obtain a complete sequence. As a result, middle-down approaches are frequently utilized to supplement the

top-down and bottom-up data through limited enzymatic digestion in order to cleave mAbs into large peptide fragments.

Utilization of high-resolution MS techniques such as high-resolution Orbitrap and FT-ICR and a variety of ion activation techniques have broadened the use of top-down and middle-down MS approaches in order to reduce mass overlapping generated from intact protein fragmentation (Fig. 10) (Mao et al. 2013). Early top-down mAb analysis demonstrated that in-source CID fragmentation and tandem MS in conjugation with TOF analyzers can rapidly characterize the variable regions of intact mAbs (Zhang and Shah 2007). Later, this approach was enhanced to acquire both sequencing data and the intact mass on a single platform by improving the transmission efficiency for intact mAb on hybrid LTQ-Orbitrap (Fig. 11) (Bondarenko et al. 2009).

Electron-driven dissociation via ion electron, i.e., ECD (Zubarev et al. 1998) and ion-ion reactions ETD (Syka et al. 2004), has enabled to generate extensive sequence-informative fragments of intact mAbs (Mao et al. 2013). Through these methods, the disulfide bonds are broken although the thermally labile PTMs are maintained for both top-down and middle-down approaches for mAb analysis (Fornelli et al. 2012; Lange et al. 2014).

Formation of single fragment pair by conventional MS techniques such as CID or IRMPD can be beneficial for de novo sequencing of mAbs; they often result in substantial internal fragmentation, extensive side chain cleavages, and limited sequence coverage. Electron capture dissociation (ECD) is an alternative method that can be applied to both denatured and native intact mAbs to overcome such challenges. ECD primarily generates *c/z* fragmentation ions by significant backbone cleavage in the light and heavy chain variable regions and is also observed to produce enhanced fragments of the segments not involved in disulfide bridge loops by not involving in frequent cleavage of intramolecular disulfide bonds (Liu et al. 2014; Tan et al. 2013). In a recent study, ECD was optimized toward de novo sequencing to obtain straightforward-to-read sequence ladders by generating ion fragments solely composed of *c*-ions from IgA1 CDR3s and their downstream regions (Greisch et al. 2021). It was also observed that IgA1 Fab isolation facilitates ion selection and reduces spectrum complexity; however, to achieve straight-forward sequence reads, the simplified precursor is not required to obtain from IgA1 CDR3s.

While ETD retains modifications and cleaves disulfide bonds—making it attractive for mAb characterization—it

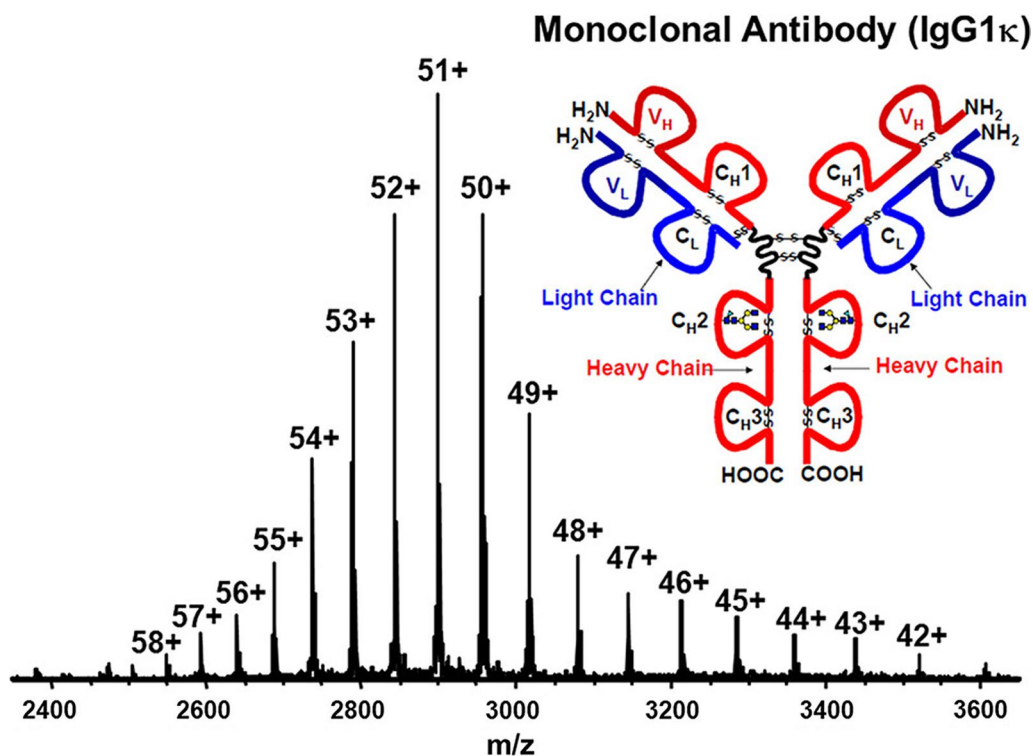


Fig. 10 A positive ESI 9.4 T Fourier Transform-Ion Cyclotron Resonance (FTICR) mass spectra for an IgG1 therapeutic antibody with a charge state distribution ranging from 42+ to 58+ was obtained from the recombinant, humanized IgG1 molecular structure (inset). Reprinted with permission from Ref (Mao et al. 2013). Copyright 2009 American Chemical Society

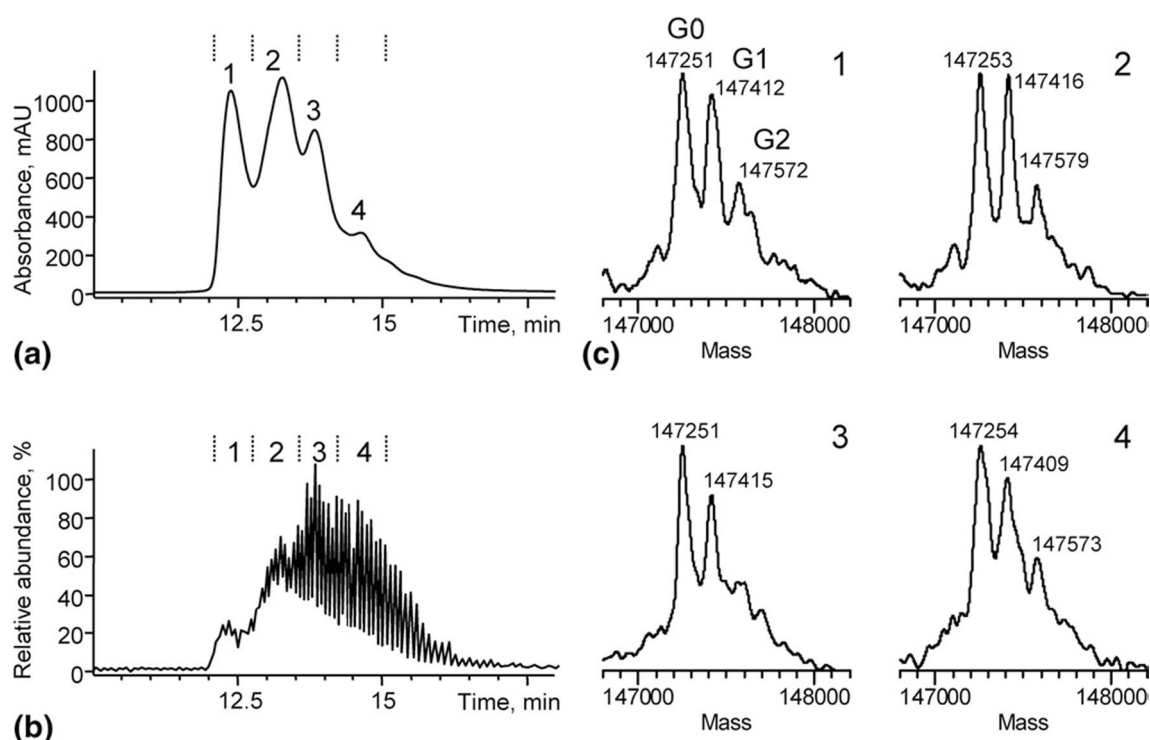


Fig. 11 RP HPLC separates IgG2 disulfide isoforms: 4 g injected. **a** UV absorbance of reversed-phase chromatogram at 214 nm. **b** Total ion chromatogram with MS scans (peaks) for mass measurement and CID scans (valleys) for top-down analysis. **c** MagTran was used to deconvolute ESI mass spectra of intact monoclonal IgG2 antibody reversed-phase peaks 1–4. Reprinted with permission from Ref (Bondarenko et al. 2009). Copyright 2009 American Chemical Society

can be less effective for precursors having high m/z values. Activated ion electron transfer dissociation (AI-ETD) uses concurrent infrared photoactivation to promote product ion generation and has proved effective in increasing sequence coverage of intact proteins. Lodge et al. demonstrated the benefits of AI-ETD for intact mAb analysis. AI-ETD outperforms EThcD as a supplemental activation method as it produces more total sequencing ions and enhances the fragmentation of disulfide-linked regions (Figs. 12 and 13) (Lodge et al. 2020).

High-order structural analysis

Biopharmaceuticals differ from small molecule therapeutics in that they must fold into higher-order structures, such as secondary, tertiary, and quaternary structures, in order to perform therapeutically effective functions. The regular substructures such as α -helices and β -pleated sheets are considered as the secondary structure, whereas the 3D structure refers to the tertiary structure. However, multi-subunit complexes of the mAbs are referred to as the quaternary structure.

Understanding the role of high-order structure in the mechanism of mAb therapies and the links between

structure and activity is crucial since the high-order structure of mAbs can affect antibody properties and functions. A thorough understanding of the effects of process conditions on protein quality may be acquired by detecting or keeping track of the high-order structure changes of mAbs during development. This information might then be used to further enhance both process and product performance.

Binding stoichiometry

The evaluation of a variety of biological species with a wide variety of features (like size, glycosylation/modifications, structures, shapes) may be consistently conducted using MS characterization of binding interactions, and it may be used to efficiently analyze heterogeneous species and particular components in intricate combinations. MS is commonly used to offer a peptide-level resolution, but it may be altered to provide better resolution by including other MS dimensions (e.g., MS/MS, MSⁿ) into the acquisition strategy to fragment and further examine certain properties of individual peptides. The analysis of the data takes longer when dimensions are added, but it could be worthwhile in order to understand complicated systems in more depth.

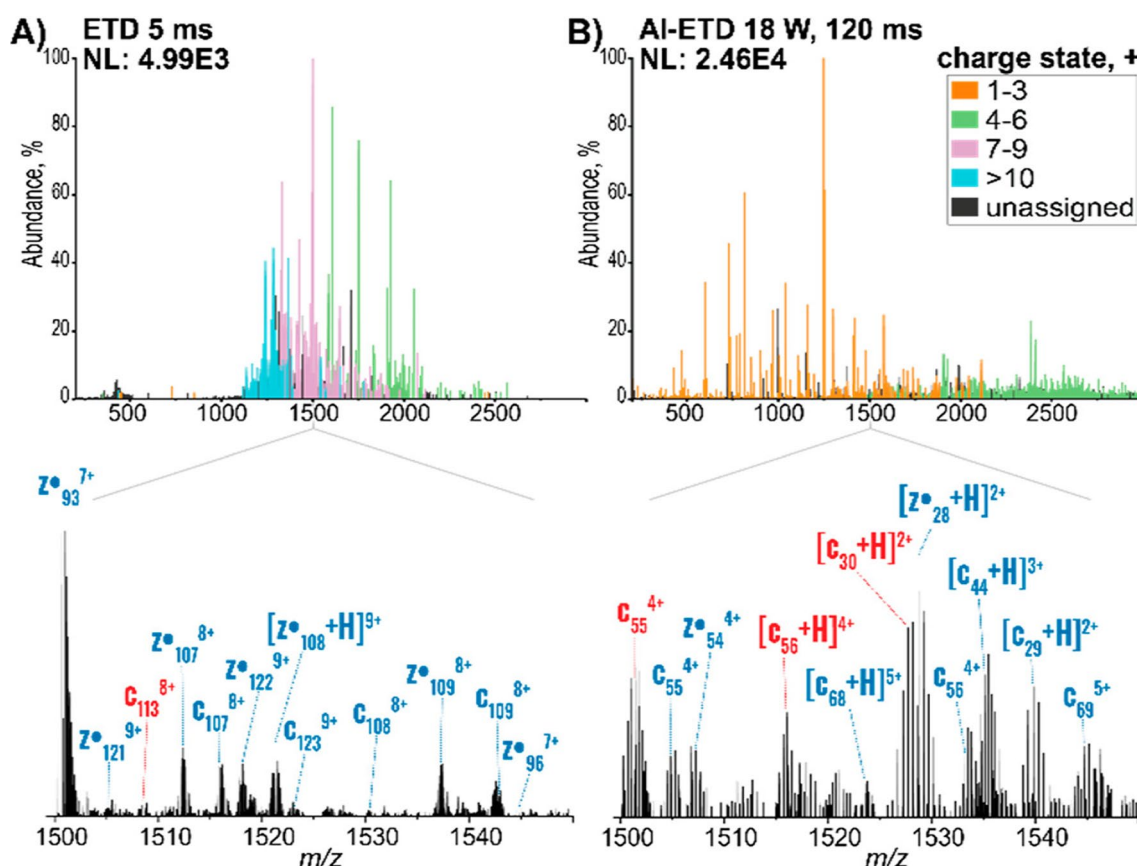


Fig. 12 Spectra produced with short and long response times showed distinct patterns. The spectra were produced by accumulating 200 scans at 240,000 resolving power at 200 m/z using **A** ETD for 5 ms and **B** AI-ETD with 18 W laser power for 120 ms. The top spectra's peaks are colored according to their associated charge states. The annotated area between 1500 and 1550 m/z represents the best matching ions for the heavy chain in blue and the light chain in red. NL stands for normalized level. Reprinted with permission from Ref (Lodge et al. 2020). Copyright 2020 American Chemical Society

In the great majority of native MS studies, ions are directly formed from native-like solutions using ESI-based techniques, where pH and ionic strength may be readily adjusted to provide conditions that maintain mAb structure and function (Kearle and Verkerk 2009). The diverse glycoforms present within a mAb sample have been detected and quantified utilizing native MS data obtained using a modified Orbitrap platform (Fig. 14) (Rosati et al. 2012). These spectra have a mass resolving power of up to 12,000 at a m/z of 6000, making it possible to identify antibody glycoforms with accuracy. It has been established that high-resolving power native MS can describe antibody mixtures both qualitatively and quantitatively in addition to identifying PTM states (Thompson et al. 2014).

The ability to preserve non-covalent interactions protein–protein interactions throughout the ESI process in native MS methods allows for the direct assessment of antibody–antigen-binding stoichiometries and stabilities. Initiating research in this field (Marshall et al.

1998) showed that complexes between the recombinant V antigen (rV), a 37 kDa protein released by *Yersinia pestis*, and its complementary mAb could be easily identified and described. These native MS measurements demonstrated the closely linked dimerization of the rV antigen at micromolar concentrations, the predominance of a 1: 2 binding stoichiometry for the antibody: antigen complex, and the specificity of the ensuing antibody–antigen binding is also evaluated. The immunological complex generated between the recombinant JAM-A protein and an antigenic protein (Ag) overexpressed in tumor cells with both murine and humanized mAbs was further studied using native MS (Atmanene et al. 2009). Similar results were found for both humanized and murine mAbs when the mAb–antigen binding stoichiometry (Fig. 15) and selectivity (Fig. 16) were calculated using these data. The investigation of antibody–antigen complexes has also taken advantage of the development of higher resolving power native MS systems (Rosati et al. 2012).

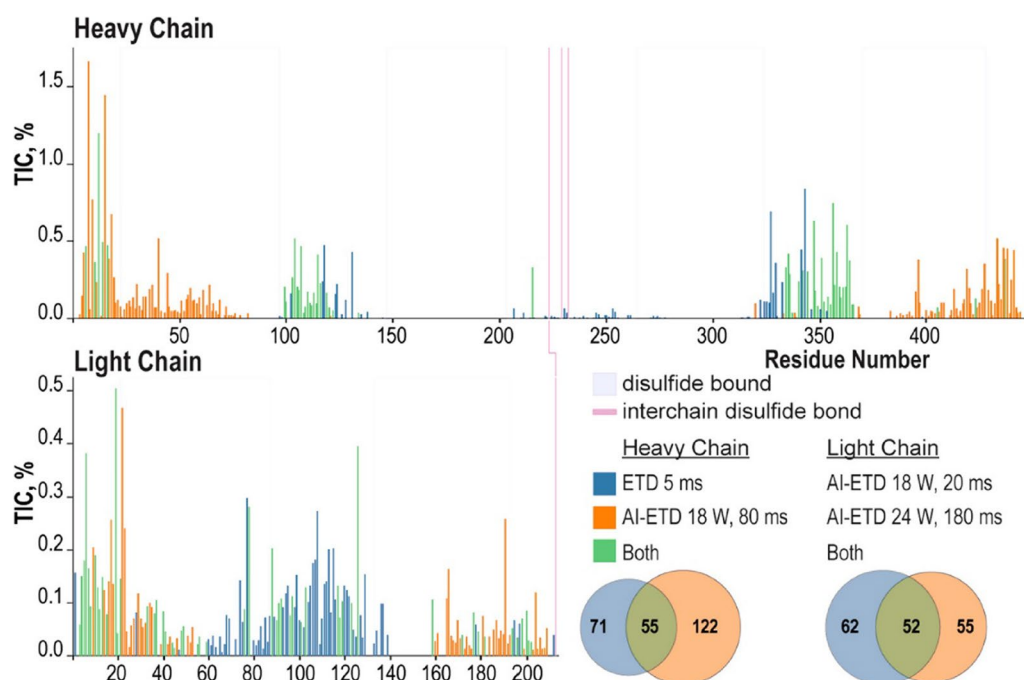


Fig. 13 Average signal (% TIC) of matched fragments obtained under the two conditions (i) amino acid residue position in the heavy (top) and (ii) light (bottom) chains. Light blue indicates the intrachain disulfide bonds, whereas pink indicates the interchain disulfide bonds. The Venn diagrams indicate the number of separate and overlapping relationships broken under each situation. Reprinted with permission from Ref (Lodge et al. 2020). Copyright 2020 American Chemical Society

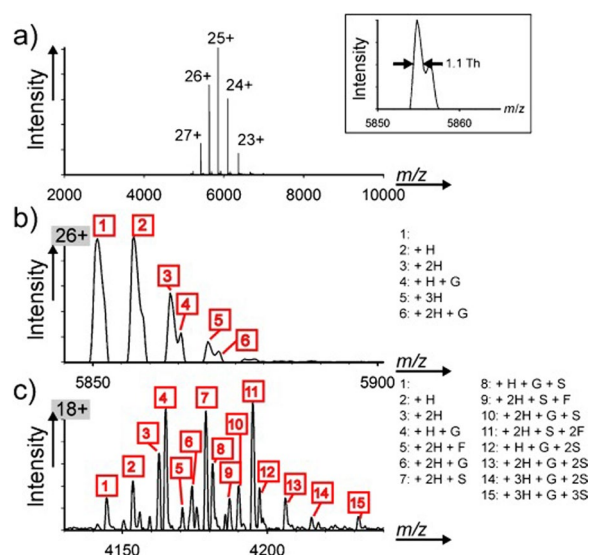


Fig. 14 Antibody glycoform profiling by native orbitrap MS. **a** Complete mass spectrum of intact mAb under native condition with FWHM to be approx. 1.1Th (inset); **b** glycosylation pattern with baseline resolved glycan peaks of intact antibody; **c** complex glycosylation pattern with baseline resolved glycan peaks for half antibody. Reprinted with permission from Ref (Rosati et al. 2012). Copyright 2012 WILEY-VCH Verlag GmbH & Co. KGaA, Weinheim

A functioning IgG hexamer's antigen binding stoichiometry has been examined using native MS (Dyachenko et al. 2015). Tandem MS was used to further describe the huge multi-protein complex that was produced, and this method was essential in revealing the location and stoichiometry of the subunits inside the assembly.

Chemical cross-linking

Chemical cross-linking coupled with MS (XLMS) has established as a complementary approach for structural elucidation of proteins. Small reactive molecules called as cross-linkers are allowed to react and link the functional groups of the amino acid side chains. Crosslinkers help to link two physically close functional groups in the native conformation of the protein or among two interacting proteins. By stably binding the drug with the antibody, they have demonstrated the potential to be applied for ADCs (Zhang et al. 2009). Bich et al. demonstrated this as an alternate technique for structural analysis of Ab–Ag interactions (Bich et al. 2010).

Antibody–drug conjugates (ADCs) analysis

Native MS procedures have also been used to describe pharmaceutical compounds that are linked to antibodies,

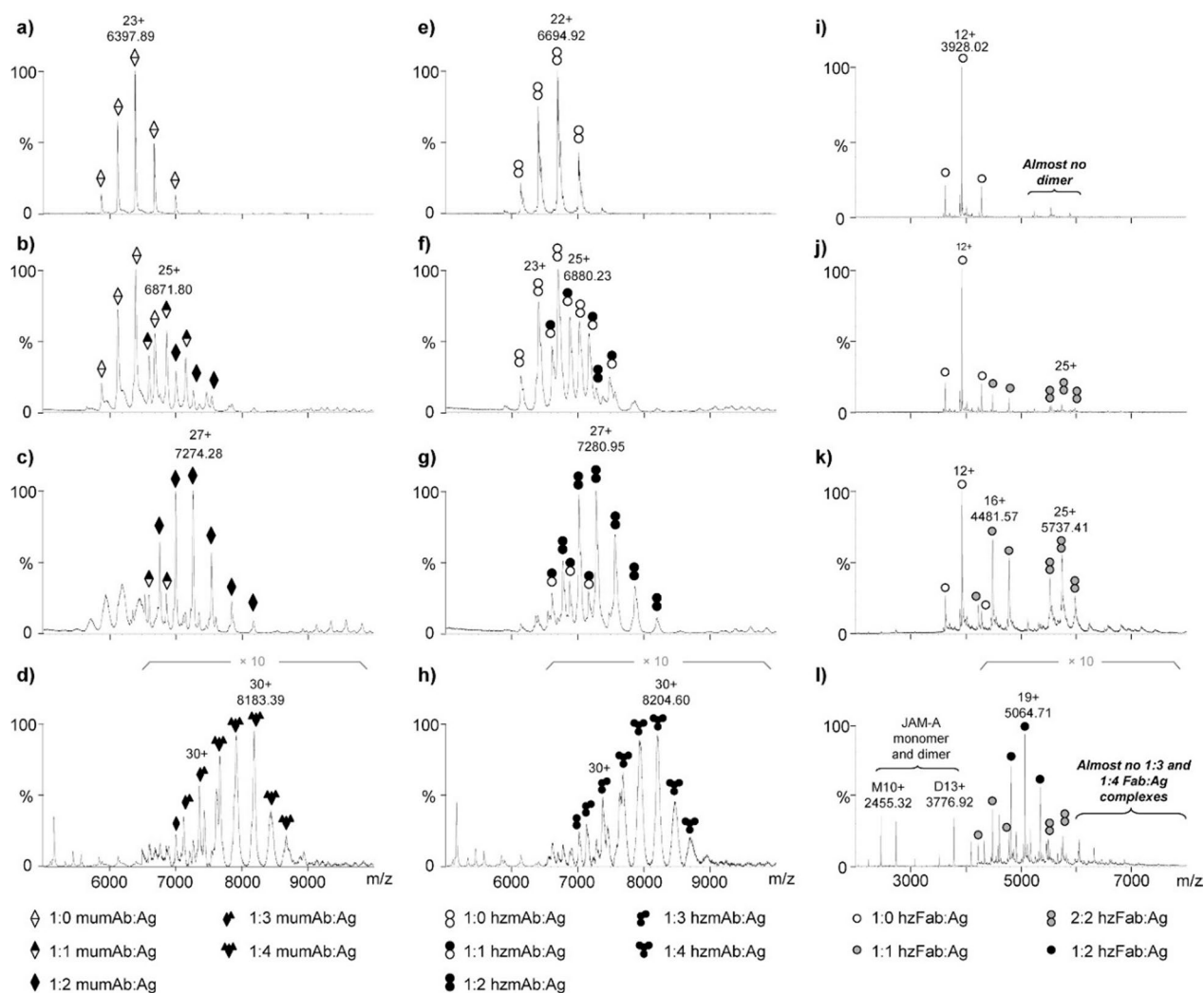


Fig. 15 Determination of mAb:Ag binding stoichiometries. **a–d** mumAb was analyzed at 5 μ M either **a** alone or in presence of **b** 5 μ M, **c** 10 μ M, and **d** 40 μ M JAM-A IB. **e–h** hzmAb was analyzed at 5 μ M either **e** alone or in presence of **f** 5 μ M, **g** 10 μ M, and **h** 40 μ M JAM-A IB. **i–l** hzFab was analyzed at 10 μ M either **i** alone or in presence of **j** 5 μ M, **k** 10 μ M and **l** 40 μ M JAM-A IB. Reprinted with permission from Ref (Atmanene et al. 2009). Copyright 2014 American Chemical Society

such as ADCs and bispecific antibodies (bsAbs). The physiological process of Fab-arm exchange, in which fragments of two IgG4 mAbs merge to produce a bsAb, was observed using native MS (Rose et al. 2011). By adding a little amount of a reducing agent, simulating the Fab-arm exchange in vitro was possible while native MS was used to track the IgG4's kinetics of dissociation. The findings demonstrated the significance of the CH3 domain in the development of the final bsAbs.

Conjugating drug molecules to cysteines in the native antibody sequence is one of the prominent methods for production of ADCs. Native MS can be used to probe the non-covalently bounded heavy and light chain structure

when the inter-chain disulfide bonds are reduced. The stoichiometry of the conjugated drug molecules with DAR values 0 to 8 depending on the number of free cysteines and the degree of completeness of the conjugation reaction can be determined by native MS. In comparison with more time consuming HIC analyses, native MS can be utilized to define cysteine-linked ADCs producing average DAR value (Debaene et al. 2014) (Fig. 17). However, native RPLC-MS as an alternative to HIC-MS was developed which reported that all the species remained intact within the DAR range of 1–8. Moreover, it was pointed out that this method has an enhanced ability to resolve multiple positional isomers of ADCs as

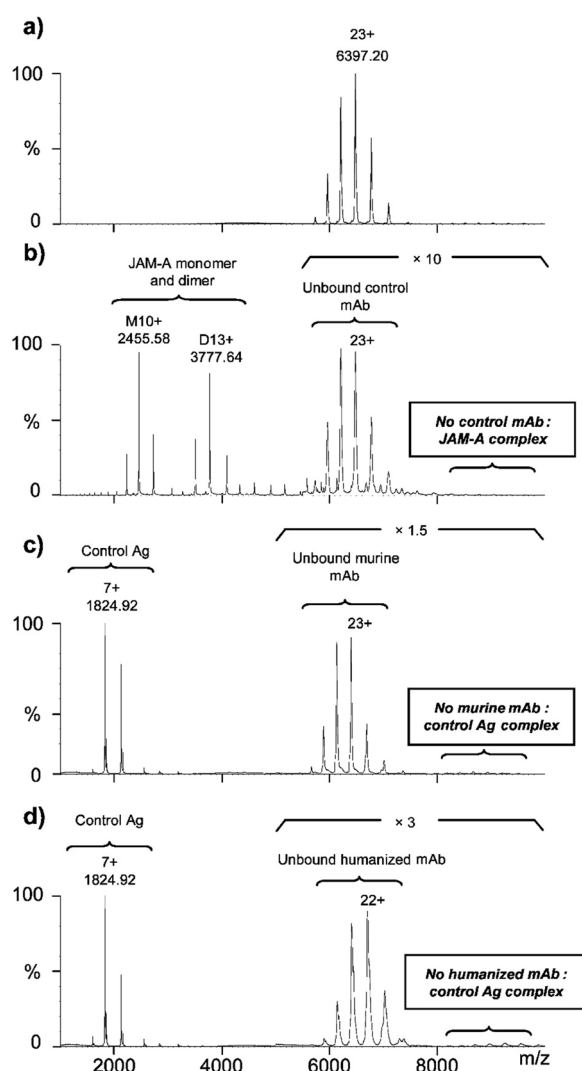


Fig. 16 Assessment of mAb:Ag binding selectivity. **a–b** Negative control mAb was diluted at 5 μM either **a** alone or **b** in presence of 10 μM JAM-IB. **c** murAb, and **d** hzmAb were incubated at 5 μM in presence of 10 μM negative control antigen. Reprinted with permission from Ref (Atmanene et al. 2009). Copyright 2014 American Chemical Society

compared to other chromatographic techniques (Chen et al. 2019).

Additionally, recent studies have revealed the benefits of native MS defining extremely diverse lysine-linked ADCs (Campuzano et al. 2018). With the use of native MS with high resolving power, average DAR values may be precisely calculated from native MS spectra obtained for deglycosylated lysine-linked ADC samples (Fig. 18). Additionally, for the broadband measurement of very precise DAR values, charge reduction approaches combined with native MS analysis of ADCs have been employed to reduce spectrum

complexity and decrease mass overlaps (Marcoux et al. 2015).

MAB aggregation

During production and storage, therapeutic proteins tend to aggregate due to fluctuations in temperature and pH, agitation, and various other factors. Several techniques such as SEC and analytical ultracentrifugation are employed in order to determine high-order oligomers and non-specific aggregations; however, it is difficult to pinpoint the precise nature of the aggregated product. The ability to characterize antibody aggregates, which are frequently formed during the breakdown of therapeutic proteins and result in activity loss, reduced solubility, and increased unfavorable immunogenicity, is another advantage of employing native MS. Native MS is perfectly suited for the detection of higher-order oligomers as it can preserve the non-covalent structures with mass-based identification of aggregates. Kükrer et al. developed a MS-based method to identify intact soluble IgG1 oligomers. HP-SEC was used to isolate monomeric and aggregated pH stressed IgG fractions, and the molecular weights of the monomers and oligomers were determined by ESI-TOF MS (Fig. 19) (Kükrer et al. 2010).

Challenges and future perspectives

The burgeoning potential of mass spectrometry (MS) in characterizing therapeutic monoclonal antibodies (mAbs) brings to light several challenges and promising future prospects. Despite its remarkable capabilities in intact mass analysis, high-throughput separation, and structural elucidation of mAbs, MS encounters hurdles in handling sample heterogeneity, achieving complete sequence coverage, and deciphering complex higher-order structures. The current limitations also lie in data processing, software integration for accurate analysis, and the need for standardization across different MS platforms. Looking ahead, the future of MS in mAb analysis seems promising. Advancements in instrumentation, including higher-resolution analyzers and improved ion activation techniques, hold the potential to address existing challenges, enabling better sequence coverage and detailed structural insights. Integrating MS with other complementary techniques, such as cryo-EM and computational modeling, offers a holistic view of mAb structures. Moreover, ongoing advancements in software algorithms and data processing tools pave the way for more efficient and accurate analysis of MS-generated data, facilitating deeper insights into mAb characteristics,

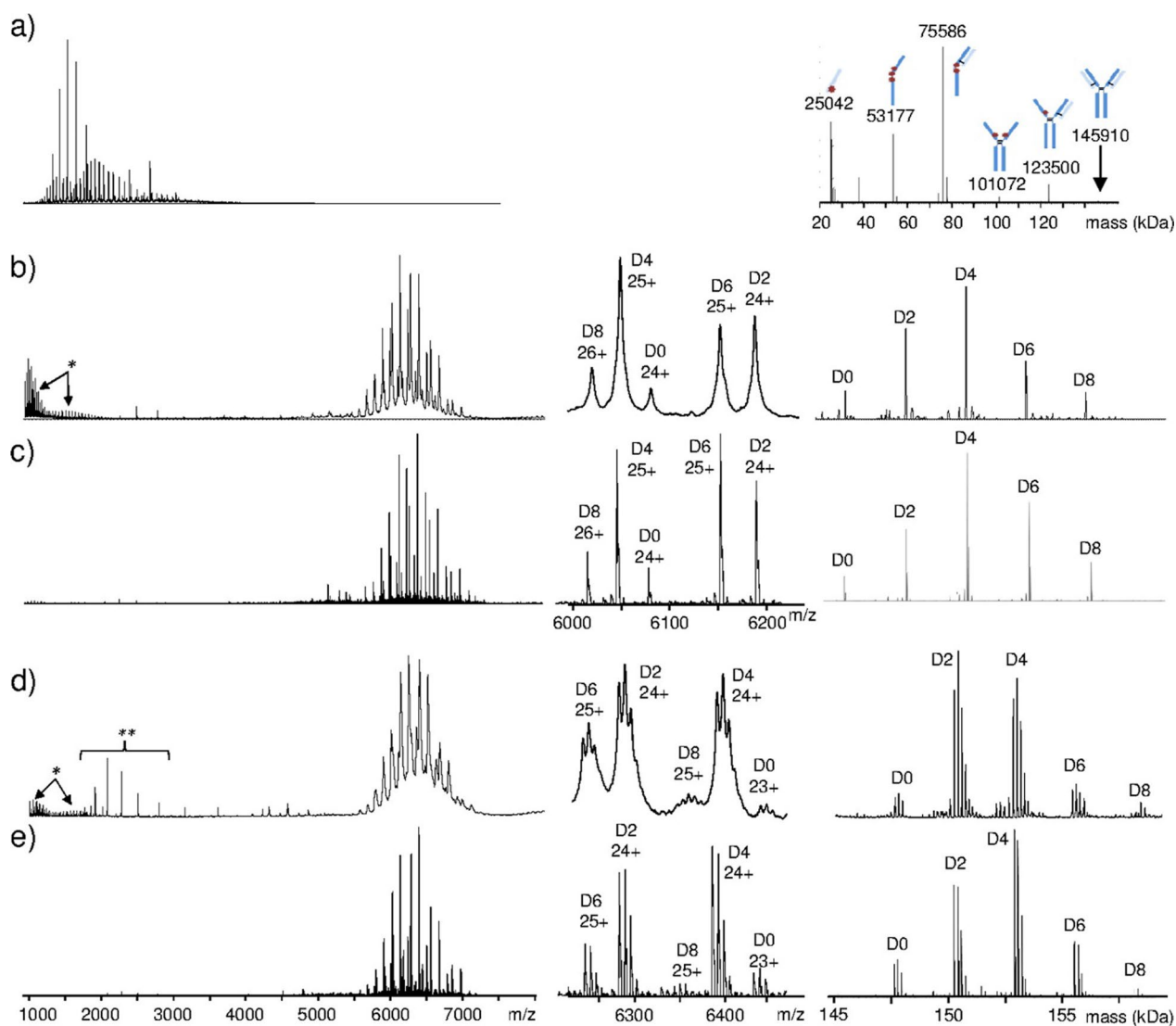


Fig. 17 Denatured and native mass analysis of brentuximab vedotin. **a** ESI mass spectra of deglycosylated brentuximab vedotin in classical denatured conditions. ESI mass spectra of brentuximab vedotin under native conditions after deglycosylation on **b** QTOF; **c** orbitrap and with glycosylation on **d** QTOF and **e** orbitrap. Left section shows the full scan spectra on the mass range of 1000–8000; the middle section shows the spectra with mass range of 6000–6250 and 6200–6500 for deglycosylated and glycosylated brentuximab vedotin, respectively; the right section shows the deconvoluted spectra for drug load profiles. Reprinted with permission from Ref (Debaene et al. 2014). Copyright 2014 American Chemical Society

interactions, and therapeutic efficacy. Standardization efforts across laboratories and platforms will be pivotal in ensuring reproducibility and comparability of results, further propelling MS as a cornerstone technology in the characterization of therapeutic antibodies.

Conclusions

In this review, we highlighted the current progress in native top-down MS for mAb characterization. Native MS has gained significant importance for the analysis of mAb, and currently, various methods are designed for high-throughput analysis and high-order structural analysis. Although the time for analysis has been reduced due

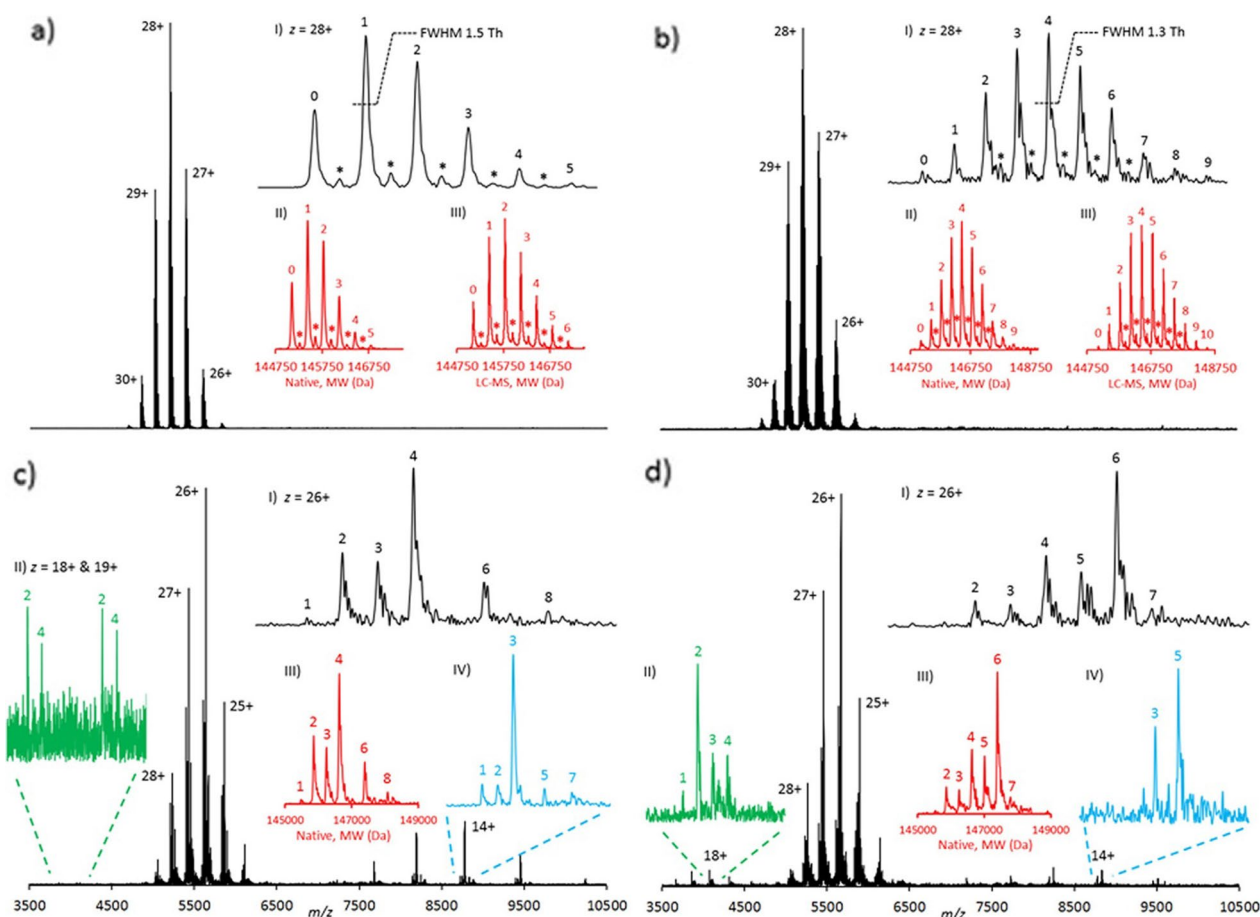


Fig. 18 Native FT-ICR MS spectra of mAb conjugate analyzed over the m/z range of 3500–10500 **a** 5 M equivalent biotin-lysine conjugate; **b** 10 M equivalent biotin-lysine conjugate; **c** 5 M equivalent TCEP, 10 M equivalent biotin-cysteine conjugate; **d** 10 M equivalent TCEP, 10 M equivalent biotin-cysteine conjugate; DAR values are annotated on selected charge states and all deconvoluted spectra. Reprinted with permission from Ref (Campuzano et al. 2018). Copyright 2017 American Chemical Society

to recent developments, however, it is still highly dependent on experienced operators.

The challenges of separations, sample preparation, ionization, desolvation, hybrid activation/dissociation techniques, high m/z transmission, adequate resolution for heterogeneity, and software development must still be

overcome to achieve a rapid, broader, and more thorough analysis of various biological macromolecules. Recent research has indicated some promising directions.

Additionally, a barrier persists in the data interpretation phases for many structural MS methods, particularly in the context of mAb studies. We anticipate that further

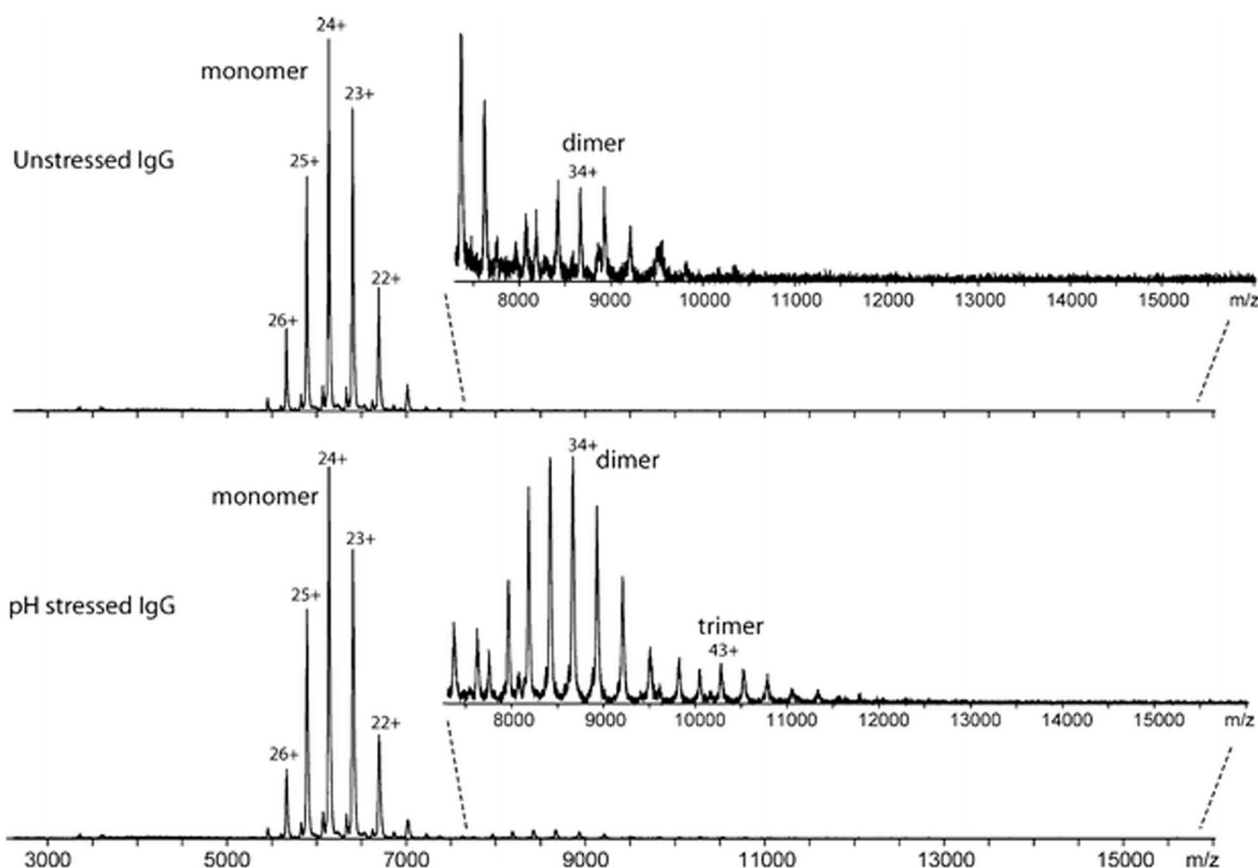


Fig. 19 ESI-TOF MS spectra of unstrained (top) and pH-stressed (bottom) IgG solutions, without HP-SEC separation. In both unstrained and pH-stressed samples, peaks between mass range of 5000–7000 represent different charged states of IgG monomers. The unstrained IgG spectrum consists of 99.8% monomers and 0.2% dimers (top inset). The pH-stressed IgG spectrum consists of 90.1% monomers, 9.5% dimer and 0.4% trimer (bottom inset). Adapted from Ref (Kükrer et al. 2010)

advancements in each of these areas will significantly advance our capacity to identify and create the next generation of therapeutic antibodies as well as our capacity to evaluate biosimilars, allowing for the continued expansion of this interesting class of therapeutics and their profound effects on human health.

Abbreviations

MS	Mass spectrometry
LC	Liquid chromatography
TOF	Time of flight
FT	Fourier transform
ICR	Ion cyclotron resonance
MALDI	Matrix-assisted laser desorption/ionization
ESI	Electrospray ionization
EID	Electron-induced dissociation
ETD	Electron transfer dissociation
ECD	Electron capture dissociation
IEX	Ion exchange chromatography
HIC	Hydrophobic interaction chromatography
PTM	Post-translational modification
mAb	Monoclonal antibody
ADC	Antibody–drug conjugate
CDR	Complementarity-determining region
DAR	Drug-to-antigen ratio
FWHM	Full width at half maximum

MME Mass measurement errors

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Author contributions

AM, HJY, DHK, and KC contributed to conceptualization; AM, HJY, DHK, JP, SJP, EJ, and SC contributed to literature gathering and data curation; AM, HJY, DHK, JP, SJP, and EJ contributed to writing—original draft preparation; and SC, JHC, MP, GB, and KC contributed to writing—review, and editing. AM and DHK contributed to visualization. All authors have read and agreed to the published version of the manuscript.

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Availability of data and materials

Not applicable.

Declarations

Competing interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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