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Recent advances in structural mass spectrometry methods in the context of biosimilarity assessment: From sequence heterogeneities to higher order structures

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Abstract

Biotherapeutics and their biosimilar versions have been flourishing in the biopharmaceutical market for several years. Structural and functional characterization is needed to achieve analytical biosimilarity through the assessment of critical quality attributes as required by regulatory authorities. The role of analytical strategies, particularly mass spectrometry-based methods, is pivotal to gathering valuable information for the in-depth characterization of biotherapeutics and biosimilarity assessment. Structural mass spectrometry methods (native MS, HDX-MS, top-down MS, etc.) provide information ranging from primary sequence assessment to higher order structure evaluation. This review focuses on recent developments and applications in structural mass spectrometry for biotherapeutic and biosimilar characterization.

Keywords: structural mass spectrometry, monoclonal antibody, biosimilar, higher order structure

1. Introduction

Monoclonal antibodies (mAb) and related compounds have emerged as a major class of therapeutic agents, with more than 120 pharmaceuticals approved by the regulatory agencies and made available on the market in 2023 [1]. No longer restricted to anticancer therapies, mAbs are being developed to treat a broad range of pathologies, including infectious (e.g. COVID-19), autoimmune, neurodegenerative (e.g. Alzheimer's), and metabolic diseases (e.g. Diabetes), and even migraines [1-3]. mAb-based formats encompass small, single-chain derivatives, IgG-based structures, immunoconjugates, bispecific antibodies, and many more [4, 5]. The first therapeutic mAb (muromonab-CD3) was accepted by the US Food and Drug Administration (FDA) in 1986 to prevent kidney transplant rejection. In 2000, the concept of "generic" mAb was introduced and termed biosimilars, as copy versions of originator mAb products produced worldwide [6-9]. The first two infliximab (anti-TNF α) biosimilar products were approved by the European Medicines Agency (EMA) in 2013 [10, 11], followed a few years later by biosimilar versions of adalimumab (anti-TNF α), rituximab (anti-CD20), bevacizumab (anti-VEGF) or trastuzumab (anti-HER2) [12-14]. Currently, 249 mAb biosimilars for 12 reference products have been approved [1].

To qualify for market entry, these 249 mAb biosimilars must follow the same rules as those that are applied to originator mAbs [15-17]. Critical Quality Attributes (CQAs) relating to structural, physicochemical and biological properties must be assessed during the process to establish biosimilarity with the originator/reference mAbs to ensure drug efficacy and patient safety [18, 19]. Biosimilars and originators are produced according to various manufacturing processes (e.g. host cells, cell culture conditions, purification procedures), which necessitates a comparability assessment of the biosimilar with its originator product. Any observed differences in comparability studies should be justified by showing that there are no clinically meaningful differences between the biosimilar and the originator molecules. Among those CQAs, in addition to identical primary sequence and correct

disulfide bond pairing, it is necessary to carefully characterise size variants (aggregates, degradation products) and charge variants mostly related to post-translational modifications (oxidations, deamidation, glycoprofiles, glycation, K-clipping, etc.). Moreover, higher order structures (HOS), meaning tertiary/quaternary structures and conformational stability, have to be addressed as they might directly impact mAb functionality (antigen binding, FcRn binding, Fcγ receptor binding, etc.) [18].

To reach this ambitious goal, a combination of orthogonal analytical techniques is employed to provide an extensive characterization of the drug products through a so-called “comparative quality study”. Mass spectrometry (MS)-based methods along with separation techniques (mainly chromatography and electrophoresis) play a key role in mAb characterization [20-22]. Among MS-based methodologies, structural MS (SMS) approaches appear to provide valuable information to not only tackle some important CQAs but to also conduct higher order structure (HOS) analyses [23, 24]. SMS comprises a series of MS-based approaches that are typically performed in “nearly native” conditions to provide information on protein and multiprotein complex assemblies [25-27]. SMS is a research area that consists of deriving structural information from MS-based data and usually focuses on purified proteins or protein complexes, including mAb-based products. SMS entails a series of methods that fall into two categories: intact protein analyses and peptide-centric approaches. For intact protein analysis, top-down MS (TD-MS) allows the sequencing of intact proteins [28, 29] and appears as a promising alternative to classical bottom-up strategies to deliver information on mAb primary sequence (including post-translational modifications). On the other hand, native MS (nMS) provides accurate mass measurements of intact non-covalent assemblies [25, 30], thus providing information on mAb homogeneity, stoichiometry and oligomeric state [31-33]. When combined with ion mobility (nIM-MS) [34, 35], nMS provides a snapshot of the diversity of conformations, providing global information on mAb shapes through CCS measurements or activation strategies (CIU). Peptide-centric methods, mostly based on labelling techniques such as hydrogen/deuterium exchange MS (HDX-MS) [36, 37], cross-linking MS (XL-MS) [38, 39], or fast photochemical oxidation of proteins (FPOP) [40, 41], are more related to 3D structure and dynamics of mAb assemblies and complement the classical biophysical techniques used in structural biology (Fig. 1).

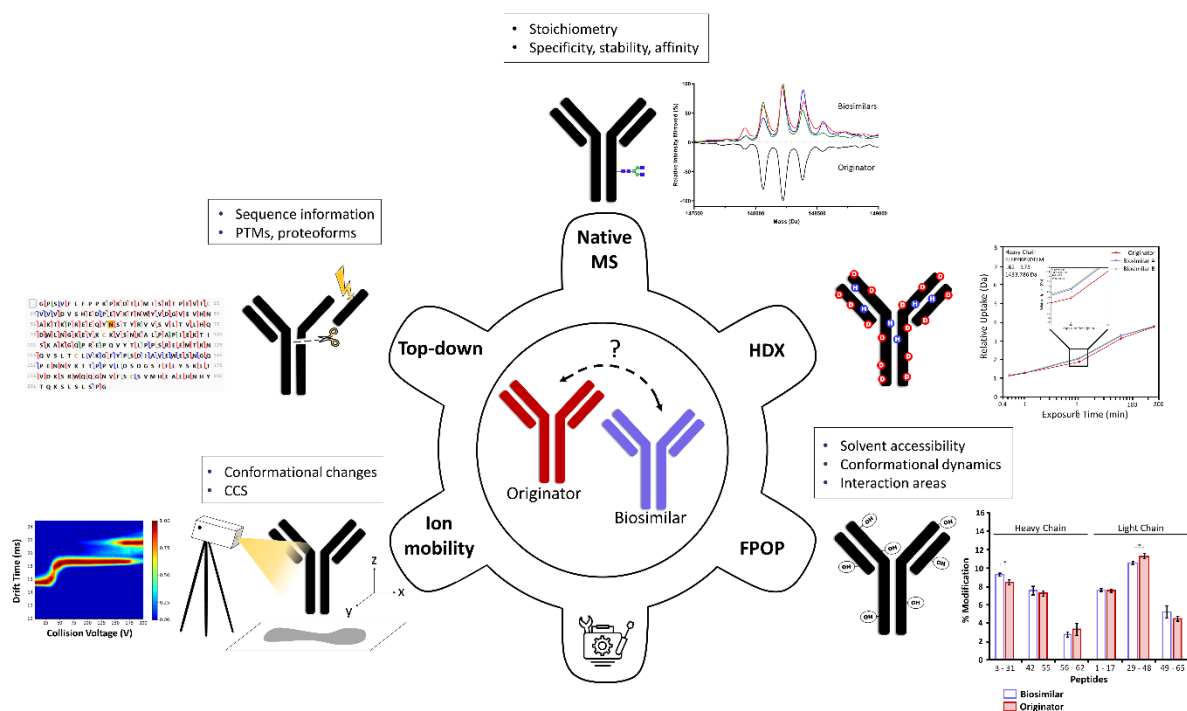


Fig. 1. Overview of different SMS approaches for biosimilarity assessment and the information obtained from each method.

While bottom-up peptide mapping strategies for primary sequence determination [42, 43] and intact mAb mass analysis in denaturing conditions [44] are well-established analytical techniques that are implemented in most biopharmaceutical companies, SMS methods appear to be a promising alternative or offer complementary workflows for the characterization of biotherapeutics. This review focuses on modern mass spectrometry approaches to assessing mAb primary sequence information (TD-MD), along with MS methods that allow “nearly native” analysis to tackle sequence micro-heterogeneities (nMS) and HOS (nIM-MS, HDX, XL-MS, etc.), with an emphasis on (though not restricted to) comparability studies between originators and biosimilar versions.

2. TD-MS: a promising alternative to peptide mapping for primary sequence assessment

Primary sequence validation is clearly the first step in biosimilarity assessment. This includes the identification of sequence variants and the quantification of the most relevant post-translational modifications (PTMs, e.g. deamidation, oxidation, pyroglutamylation, glycosylation). The gold standard for MS-based strategies for primary sequence assessment is currently peptide mapping, which relies on the enzymatic digestion of the mAb followed by MS/MS identification of the peptides [45]. Peptide mapping generally affords comprehensive sequencing along with the identification and quantification of important PTM hot spots that might affect stability and/or immunogenicity [18, 46]. Peptide mapping approaches have even become well-established as multi-attribute-methods (MAM) for monitoring CQAs in non-Good Manufacturing Practice environments for antibody-based products and process characterization purposes, and they tend to enter quality control environments [47, 48]. One of the main limitations of peptide mapping is that proteins are inferred from peptides, hampering a direct link between the abundances of peptides and coexisting mAb variants [49]. Thus, these proteoforms and the linkages among different PTMs are often lost in bottom-up experiments [50], a key factor to address in characterizing mAbs biosimilars.

Alternative and complementary MS-based intact-protein approaches have emerged and attracted increasing attention. These approaches are classified as top-down (when considering intact ~150 kDa mAbs) [49, 51-53] and middle-down mass spectrometry (MD-MS, for ~25-50 kDa mAb subunits investigation obtained after either S-S reduction or specific enzymatic digestion). In practice, TD/MD-MS approaches entail the direct infusion or LC-MS/MS analysis of intact proteins/subunits, followed by the selection of ions to be sequenced for further activation in the gas phase of mass spectrometer and protein sequencing. A schematic representation of the method is provided in Fig. 2A. Contrary to bottom-up approaches, these strategies allow the fragmentation spectra of each species to be correlated with their intact mass measurements in the MS1 stage. However, fragmentation of relatively “large” proteins and proteoforms (greater than 40-50 kDa) remains an analytical challenge and requires the combination of alternative and complementary fragmentation techniques under multiple experimental conditions [51, 54].

The MS fragmentation step is thus central to efficient and successful TD/MD-MS analysis. Most of the popular activation techniques comprise either collision-induced dissociation (CID) and its higher-energy version (higher energy collisional dissociation, HCD) or electron-driven methods such as electron transfer (higher-energy)/capture/activation dissociations (ET(hc)D/ECD/EAD) (reviewed in [55]). Alternative fragmentation methods using higher energy activation, such as ultraviolet photodissociation (UVPD), performed at different wavelengths (mainly 193 and 213 nm) are also gaining in popularity [56, 57]. As each fragmentation technique leads to the cleavage of specific bonds in the backbone of the protein (generation of a , b , c , and y , x , z ions), this combination of fragmentation spectra from separate TD/MD-MS runs contributes significantly to increased and maximized sequence coverage [58, 59]. By combining EThcD and UVPD, Fornelli *et al.* reached ~40% sequence coverage for the heavy chain of intact rituximab in TD-MS compared to 25% with only ETD fragmentation [58]. Other fragmentation strategies are constantly being developed, such as activated ion ETD (AI-ETD). This method uses infrared photoactivation to promote product ion generation, with a first application to mAb sequencing [53]. The study pinpointed an effective increase in sequence coverage from 28% for ETD and 35% for EThcD to 43% for AI-ETD considering the heavy chain of the reference NIST mAb. AI-ETD also improved the fragmentation of disulfide-enclosed regions and yielded a greater number of total fragment ions.

TD/MD-MS approaches are also able to provide precise information for the determination of PTM type and position. Contrary to peptide mapping bottom-up strategies, TD/MD-MS workflows do not require extensive sample preparation (i.e. proteolytic digestion) before MS analysis. This not only dramatically reduces sample preparation time but, more importantly, it lowers potential experimental artifacts caused by the use of enzymes and chemicals [60-62]. Secondly, TD/MD-MS uniquely enables the discrimination and quantifying of mAb proteoforms (from relative peak intensities of intact mass-separated proteoforms). For PTM analysis by TD/MD-MS, the most commonly detected PTMs are, among others N-linked glycosylation, C-terminal Lys clipping, and N-terminal formation of pyroglutamate [63, 64]. Serial combination of ECD and UVPD also enables the monitoring of antibody chain pairing by TD- and MD-MS by cleaving intermolecular disulfide bonds linking the HC and the LC of a mAb at the intact and fragment levels [65]. TD/MD-MS analyses that combine CID and EAD fragmentation methods have also been reported for the detection and quantification of mAbs in serum samples [66], paving the way for tracking the progress of PTM dynamics and potential TD/MD-MS applications in pharmacokinetics (PK), biodisponibility or biotransformation studies.

In practice, TD/MD-MS approaches are still tricky to handle and require extensive expertise with regard to running experiments on the mass spectrometer as well as data treatment. However,

complementary bottom-up approaches are still necessary, as dynamic ranges and limits of detection of traces of proteoforms in highly pure mAb matrix are still limited in TD/MD-MS. For instance, Dekker *et al.* associated bottom-up and TD-MS strategies to study antigen-binding fragments of antibodies [67]. More recently, Wang *et al.* demonstrated the benefits of combining a bottom-up and TD-MS method for the characterization of a recombinant human growth hormone (hGH). This study can be considered as proof of concept in the context of biosimilar development, highlighting the high similarity between biosimilar products and those of innovators [68].

In terms of applications, a significant number of MD-MS (rather than TD-MS) studies have been carried out on mAbs [69-72] and antibody-drug conjugates (ADCs) [73-75] because mAb-based formats of smaller sizes are more efficiently sequenced. TD/MD-MS methods have yet to reach the holy grail of 100% sequence coverage or the quantification of major PTM hot spots required for mAb biosimilarity assessment. Fig. 2B and Fig. 2C show representative data obtained when using MD-MS approaches. Antibody sequence coverage using TD/MD-MS remains incomplete, reaching at best 86% for light chains and 76% for Fd subunits of NIST mAbs [76]. This sequence coverage obtained by TD/MD-MS is lower because it is particularly arduous to obtain backbone cleavages at each amino acid as well as identify and assign MS/MS fragments in highly populated spectra. For more heterogeneous ADCs (number and locations of payloads), TD/MD-MS is even more complicated. Hernandez-Alba *et al.* reported for the first time the MD analysis of a third-generation site-specific DAR4 ADC using different fragmentation techniques for primary sequence validation, drug conjugation, and glycosylation site assessment [74]. Watts *et al.* used in the same way a combination of UVPD, ETD, and ETHcD to maximize the sequence coverage of each subunit of the ADC up to 60-80% [75]. These combined fragmentation patterns provided sufficient information to allow confirmation of both the sequence of the CDR regions and the payload conjugation sites.

Although TD/MD-MS strategies currently present a series of limitations that hamper their routine implementation in biopharmaceutical R&D teams, many improvements have been made in this field. In 2020, an interlaboratory study showed the importance of experimental developments (fragmentation methods, MS parameters, etc) for the characterization of intact mAbs using TD/MD-MS approaches [63]. In the same way, comprehensive studies revealing a combination of parameters and leading to enhanced mAbs sequence coverage are highly beneficial to the scientific community [77, 78]. More importantly, TD/MD-MS approaches require robust downstream data treatment software that is accepted by a broad community. Indeed, TD/MD-MS data analysis can be critical due to complex MS/MS spectra and the specialized software required for fragment ion mass determination and database searching. Extensive efforts have been made in the area of data treatment. Several tools have been set up to facilitate the deconvolution, such as mMass [79], YADA [80], MSDeconv [81], or MASH [82], and to automate TD mass spectra assignment with better accuracy as MS-Align+ [83], ProSight Lite [84] and ProSight PTM 2.0 [85] or TopPIC [86]. De novo TD-MS sequencing has also been experimented with [87]. Recently, Lantz *et al.* developed an algorithm, ClipsMS, which can also assign internal fragments generated by TD-MS fragmentation, resulting in increased sequence coverage and better confidence in PTM site localization [88]. However, caution should be paid for putative false positive results [89].

Further development of analytical methods is still required to boost the implementation of TD/MD-MS in biopharmaceutical environments in the future. Multi-level mass spectrometry approaches with online hydrophilic interaction chromatography before TD analysis (HILIC-TD-MS) have been reported to improve N- and O- glycosylation characterization [90], notably in the context of SARS-CoV-2 receptor-binding domain (RBD) characterization [91, 92]. In addition, methods such as proton transfer charge reduction [93] and additional fragmentation might have a considerably positive effect

on the comprehensive characterization of mAb-based products, especially in the context of biosimilarity assessment.

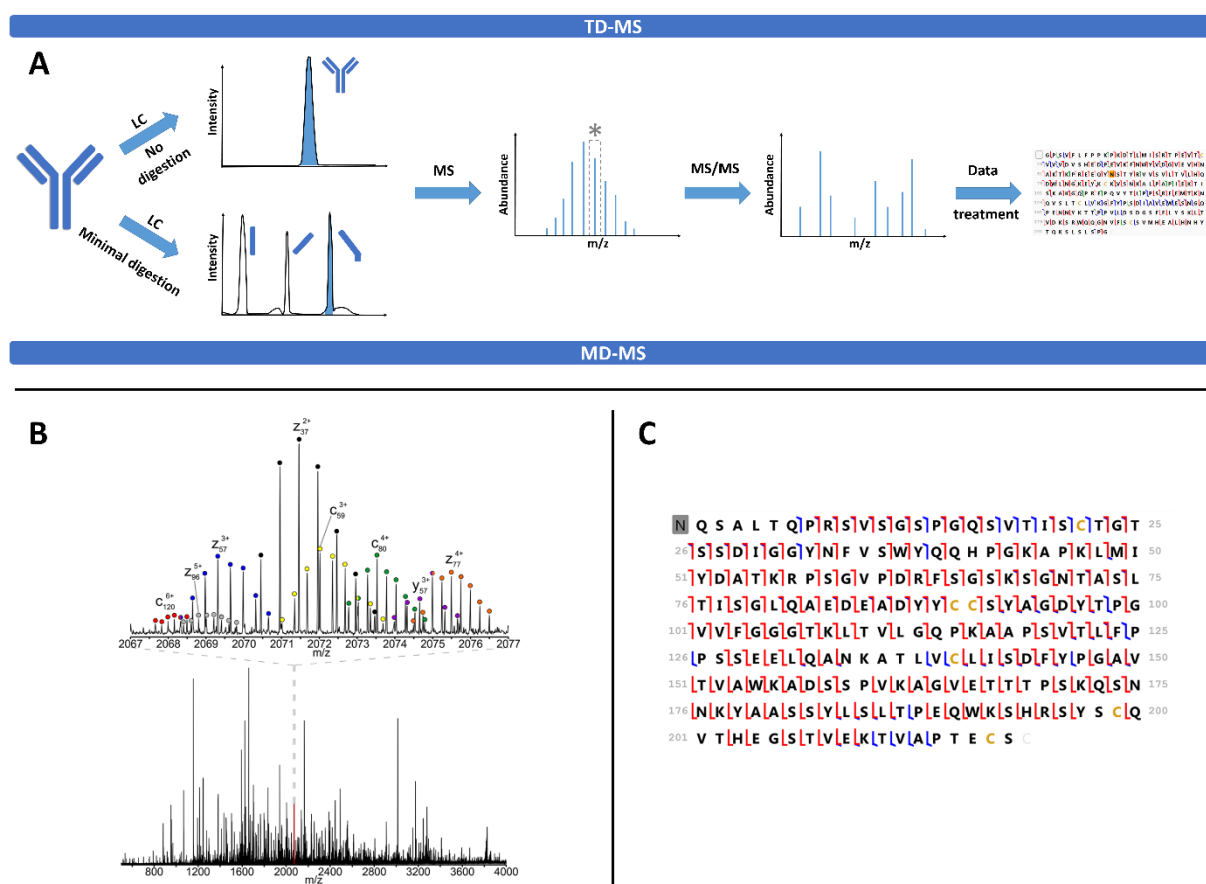


Fig. 2. (A) Schematic workflow of TD/MD approaches. (B) Example of an MD MS application to mAb analysis: sequencing of a light chain of SiLuLite mAb with a 21 T ESI FT-ICR MS employing ETD/PTR MS/MS. The inset shows an expanded view of a tandem mass spectrum with isotopic envelopes of product ions, which have been assigned and color-coded for facile visualization. Reproduced with permission from [56]. Copyright 2020 American Society for Mass Spectrometry. (C) Total sequence coverage of 85% achieved for the analysis of the disulfide bond-reduced light chain of SiLuLite mAb with a 21 T ESI FT-ICR MS, based on middle-down MS/MS (combination of results from two tandem mass spectra). Included are product ions identified from CID/PTR MS/MS (10 transients averaged, b/y-ions, cleavage sites shown in blue) and of ETD/PTR MS/MS (10 transients averaged, c/z-ions, cleavage sites shown in red). Reproduced with permission from [63]. Copyright 2020 American Society for Mass Spectrometry.

3. Intact mass measurements using native MS for size-, charge- and hydrophobic variant analysis

Among the intact-level protein analysis methods, classical intact/subunit mass measurement in denaturing conditions (water/acetonitrile/formic acid) either by direct infusion or through reversed phase LC-MS analysis are standard methods in most biopharmaceutical companies [46, 94] (for review see, [44]). More recently, nMS has entered R&D labs for the characterization of mAb-based formats as a powerful tool that allows the analysis of the protein in a “native-like” state. Compared to classical denaturing conditions, a narrower charge state distribution (CSD), in addition to lower charge states obtained in nMS conditions (usually electrosprayed in ammonium acetate volatile buffers), allows higher MS spectra resolutions and subsequent fewer interferences between overlapping CSDs [21, 33, 95]. This is of particular interest with regard to mAb-based formats of different sizes and complexities [20, 96]. A schematic representation of the method is provided in Fig.

3A. nMS enables the assessment of some relevant CQAs, including purity and homogeneity, as well as bispecific formation, drug load distribution, the determination of the average drug-to-antibody ratio (DAR) for ADCs, and even mAb mixture analyses. Regarding biosimilar simplified approval procedures, nMS provides a fast and reliable approach for the direct profiling of mAbs at the intact level, assessing CQAs related to homogeneity, size variants, charge variants, drug load distribution (for ADCs), and HOS [97]. For instance, nMS was reported for intact mass measurement and assessment of glycosylation profile differences between Avastin® (Bevacizumab) and its biosimilar versions [98]. However, the broader application of nMS has been long hampered by its lack of automation and thus reliance on manual buffer exchange methods. In recent years, the development of online separation techniques, both chromatographic and electrophoretic, which enable direct streamlining with nMS analysis, has allowed for quicker and more accurate analysis of biotherapeutics variants and has fostered its implementation in biopharmaceutical environments [20, 99].

Among such separation techniques, size exclusion chromatography (SEC), which separates species based on hydrodynamic volumes, has been directly coupled to nMS for rapid and automated buffer exchange using volatile ammonium acetate as mobile phase [20] or for identification of aggregates (or high molecular weight species, HMWS) and degradation products (or low molecular weight species, LMWS) [100, 101]. For instance, within the same SEC-nMS runs, several CQAs can be assessed within a few minutes, rendering intact mass measurement of the main product, purity, glycosylation profiles, HMWS, and LMWS. This has boosted the development of devoted integrated automatized analytical LC-nMS, precluding the democratization of SEC-nMS in both R&D and QC environments. SEC separation can also be implemented in 2D SECxSEC-nMS setups for the optimization of both separative and preparative coupling to native MS [102]. Fig. 3B illustrates the SEC-nMS biosimilarity assessment of four trastuzumab samples, exhibiting overlapping MS profiles between Herceptin® and biosimilar versions (Herzuma®, Ontruzant®, and Trazimera®).

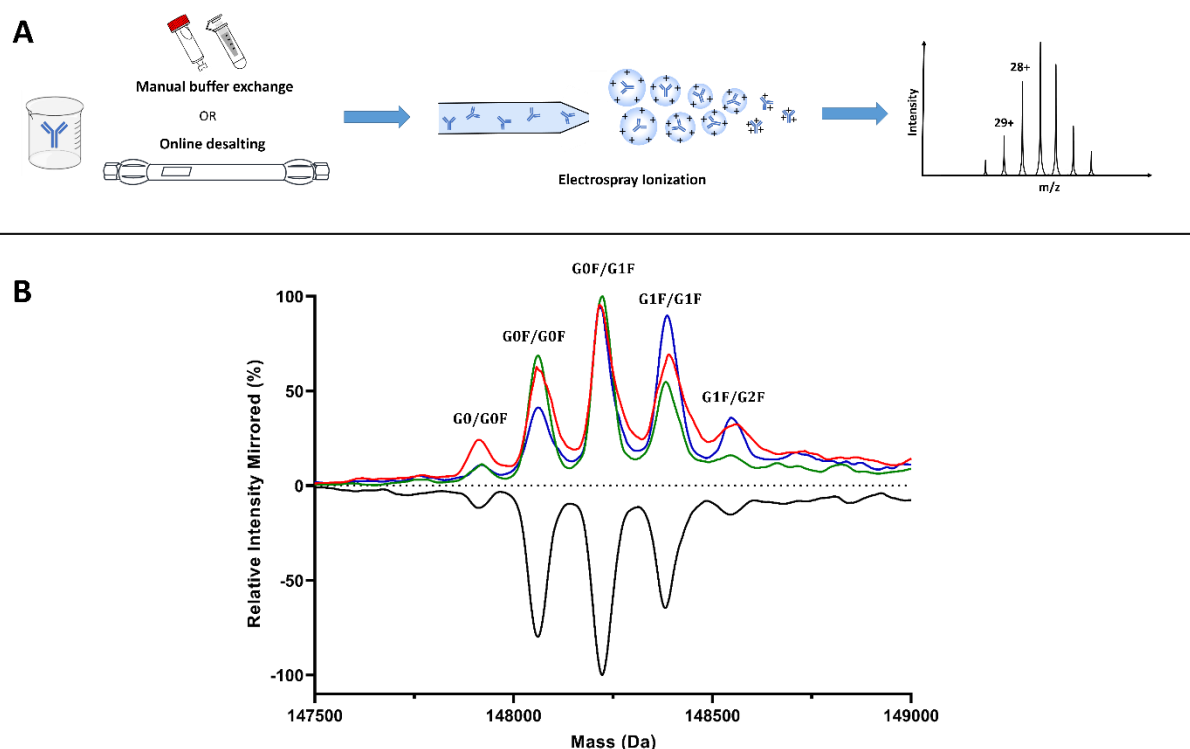


Fig. 3. (A) Schematic workflow of MS native approaches. (B) Deconvoluted native mass spectrum showing an overview of the glycosylation heterogeneity of trastuzumab, comparing superimposed biosimilar spectrum of Trazimera® (green), Herzuma® (blue), Ontruzant® (red), to mirrored originator Herceptin® (black).

Similarly, ion exchange chromatography (IEX), which separates molecules based on their surface charge profiles and is the gold standard for charge variant analysis [103], can be performed in nMS-compatible mobile phases. Charge variations can result from C-terminal lysine truncation, N-terminal pyroglutamate formation, deamidation, or various glycation profiles. Size variants can also be observed by IEX due to disulfide bond heterogeneity, or aggregation. Similar to SEC-nMS method development, the demonstration of direct IEX-nMS coupling using ammonium acetate as a mobile phase has been reported by several groups using either a pH gradient [104, 105], salt gradients [106], or a combination of both, called hybrid salt pH gradients [107]. The reliability of pH-based IEX separation depends on the robustness and linearity of the pH gradient, which can be difficult to obtain using volatile ammonium acetate buffer mobile phases. This can require the use of low amounts of organic modifiers or additives that can question the non-denaturing conditions of the separation (e.g. methanol [108]; acetic acid) but can be implemented at lower salt concentrations than salt-mediated gradients, providing better compatibility with nMS. Hybrid pH-salt gradients can be more difficult to implement but provide better chromatographic resolution and an additional separation dimension [109]. Recently, Murisier *et al.* [110] implemented a hybrid salt-mediated pH gradient, on an integrated LC-nMS platform, to provide a straightforward and rapid analysis of the charge variant profiles of Remicade® biosimilars (Infliximab). This approach allowed for the identification of pyroglutamate from N-term Glu/Gln residues, the presence of sialylated glycans, and the truncation of C-term lysine. The accurate identification of minor peaks representing 1-2% of relative abundance was achieved. Their results showed that such workflows can provide good separation, enabling the identification of small mass differences between PTMs even on a “low resolution” mass spectrometer [110]. In addition, anion exchange chromatography (AEX) hyphenated to nMS has been described for the study of highly glycosylated biotherapeutics erythropoietins (EPOs) [111]. AEX-nMS was also applied to identify the complex glycopattern of r-hGAA protein,

suggesting a wider application range for proteins of higher complexities in their glycosylation and/or phosphorylation profiles [112]. As IEX separation efficiency is related to the pI of the protein/fragment analysed, different modes of ion exchange can be considered to optimize the separation. For proteins with more basic averaging pIs, CEX tends to provide better separation, whereas AEX can be better suited for more acidic averaging pIs [113]. Liu *et al.* [114] applied a salt gradient on both intact and subunits levels of mAbs, with direct nMS coupling. AEX separation proved to be more efficient for IgG4 mAbs that tend to have generally more acidic pIs. Moreover, the separation of digested and deglycosylated fragments enabled the identification of site-specific deamidation variants and deamidation isoforms and provided comprehensive charge heterogeneity characterization [114]. More recently, Deslignière *et al.* also reported on the benefits of CEX-nMS at a middle level for improved characterization of Cys-engineered homogeneous DAR8 trastuzumab-deruxtecan ADC [115].

Finally, the coupling of hydrophobic interaction chromatography (HIC) to nMS is the most challenging technique. HIC is the gold standard for drug load distribution and average DAR assessment of Cys-ADCs [116] as well as for tackling degradation, fragmentation, misfolding, oxidation, carboxy-terminal heterogeneity, aspartic acid isomerization, unpaired cysteins, free thiols, and others [117]. HIC separates molecules based on the adsorption of the molecule through hydrophobic interaction with the stationary phase, which usually consists of mildly hydrophobic components, most commonly, relatively short n-alkyls, phenyl or ether, linked onto silica or polymeric matrix [118]. The elution from the stationary phase is driven by a decreasing salt gradient. Although the use of organic modifiers usually improves the chromatographic separation, this approach can be more difficult to couple to nMS directly. Despite the limitations inherent to the HIC separation performances, direct HIC-nMS couplings have been reported using high to medium volatile salt concentration gradients, both with and without the presence of organic modifiers, and thus questioning the “real” native-like separation of the species [119, 120]. A way to circumvent the use of “non-native” organic modifiers for HIC-nMS coupling is to consider multidimensional chromatography. Ehkirch *et al.* first reported on the comprehensive bi-dimensional HICxSEC-nMS setup [116] using a HIC column as the first dimension followed by an SEC column used as the second dimension for fast buffer exchange followed by nMS or nIM-MS. This straightforward setup allows direct coupling of HIC to nMS without compromising HIC separation performances for more accurate Cys-ADC analysis and detection of positional isomers that cannot be identified by both methods as a standalone [116]. More recently, Sarin *et al.* [121] reported on an automated method for the monitoring of charged-based heterogeneity of recombinant mAbs. This was achieved by implementing a first separation by HIC followed by weak cation exchange chromatography (HIC-WCX-MS) to provide a bi-dimensional separation, with a second WCX separation step directly compatible with nMS analysis (WCX mobile phase A: 25mM Ammonium Acetate pH = 6.0; mobile phase B 100mM ammonium acetate pH = 9.0). Such 2D LCxLC combinations can provide a way to increase the number of variants identified from a single separation technique, particularly on low abundance variants. The use of complementary separation techniques can differentiate variants such as pyroGlu, oxidation, or lysine, which would usually co-elute when applying either HIC or WCX individually [121].

Beyond chromatographic methods, non-denaturing separation techniques such as capillary electrophoresis (CE) can also be implemented online with nMS [122, 123]. In the context of ADC studies, for instance, DAR determination can be assessed using online CE-nMS [122]. Füssl *et al.* [124] provided an example of the profiling of a mAb charge variant, comparing CEX and CE-nMS as orthogonal separation techniques for intact protein analysis. Both techniques demonstrated the separation of an identical number of main charge variants, but CE-MS showed enhanced separation of isoforms within individual charge variants peaks, based on differences in glycoform complexity

influencing the hydrodynamic radius. Overall, CEX-nMS showed better adaptability to specific drug formulations, but CE-nMS tended to be more robust and less sample-consuming [124].

To summarize, nMS methods can provide key insights into biosimilar analytical characterization by assessing several CQAs, including size-, charge- and hydrophobic variants along with drug load distributions and average DAR.

4. HOS characterization

The analytical characterization of mAb-based format HOSs is challenging due to many sources of heterogeneities related to charge- and size- variants (multimers and aggregates), or modifications of primary sequences. However, while HOS and conformational heterogeneity are important for mAb stability, efficacy, safety, and structure-function relationships, analytical techniques that address these points both qualitatively and quantitatively are still lacking [18]. Several SMS approaches, including IM for global conformation characterization, to labelling methods for more in-depth structural investigation (HDX-MS, FPOP, XL-MS, etc.), are of major interest to tackle HOS from macro- to peptidic-resolution perspectives.

4.1. HOS at the intact level: ion mobility methods for biosimilarity assessment (IM-MS and CIU)

The use of ion mobility coupled to nMS (IM-nMS) enables the in-depth characterization of global protein conformation in the gas phase and is progressively being added to the nMS-based analytical toolbox. As IM measures ion drift under the influence of an electric field in the gas phase, it offers an additional dimension of ion separation in gas phase separation, based on charge and conformation [125-127]. The first generation of high-resolution instruments, introduced by Dugourd *et al.* [128], was based on linear drift tube IM-MS (DTIMS). In 2006, IM-MS platforms based on traveling wave IM-MS (TWIMS) were released, which helped make the technology more accessible to the research community [129, 130]. The focus was on improving the resolving power, and so different high-resolution technologies/platforms were developed after 2010, including trapped IM-MS (TIMS) [131, 132], cyclic TWIMS (cIM-MS) [133], as well as several structures for lossless ion manipulation (SLIM) designs [134-136]. In the meantime, other IMS techniques that rely on spatially-dispersive IM-MS separation have been developed and are currently being applied, such as the high-field asymmetric waveform IMS (FAIMS) [137].

For small molecule drug characterization, IM-MS is used as an additional dimension of separation and relies on collision cross-section measurements (CCS) [138, 139]. In the case of mAb-based formats, CCS calculation is neither straightforward with first-generation instruments nor with high-resolution IM cells [140]. Indeed, mAbs exhibit extremely complex conformational spaces that are incompatible with currently achievable IM resolutions. As HOSs are difficult to address for mAb validation, legal agencies have reported on the robustness, reproducibility, and repeatability of IM-MS in a comparative study involving five different mAbs, and concluded that the technique fulfilled legal agency requirements from the analytical point of view [141]. ADC characterization and DAR determination have also been performed thanks to IM-MS [142] and other IM-based technologies such as SLIM separation [143]. From an analytical point of view, IM analysis can be integrated into native LC-nMS methods previously reported. Etkirch *et al.* took the benefit of SEC-IM-nMS to separate and identify two monomeric conformers of pembrolizumab, which could be correlated to the coexistence of oxidized and non-oxidized mAb monomers [20].

The implementation of IM-MS technology can contribute to the establishment of an analytical method that aims to monitor and validate the glycan variations of batch production during the process of antibody development. Thus, the assessment of lot-to-lot heterogeneity in the glycosylation profile of the commercial mAb trastuzumab has been made possible thanks to nMS and IM-MS analysis [144]. In this work, four different production batches of trastuzumab were analysed and compared at intact and middle levels using online LC/MS. IM-MS analysis enabled the confirmation of the structures of glycan moieties of the glycopeptides. This approach allowed the parallel recording of all first- and second-generation product ions. In addition, with the two-step sequential fragmentation process, an optimization of the collision energy for glycosidic and peptide fragmentation can be conducted separately. The information obtained is maximized in each step by avoiding the loss of glycan sequence information due to the higher energy applied for peptide backbone fragmentation. More recently, the study of an originator (infliximab) and its biosimilars (Remicade® and Inflectra®) has been carried out [145].

Moreover, as CCS measurements of mAb products are often not informative and because very close conformation ($< 2\%$ in ΔCCS) cannot be differentiated by standard IM-MS measurements, due to a lack of resolution and poor IM separation, alternative IM-based methods have been developed. Ruotolo and colleagues introduced collision-induced unfolding (CIU) experiments in 2009 as a way to circumvent the lack of resolution of first-generation IM-MS instruments and provide information on gas phase collision-induced unfolding behaviour [35, 146]. CIU experiments are performed by a sequential increase of an accelerating potential difference to induce ion activation before IMS separation. As the accelerating voltage is raised before the IM cell, collisions with the buffer gas become more energetic and induce progressive gas-phase unfolding (Fig. 4A) [147]. Thus, CIU has already been able to reveal differences in mAb structure and stability based on changes in their sequence [148]. This allows the monitoring of domain exchanges in antibodies [149] or the differentiation of stability shifts associated with antibody disulfide [150], glycosylation patterns [151], ADC conjugation [152, 153], or multispecific mAbs [154, 155]. Interestingly, depending on the question being addressed, middle-level CIU experiments performed on Fab or Fc domains [140] have proved to be more informative than on intact mAb CIU. In addition, when combined with SEC [156] or CEX [157], CIU experiments can be performed in more straightforward and automated workflows, paving the way for size and charge-variant CIU analysis. In the context of biosimilarity, IM-MS and especially CIU approaches performed with high-resolution IM instrumentations could detect subtle conformational differences that had only been poorly resolved on early-generation IM platforms [154].

Several studies [98, 158] used IM-MS strategies to compare drift times generated from TWIMS analysis of originator and biosimilar mAb and revealed similar highly charged species and homogeneous IMS driftscope plots. In agreement with NMR, circular dichroism, and infrared spectroscopy, the approach of Montacir *et al.* confirmed the similar primary and higher order structure of Rituximab biosimilar and originator. Pisupati *et al.* [159] applied IM-MS for the characterization of HOSs of the originator Remicade® (infliximab) and its biosimilar version (Remsima®). To enhance higher order structural characterization, intact mAbs were first analysed by IM-MS under native conditions. The two mAbs had similar IM drift times with discrete positions in drift time versus m/z space for antibody fragments, monomers, and dimers. Concerning CIU analysis, although a significant similarity can be assessed at high collision voltages, a marked difference was observed at collision voltages below 50 V (Fig. 4B). Previous evidence has linked this region of mAb CIU plots to the Fc region and glycoform variations [150]. In a study carried out by Kang *et al.* in 2020 [160], three different innovator and biosimilar pairs – rituximab, bevacizumab, and trastuzumab – were characterized by IM-MS and CIU approaches. They compared the difference in folding stability

regarding the transitions of the CIU fingerprints by calculating the CIU50, the voltage at which 50% of one more compact transition state converts into the next more unfolded population. While no significant differences in CIU50s were observed between the trastuzumab and bevacizumab biosimilar and original products, the CIU50 value for the second transition (CIU50-2) was higher for the rituximab biosimilar than the originator. They also looked at structural differences after thermal stress IM-MS analysis revealed several subtle, but statistically significant changes after 4 weeks of incubation. For instance, concerning the bevacizumab pair, the CIU50-1 changed significantly for the originator but not for the biosimilar. The latter had a higher CIU50-2 (a 7% difference) than the original mAb after stress. In summary, CIU-IM-MS analysis provided a gas-phase folding stability profile for the original and follow-on products, confirmed the same year by a comparable study [161].

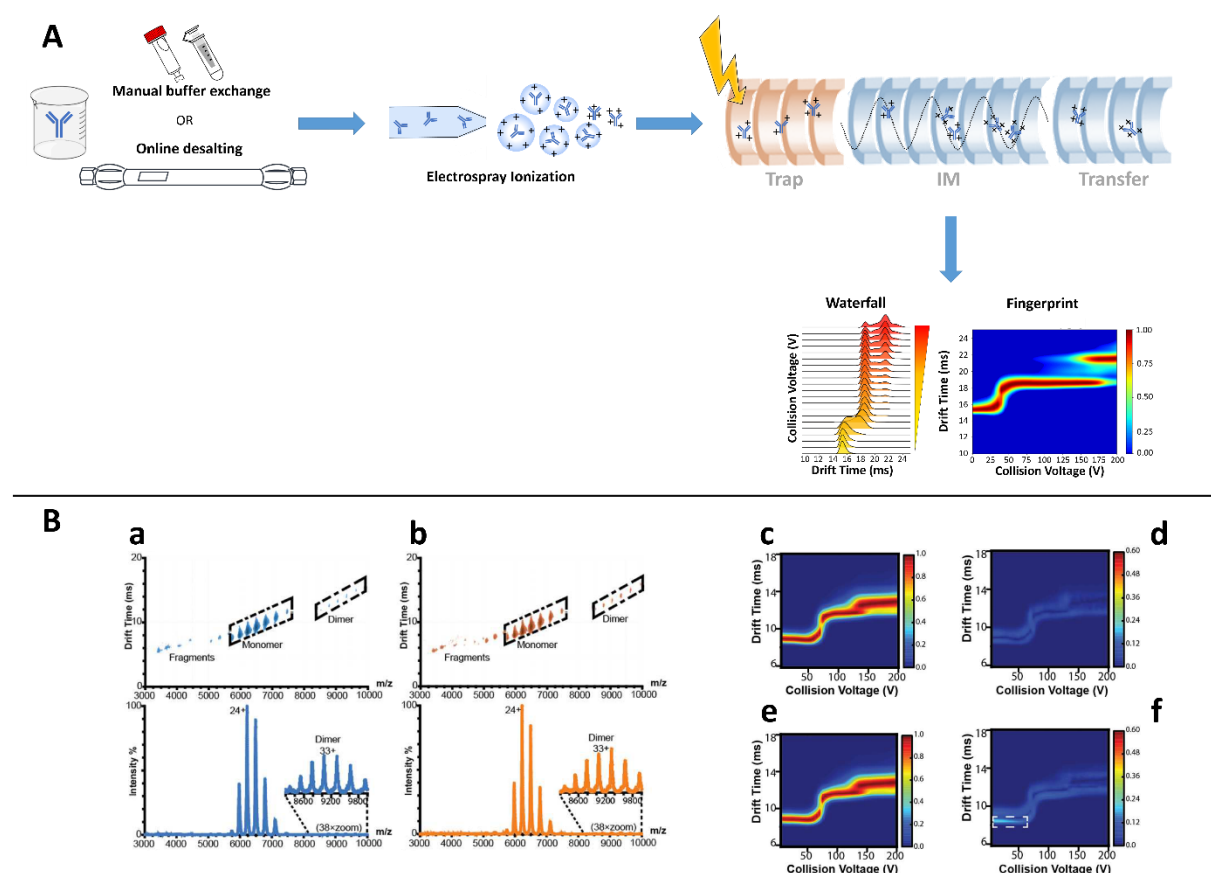


Fig. 4. (A) Schematic workflow of IM-MS and CIU approaches. (B) Ion-mobility mass spectrometry of Remicade® (RC) and Remsima® (RS). Representative IM-MS spectra of a, RC and b, RS with annotated fragment, monomer, and dimer species. Average collision induced unfolding (CIU) fingerprint of c, RC and e, RS and standard deviations of d, RC and f, RS CIU fingerprints (n = 4 lots). Reproduced with permission from [159]. Copyright 2017 American Society for Mass Spectrometry.

As seen in the previous study [160], comparing biosimilar and reference products in forced degradation studies using thermal, mechanical, or chemical stressors [162] appears to be a strategy for evaluating biosimilarity. Forced degradation studies generate valuable analytical data, in relatively short periods, to compare biosimilar and reference products and to help link protein structural information with product quality, safety, and efficacy. Protein product assessment under stressed conditions gives insights into the mechanisms of how these proteins may unfold, lose efficacy, aggregate, and become immunogenic, etc. The power of CIU to monitor such differences has been highlighted in a recent study comparing antibodies with different CDR regions under pH and heat degradation conditions [163].

4.2. Reversible labelling methods for HOS characterization: HDX-MS

HDX-MS is mostly used as a differential approach that compares the deuterium incorporation within a protein in two different states (e.g. free versus bound, stressed versus unstressed, etc.). Deuteration consists of the dilution of samples in a deuterated solvent (usually D₂O) and labile amide backbone hydrogens are exchanged with deuterium. The deuteration reaction is quenched using acidic pH (2.5) and low temperature (0°C) to avoid back-exchange. Then, the protein is enzymatically digested with adapted proteases (usually pepsin), followed by LC-MS/MS analysis. The deuteration step is realized at different time points to compare deuterated profiles and deuterium incorporation in the absence or presence of protein partners [37, 164]. A schematic representation of the method is provided in Fig. 5A. HDX-MS is particularly suitable for the study of protein conformational dynamics by assessing solvent accessibility as a function of time. It can also provide information on interacting regions between protein partners, even enabling differentiation of allosteric from orthosteric binding. HDX-MS is now a well-established method for characterizing individual proteins as well as large protein complexes. In the booming market of biotherapeutics, reviews referring to the use of HDX-MS in the context of drug discovery and therapeutics are currently flourishing [165-168].

In the context of biotherapeutics development, HDX-MS has played an important role in mapping epitopes of antibody–antigen interactions [169-172]. The HDX-MS approach is particularly useful for epitopes that span multiple protein binding sites, as these are particularly challenging to study through other structural approaches [173]. HDX-MS has emerged as one of the techniques of choice for mapping epitopes of mAbs [174] as well as for protein therapeutics with payload conjugations [175] or more complex samples such as bispecific antibodies [176].

Conformational analysis of proteins is used in the pharmaceutical industry not only for epitope mapping but also for the development of biosimilar products. Although a review published in 2015 [21] stressed the benefits of HDX-MS for originator and biosimilar comparison, there are few studies on this application in the literature. In 2013, Visser *et al.* [177] used HDX-MS to compare a rituximab biosimilar and its originator and found a high similarity between the two mAbs at the level of their primary structures, HOS, PTMs, and biological properties. Even when using different cell lines and manufacturing, the study showed that the biosimilar and the innovator had the same conformations. Other studies [145, 161] reached the same conclusions a few years later. In comparing Remicade® (reference) and Inflectra® (biosimilar), Fang *et al.* [145] observed very small differences, close to the location of the glycan site (Fig. 5B). These changes suggested slight differences in glycan composition at the glycosylation site lead to small conformational changes between the innovator and the biosimilar that do not affect the function of the biosimilar. A report [178] published in 2017 dealt with direct comparisons of reference versus biosimilar proteins using, among others, HDX-MS. This quality assessment was performed according to the International Conference on Harmonization (ICH) comparability guidelines and the biosimilar guidelines of EMA and FDA, thus showing the importance of this analytical technique, which was officially recognized for the assessment of HOS.

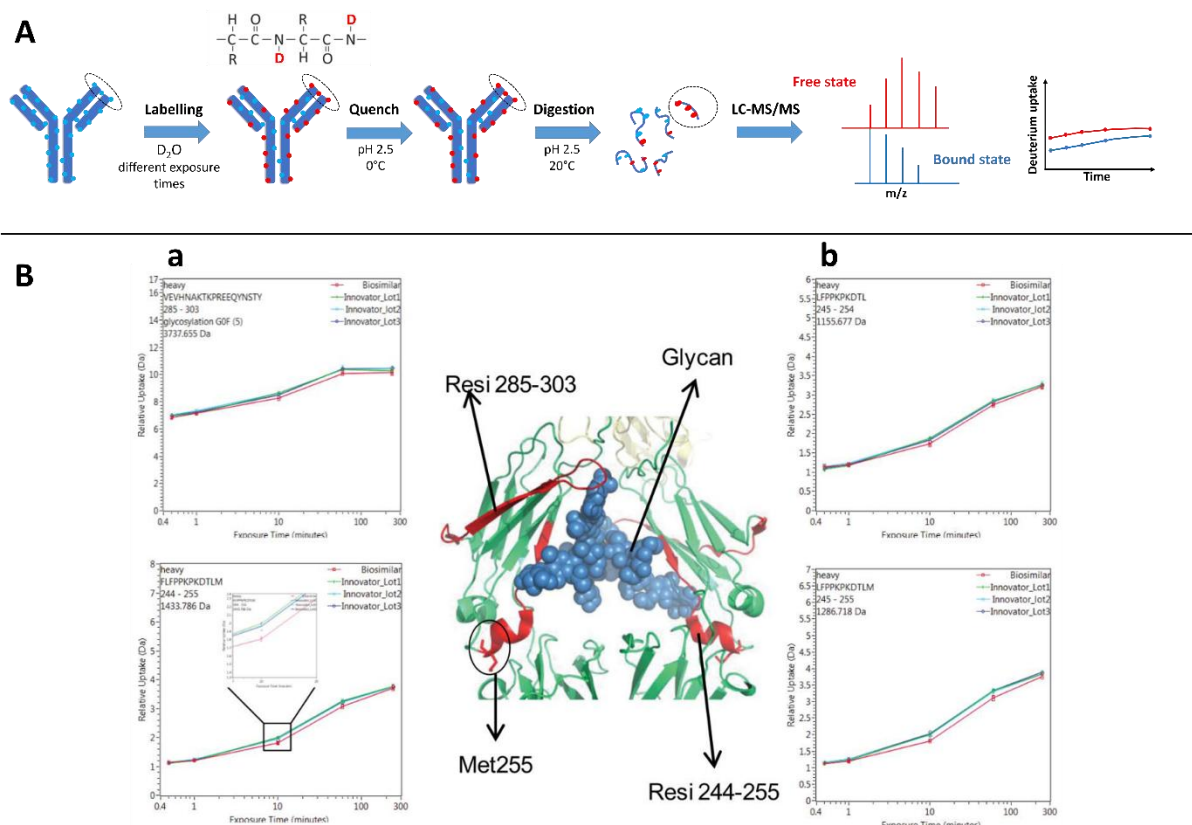


Fig. 5. (A) Schematic workflow of the HDX approach. (B) Comparison of deuterium incorporation of innovator and biosimilar samples. (a) Representative deuterium incorporation profiles of regions (residues 244–255, 245–254, 245–255, 285–303) in CH2 domains shows a minute difference. The red line represents the data from the biosimilar product; the green, cyan and blue lines represent the data from the 3 batches of the innovator samples. The experiments have been repeated in triplicate runs. (b) The location of the region that displayed minor difference among biosimilar and innovator samples are coloured in red in the model structure of IgG1 (PDB: 1HZH). Glycosylation is shown in blue. Met255 is circled and shown in stick notation. Reproduced from [145].

For the growing area of mAb development, Madjumbar *et al.* [179] outlined the use of HDX-MS for monitoring dynamics and conformational changes in mAbs as a result of modifications or degradation [180], etc. Moreover, the production of biotherapeutics requires novel bioanalytical techniques to carry out batch-to-batch quality control [181–183], where again HDX-MS brings an additional level of in-solution conformational characterization. In addition, HDX-MS appears to be particularly useful for routine analysis to assess effector functions [184, 185] or the impact of post-translational modifications (PTMs) on the structure of a mAb. HDX-MS was reported to examine how PTMs of IgG1 antibodies, specifically methionine oxidation, impact their ability to bind with the FcγRIIIa receptor [186]. Several papers have been published regarding protein glycosylation, one of the most common PTMs [187]. Thus, Anderson *et al.* [188] reported the stabilization of a complex IgG1-FcγRIa, thanks to intramolecular glycan-protein bonds.

In addition, new methodologies are also emerging to facilitate HDX-MS analysis of disulfide-bonded proteins with, for example, the introduction of electrochemical reduction [189–191], the implementation of a SEC-HDX coupling [192] or the use of dual protease columns for enhanced sample digestion during the analysis [193]. Another paper [194] promotes the establishment of an accelerated workflow evaluating lead monoclonal antibodies.

4.3. Irreversible labelling method for HOS characterization

4.3.1. Chemical cross-linking MS (XL-MS)

XL-MS is an additional powerful tool for mapping protein/protein or protein/ligand interactions and elucidating the architectures of protein complexes, which crucial to refining 3D structural models generated from conventional biophysical techniques [195, 196]. The chemical cross-linking of proteins or complexes in their native states creates new covalent bonds between proximal residues [197]. Cross-linkers are composed of different elements: the spacer indicates the distance between the two-targeted residues and the reactive end groups dictates which amino acids are targeted [198]. After the cross-linking reaction, the cross-linked proteins and protein complexes are usually enzymatically digested and analysed by high-resolution mass spectrometry [199]. The identification of cross-linked peptides enables the determination of spatial proximities, either between residues located on two different proteins (interprotein cross-links) or within one protein (intraprotein cross-links). Dead-end cross-links can also provide information on solvent accessibility.

Considerable effort has been made to optimize each step of the workflow with the implementation of numerous methodological developments throughout the analysis. The review by Piersimoni *et al.* published in 2022 provides a comprehensive description of these strategies [200]. Some of them refer to the chemical nature of the cross-linking reagents themselves (photoactivable, MS-cleavable, wide range of sizes for the spacer, etc.) [201]. The advent of XL-MS is also closely related to the massive technical advances in MS instrumentation. Currently, available instruments offer capabilities in mass accuracy, resolving power, sensitivity, and dynamic range that allow biological questions to be favourably addressed by XL-MS. Recently, the implementation of different modes of MS fragmentation [202, 203], DIA acquisition, or ion mobility [204-206] has significantly helped improve the efficiency and confidence of cross-link identification. Along with instrumental developments, major efforts have been made to establish bioinformatics tools using specialized algorithms and software [197]. Finally, enrichment strategies have been implemented to enhance cross-linked peptide detection. The enrichment of specifically cross-linked peptides before MS analysis offers another possibility to preselect modified peptides. They can be enriched by pre-fractionation (with SCX or SEC) [207] or by affinity purification [208, 209]. These developments are particularly relevant for *in vivo* XL-MS analysis of protein complexes [210].

While XL-MS is widespread for protein-protein interaction analysis, it is scarcely used for mAb analysis. To date, only a few works mention the use of crosslinks in a study involving biotherapeutics. Tremblay *et al.* show how XL-MS can clarify HDX-MS data in the context of antibody–antigen interactions [211]. The results highlight the synergistic information given by the two methods suggesting their potential for studying HOS of protein therapeutics and thus for accelerating therapeutic protein development.

4.3.2. Fast photochemical oxidation of proteins MS (FPOP-MS)

Another technique that is increasingly gaining importance and deserves attention is FPOP as shown by the number of recent reviews [212-215]. FPOP footprinting is a structural mass spectrometry method that maps proteins by fast and irreversible chemical reactions. This analytical strategy belongs to the family of hydroxyl radical footprinting (HRF) techniques, which consist in exposing a protein in solution to hydroxyl radicals $\text{OH}^\bullet$. To date, several strategies have been designed to generate hydroxyl radicals such as radiolysis of water [216], Fenton chemistry [217], the use of synchrotron [218], high voltage electrical discharge [219], plasma-induced modification of biomolecules [220] or electrochemistry [221, 222]. First developed in 2005 by Hambly and Gross [223], FPOP is based on laser photolysis of peroxide H_2O_2 [224, 225]. In practice, a solution of protein and hydrogen peroxide is flowed through a fused silica capillary and irradiated by a 248 nm KrF

excimer laser [226]. Thus, the protein sample is exposed to oxidation during a short time window. FPOP-labelled proteins are then typically analysed after enzymatic digestion using bottom-up approaches by LC-MS/MS. The ratio of oxidatively-labelled and unlabelled peptides obtained under different conditions provides information on protein conformation, structural dynamics, and interactions [212, 227]. A schematic representation of the method is provided in Fig. 6A.

In contrast with HDX-MS, FPOP oxidative labelling is irreversible. It allows for a more flexible post-labelling workflow. Indeed, extensive and optimized sample handling as well as LC separation before MS analysis is possible without concern for losing structural information [227, 228]. Another advantage is the short microsecond timescale of FPOP labelling which allows monitoring faster structural changes of the molecule closer to its native environment [224, 229]. This is made possible by the introduction of an appropriate scavenger that controls the radical lifetime in competing with its self-quenching [41]. Thus, it enables the study of rapid kinetics [230] and ensures that protein labelling is occurring faster than proteins can unfold [231]. Similar to HDX-MS, oxidative modifications induced by FPOP reflect solvent accessibility but hydroxyl radicals react primarily with side chains of amino acids, and not with the protein backbone [232]. In addition, as radicals can oxidatively label side chains of many different amino acids [212] and as FPOP cannot suffer from label scrambling during MS fragmentation (in contrast to HDX), good spatial resolution with residue-specific information can be achieved.

Several types of applications are now covered with FPOP analysis such as conformational dynamics, aggregation, or epitope mapping. Numerous studies have shown the interest of FPOP to study antibody-antigen interactions [233-237]. For instance, Zhang *et al.* used FPOP to interrogate the HOS of biotherapeutics in mapping the binding interface between a monoclonal antibody Fab fragment and the vascular endothelial growth factor protein [234]. The changes observed in solvent accessibility between the free and the complexed Fab-1 highlighted regions involved in the binding interaction, corroborating the X-ray crystal structure information. More recently, Schick *et al.* demonstrated the benefit of FPOP not only for drug-target interactions but also for host drug response in probing the epitopes for polyclonal anti-drug antibodies raised against a bispecific mAb [176]. FPOP can also be used to report local and long-range conformational changes in mAbs associated with high glycosylated protein [238], reversible self-association [239], and Fc binding [184, 240]. These studies demonstrated the sensitivity of FPOP to identify minor solvent-accessibility differences, otherwise challenging to identify using other structural and footprinting approaches [184]. In the context of biosimilarity assessment, FPOP has also been used to investigate the effect of different formulation buffer conditions on the structure of adalimumab and its thermal stability and aggregation properties. Conformational changes and aggregation have been detected, along with the identification of the affected specific regions [241].

Although FPOP has proven useful to the study of biotherapeutics, it still has limitations that must be overcome. For instance, amino acids have different reactivities with hydroxyl radicals [242]. Because of these differences, highly reactive residues such as methionine may be modified whether they are solvent-accessible or not. It is now possible to account for these differences to calculate a protection factor, as reactivity rates of the amino acids are better known [243, 244]. Other adaptations and improvements to FPOP have focused on more accurate measurement of the radical dose to ensure reproducibility and consistency. Strategies have been developed to more precisely measure the hydroxyl radical dose experienced by the protein analytes by inline dosimetry, measuring changes in the UV absorbance of either adenine [245] or Tris buffer [246]. Consequently, this allows FPOP comparisons between different solution conditions where the OH[•] scavenging ability of the buffer may vary [241, 246, 247]. Finally, despite advances in analysis software [248-251] and quantitation

methods [252], FPOP methods lack intuitive and easy-to-use data processing tools to streamline and facilitate data analysis. This is also one explanation for the near absence of this technique in the analytical toolbox of biopharma industries. In the last decade, important advances have been made, especially in throughput and accessibility, with several footprinting platforms now available [253-255]. Sharp *et al.* [253] showed the effective determination of the epitope of TNF α recognized by adalimumab using the commercially available flash oxidation (FOX) protein footprinting system (results are depicted in Fig. 6B). The development of these easy-to-use and automated platforms would pave the way for the adoption of FPOP to a wider audience.

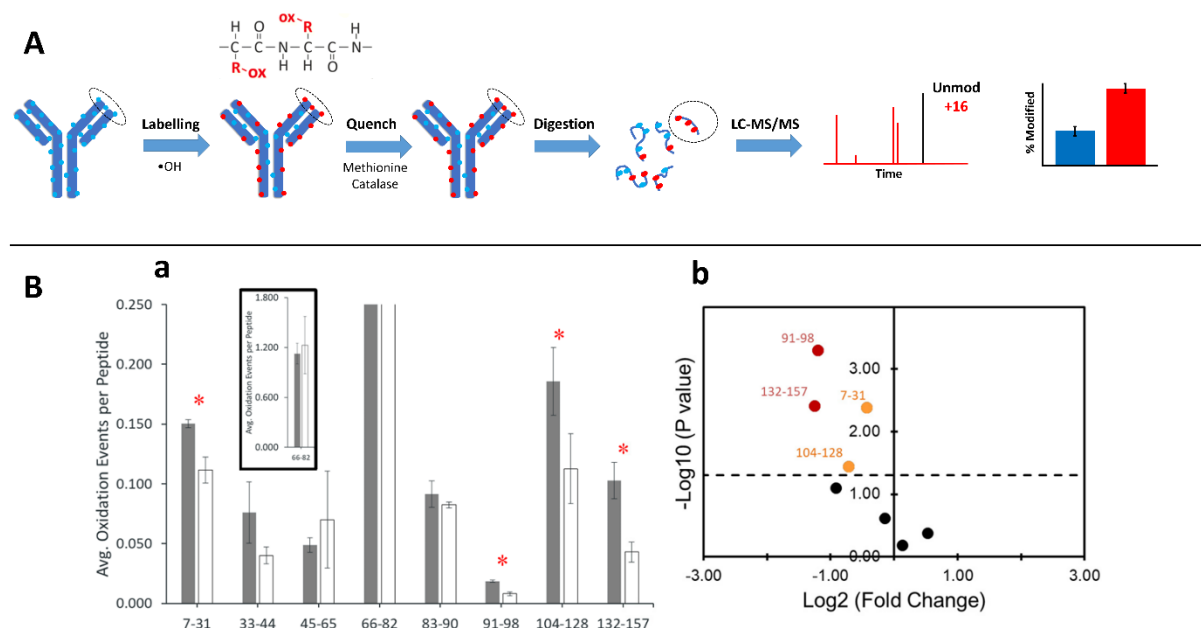


Fig. 6. (A) Schematic workflow for FPOP analysis. (B) FOX system HRPf of TNF α in the presence or absence of adalimumab. (a) Histogram of FOX system HRPf peptide level results for TNF α alone (filled) or in the presence of adalimumab, compensated for scavenging (open) as reported by FoxWare Protein Footprinting software. (Inset) Peptide 66–82 results rescaled. Error bars represent one standard deviation from a triplicate measurement. Peptides that showed statistically significant protection upon adalimumab binding are marked with a red asterisk ($p \leq 0.05$). (b) Volcano plot of changes in TNF α FOX system HRPf results upon binding of adalimumab. Peptides that showed a statistically significant change that was at least a two-fold reduction in oxidation are in red, while peptides that showed less than a two-fold reduction in oxidation are in orange. Reproduced with permission from [253]. Copyright 2021 American Society for Mass Spectrometry.

5. Combination of different structural MS methods for in-depth characterization

The benefits and strengths of structural MS methods for biosimilarity assessment are even more obvious when used in combination with, or to complement, other structural biology biophysical techniques. For instance, HDX-MS is increasingly being used to complement MS methods such as native MS [256] and orthogonal techniques such as X-ray crystallography [257], SPR [258], or cryo-EM [259]. Pan *et al.* [260] described a comparison of HOS analysis of mAbs using, for the first time, bottom-up and top-down HDX-MS methods. They analysed two bevacizumab biosimilars to the originator using two different sequencing methods. The bottom-up and top-down sequencing approaches both indicated that the three samples had no differences in HOSs (zero difference at various HDX time points). It should be mentioned that neither of the two strategies were able to obtain 100% sequence coverage. However, it was also concluded that top-down sequencing, combining ETD/ECD analysis, was better suited for detecting subtle differences as it presents a significantly lower back exchange ($\sim 2\%$) and a close-to-residue-level spatial resolution compared to classical bottom-up HDX-MS [260]. In 2020, in the work of Kang *et al.* [161], the structural similarity

determined by IM-MS was supported by HDX as well. Yet the authors quite rightly mentioned that structural perturbations were likely below the limit of detection for HDX-MS (peptide level resolution) but may still be present. The same year, another study employed epitope mapping to assess epitope similarity [261] and provided a rapid method for the comparison of binding epitopes, which is critical for the assessment of similarity. Once again, Avastin® (bevacizumab) and its biosimilar were shown to have highly similar binding epitopes with the target. Although subtle differences were observed in the target conformational dynamics after binding, it would be arduous to attribute these subtle differences to functional variations. Moreover, with the current progress in computational structure prediction [262, 263], incorporation of HDX-MS data into antibody-antigen complex docking raises challenges that must be tackled to go toward a more reliable and high throughput epitope identification [264, 265].

Finally, numerous studies notably carried out both FPOP and HDX to characterize protein structure [184, 233, 235, 266, 267]. This synergy can be explained in terms of labelling timescale and labelling targets (i.e. side-chain vs backbone) [232, 233]. By using several orthogonal structural techniques including HDX-MS and FPOP, Li *et al.* [235] pointed out the identification of an energetic epitope by determining the interfacial hot spot that controls the binding affinity for an anti-interleukin-23 antibody. The results of this work demonstrated the complementarity of FPOP and HDX. On one hand, HDX was able to inform on regions that were unreactive to hydroxyl radicals (no production of oxidation products) for which FPOP could not provide information. On the other hand, FPOP, which has a faster labelling time scale, could detect changes occurring more rapidly.

In another study, the combination of FPOP, IM-MS, and top-down enabled the reporting of structural differences between IgG2 isomers that have distinct disulfide isomeric structures [268]. TD-MS and FPOP-MS detected localized conformational changes and suggested low flexibility in the N- and C-terminus of the light chain, owing to structural constraints of the disulfide bond. In addition, the binding region on IgG2 antibody, which is responsible for antigen specificity and affinity, displayed no significant FPOP differences for mutants versus wild type, suggesting that antigen-binding affinity of the mutants and wild type is probably similar.

6. Concluding Remarks and Perspectives

While already well established in the field of structural biology, structural MS approaches have also entered biopharmaceutical R&D laboratories. This review highlights the many MS-based analytical techniques that are available for the in-depth characterization of different therapeutic mAb-based formats, from the discovery phase to the development process as well as for biosimilarity assessment. Molecular biosimilarity assessment made considerable progress in the past ten years, namely with the advent of state-of-the-art MS-based approaches performed in a “native-like” state. Hyphenation of non-denaturing LC methods with native MS approaches has fostered the routine use of advanced structural MS methods. Intact-level strategies (TD/MD-MS and nMS) can assess primary structures, size variants, and drug load distributions. In addition, HDX-MS has its place in tackling the dynamics of antigen binding, epitope/paratope mapping, and batch-to-batch structural comparison. Although it is not an MS-based method, mass photometry (MP) is a recent addition to the field of structural biology that affords direct mass measurements of intact noncovalent assemblies. This label-free technique is an optical method that quantifies molecular weights of biomolecules at the single-molecule level by interferometric detection of scattered light [269]. MP is a handy and rapid tool that provides insight into therapeutics and by-products under different conditions and is already finding its place in the analysis of therapeutics. One of the main advantages is that a broad range of volatile and nonvolatile buffer solutions can be analysed without any sample preparation step. In addition, MP is more sensitive than native MS, allowing it to analyse proteins in a 100 pM – 100 nM

concentration range, while micromolar concentrations ($> 5 \mu\text{M}$) are necessary in nMS [270]. Even if MP mass measurements are far less resolutive and accurate than nMS, this method offers a rapid snapshot of sample heterogeneity, as well as assessing binding affinities of mAb/antigen complexes [271, 272]. The major drawbacks of this technique are the resolution and the limited low molecular weight range, which will most likely be eliminated shortly through instrumental improvements [273]. MP could undoubtedly become an essential routine tool for quality control of mAb-based products, particularly to provide fast and easy monitoring of forced degraded studies and biosimilarity assessment.

Contrary to molecular biosimilarity assessment, HOS biosimilarity demonstration is much more challenging and still needs method development. While atomic resolution biophysical techniques can serve for drug design, they still fail to provide large-scale 3D structures of mAb-based formats. This is mostly due to the high “heterogeneity” of mAb compounds (in terms of variants, PTMs, drug conjugation, etc.) as well as to the high flexibility related to the hinge region of those types of biologics. To circumvent these drawbacks, innovative approaches combining preparative MS deposition before EM acquisition are emerging. Approaches based on electrospray ion-beam deposition (ES-IBD) [274, 275] have been developed for the precisely controlled preparation of materials on surfaces. This preparative MS technique is based on *in vacuo* mass selection of the species to be deposited, allowing the preparation of homogeneous material for further imaging. Folded protein ions are generated by native before mass-to-charge ratio filtering (separation of species of interest from other proteins, contaminants, aggregates, fragments, etc.) and deposited with a controlled landing energy onto cryo-EM grids for instance [276, 277]. ES-IBD is often referred to as “soft landing” at lower collision energies or as “reactive landing” at higher collision energies or if the collision results in the formation of a covalent bond to the surface [278, 279]. These new methods would accelerate the screening of sample conditions as well as interpreting and refining 3D structures of gas-phase purified biotherapeutics.

Finally, another promising breakthrough lies in structure prediction tools adapted for biosimilarity assessment. Protein modelling recently made a major leap in successful structure prediction with artificial intelligence tools such as AlphaFold [280, 281]. This is a deep-learning algorithm designed to predict with high accuracy the three-dimensional structure of proteins and thus tackle the issue of protein folding [282, 283]. AlphaFold incorporates physical and biological knowledge about protein structure, leveraging multi-sequence alignments, into the design of the deep-learning algorithm [281]. It takes advantage of the observation that residues in spatial contact tend to show patterns of correlated mutations [284]. This advance in computational tools may particularly assist with the development of biosimilars [285]. The impact of subtle structural differences could be verified by running a 3D structure check, thanks to AlphaFold software. Combining sensitive structural techniques with AlphaFold predictions would give insight into the 3D structure of a biosimilar product. However, trained on the Protein Data Bank (PDB) data, some bias may occur towards under-represented protein classes (especially antibodies representing only 3.5% of the PDB released structures in 2023) as many diverse targets of pharmacological interest are represented. To overcome this issue, Deane *et al.* implemented the Structural Antibody Database (SAbDab) containing updated information such as curated affinity data or sequence annotations [286, 287]. In 2020, they went further to develop the Therapeutic Structural Antibody Database (Thera-SAbDab). This database records all mAb-based formats recognized by the World Health Organisation (WHO) and identifies any corresponding structures in SAbDab with near-exact or exact variable domain sequence matches. More recently, Ruffolo *et al.* set up a new deep-learning method for antibody structure prediction called IgFold [288]. IgFold consists of a pre-trained language model trained on 558 million natural antibody sequences followed by graph networks that directly predict backbone

atom coordinates. Although other antibody-specific deep learning methods such as DeepAb [289] or ABlooper [290] have significantly improved CDR loop modelling accuracy, IgFold is said to predict structures of similar or better quality than those alternative methods (including AlphaFold) in significantly less time. Ruffolo *et al.* were able to predict structures for 1.4 million paired antibody sequences, providing structural insights into 500-fold more antibodies than have experimentally determined structures. All these developments in AI-based bioinformatic models demonstrate the ability to bring solid evidence of 3D structures, enabling the confirmation of biosimilarity without necessitating in vivo studies.

Altogether, we expect that empowered, fast, and robust structural MS workflows combined with deep learning and artificial intelligence will boost biosimilarity assessment and, more generally, therapeutic protein characterization.

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