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The impact of mass spectrometry on the study of intact antibodies: from post-translational modifications to structural analysis

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Monoclonal antibodies (mAbs) are important therapeutics, targeting a variety of diseases ranging from cancers to neurodegenerative disorders. In developmental stages and prior to clinical use, these molecules require thorough structural characterisation, but their large size and heterogeneity present challenges for most analytical techniques. Over the past 20 years, mass spectrometry (MS) has transformed from a tool for small molecule analysis to a technique that can be used to study large intact proteins and non-covalent protein complexes. Here, we review several MS-based techniques that have emerged for the analysis of intact mAbs and discuss the prospects of using these technologies for the analysis of biopharmaceuticals.

1. Introduction

Therapeutic monoclonal antibodies (mAbs) have moved to the forefront of pharmaceutical and biotechnological research for development as treatments against cancers, neurodegenerative, and immunological diseases as well as for possible usage as antibacterial agents.[1–3] Antibodies are an enticing treatment option due to their high target specificity which generally results in decreased side effects.[4] Since 1986, approximately 30 mAbs have been introduced for clinical use,[5] and many more are in research, developmental, and pre-clinical trial stages.[6,7] While initial forays into therapeutic mAb development used murine antibodies, it was found that chimeric and humanised mAbs provided better immunogenic profiles, and, currently, most research focuses on fully human mAbs.[8] In 2011, 5 of the top 12 best-selling pharmaceuticals were mAbs. Moreover, 3 of the top 10 most valuable R&D projects, as ranked by net present value, are based on mAbs.[6] This drive by the pharmaceutical and biotechnological industry indicates how important the development of therapeutic mAbs is. Parallel with these

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developments, rapid, sensitive, and robust analytical methods are needed for detailed characterisation of these structurally complex, large molecules.

Antibodies, also known as immunoglobulins (Ig), have a general Y-shaped structure that is conserved over all classes of antibodies: IgA, IgD, IgE, IgG, and IgM. While there is commonality in the individual unit structure, *i.e.*, dimerisation of two heavy chain–light chain heterodimers *via* heavy chain interaction, differences in the heavy chains result in different properties, including the propensity to form higher-order oligomers, specifically, a dimer for IgA and a pentamer for IgM. As currently most therapeutic mAbs are based on IgG derivatives,[4] this review will focus on the structural characterisation of IgGs by mass spectrometry (MS).

Each IgG molecule consists of two heavy chains and two light chains which are held together by disulphide bonds (Fig. 1). The variable domains on the light and heavy chains, VL and VH, respectively, account for the high antigenic specificity. The constant region of the light chain, CL, interacts with the first invariable region of the heavy chain, CH1, to form the antigen binding fragment (Fab) in combination with the variable domains. The dimerisation between the two heavy chains is secured by the intermolecular disulphide bonds in the hinge region, but further governed by the crystallisable fragment (Fc). The Fc consists of two heavy chain constant domains, CH2 and CH3, whereby the CH3 domains of the two heavy chains form strong non-covalent bonds with each other. The hinge region, which connects the Fab and Fc regions, accounts for much of the difference between the IgG subclasses (IgG1, IgG2, IgG3, and IgG4), specifically in the length of the hinge region and number of disulphide bonds between the heavy chains. The IgG subclasses also differ in the amino acid sequence in the constant regions of the heavy chain.

The molecular composition of mAbs, even when produced by recombinant expression in well-defined hosts, can be very heterogeneous, due to a variety of modifications. Foremost, the glycan chains attached to the CH2 domain can be very complex, but also the occurrence of variable C-terminal lysine cleavage and other side-chain post-translational modifications, such as de-amidation or oxidation, lead to a variety of different molecular entities.[9] These sources of heterogeneity, which are often relatively small in mass and abundance compared to the intact mAb (150 kDa), pose serious challenges for the analysis of intact antibodies.[10,11]

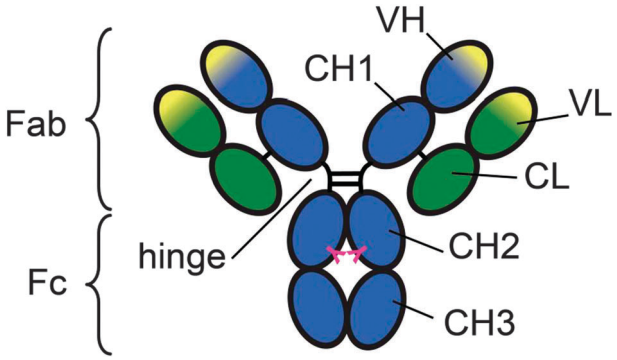

Fig. 1 Cartoon of an IgG1 antibody highlighting the various domains and fragments. The light chains are shown in green, and the heavy chains in blue, with two disulphide bonds linking the CL and CH1 domains and two disulphide bonds linking the heavy chains in the hinge region. The antigen binding regions are highlighted in yellow on the variable domains of the heavy and light chains. The glycan chains attached to the CH2 domain are represented by the pink structures. The CH3 domains on the heavy chain interact strongly with each other *via* non-covalent interactions.

A powerful method that can address the structural complexity of biomolecules is mass spectrometry (MS). MS was long restricted to the realm of small molecule analysis, due to the inability to transfer large molecules and complexes to the gas phase efficiently. With the introduction of matrix-assisted laser desorption ionisation (MALDI)[12] and electrospray ionisation (ESI),[13] large molecules could successfully be introduced to the gas phase, making the biological molecular arena accessible for MS. Sensitivity and mass resolution initially lagged behind for these larger molecules, but these limitations have been addressed by new generations of mass analysers. Next, the retention of non-covalent macromolecular complexes following ionisation by ESI

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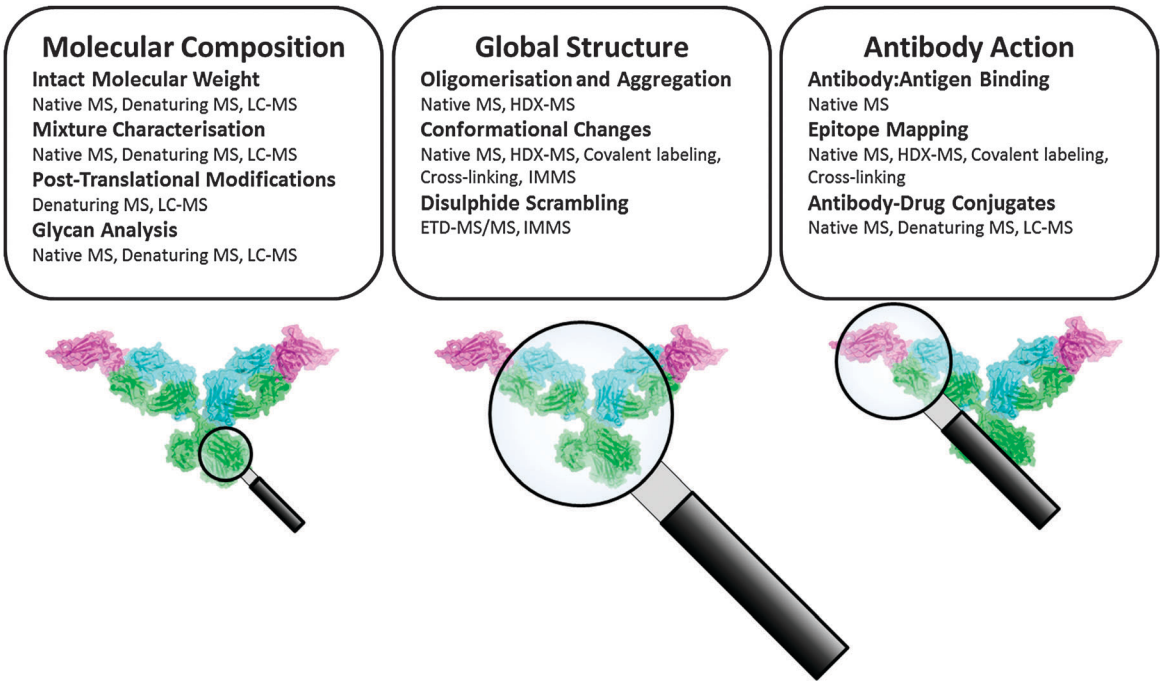

Fig. 2 Overview of how different MS-based approaches can provide structural information on intact antibodies.

was achieved with the development of native MS[14] which uses a volatile buffer at a neutral pH as ESI solvent, *e.g.*, aqueous ammonium acetate, in conjunction with nanoflow elution for very "gentle" ionisation. NanoESI under these "native" conditions in combination with time-of-flight (TOF) instruments, possessing a theoretically infinite mass range, has brought MS to the forefront of the analysis of large, intact biomolecular complexes.[15]

In this review, we will focus on recent progress in the applicability of MS for the characterisation of intact mAbs (summarised in Fig. 2). The discussed applications reveal MS to be a versatile technique with the ability to determine many molecular and structural attributes of mAbs. These include the analysis of post-translational modifications, conformational changes, binding interactions and epitopes. Here, we highlight a wide range of MS-based techniques that have been used to probe complex mAb's various structural levels.

2. Mass spectrometry of intact antibodies under denaturing conditions

Analysis of the mass of intact antibodies allows the assessment of the precise molecular weight (ideally confirming the protein sequence), the purity, and the heterogeneity due to protein modifications. Early examples used MALDI-TOF MS or triple quadrupole instruments for such analyses;[16–18] however, the high m/z requirements make such experimental approaches challenging. Currently, analysis of intact proteins is generally in the realm of ESI-TOF-like instruments due to the accessibility of a suitable m/z range, *i.e.*, high m/z, whereby the maximum m/z required is typically 5000 for denatured proteins (Fig. 3 and 4a). In the biopharmaceutical industry, it is now routine to check molecular weight and heterogeneity of intact antibodies (glycosylated or deglycosylated), partially reduced antibodies, *i.e.*, light chain and heavy chain, or antibody fragments (Fab and Fc), by ESI-TOF using either direct infusion or a chromatographic step, typically reverse-phase high-performance liquid chromatography (RP-HPLC) or size exclusion chromatography (SEC).[4] The addition of organic solvents and acid results in the protein becoming unfolded, acquiring many positive charges during ESI, thus keeping the m/z in a range easily attainable by most mass analysers. With modern instrumentation, the mass accuracy attainable for a protein of the size of an intact antibody, approximately 150 kDa, can range from 10–100 ppm, *i.e.*, 1.5 to 15 Da. In terms of mass resolution, current state-of-the-art TOF and quadrupole-TOF instrumentation ranges from 5000 to 10 000 (full width half maximum resolution, FWHM). As described in Zhang *et al.*, due to the natural width of the isotopic envelope of an antibody's molecular formula (approximately 25 Da), resolution higher than 8000–10 000 is not particularly advantageous for mass measurement,[4] as it can also compromise sensitivity in these types of instruments. Resolving relatively small mass differences, less than 25 Da (*e.g.*, oxidation or deamidation), is currently best addressed by analysing fragments or peptides of the antibody or by including an additional separation step.

The ability to obtain the precise molecular composition of mAbs directly by analysis of the intact molecule is an attractive option, since it may remove the necessity for extra sample preparation steps, *e.g.*, denaturation, enzymatic digestion, deglycosylation, which often come at the expense of sample losses, not only speeding up the analysis but also reducing the likelihood of protein modification during sample handling and thus increasing reproducibility. These approaches, in which the mAbs are denatured prior to MS analysis, have been extensively

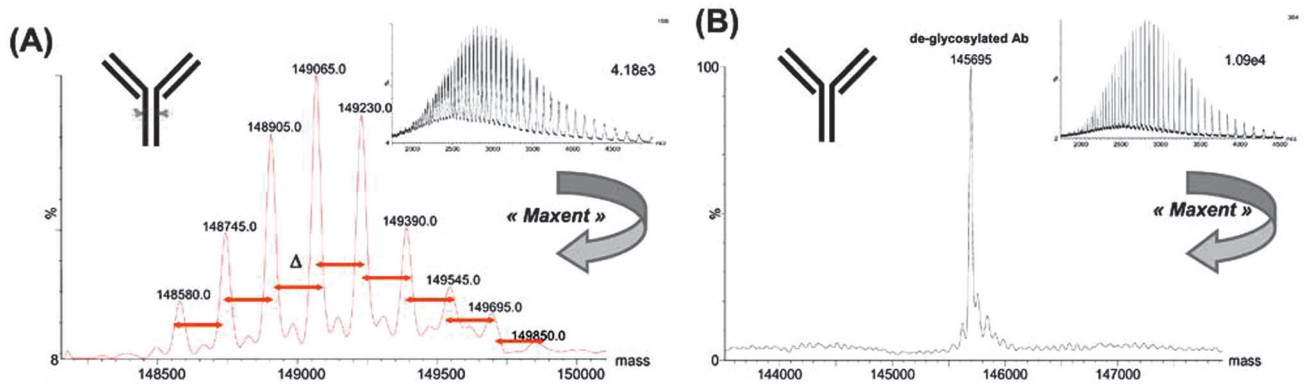

Fig. 3 ESI-mass spectra of intact humanized mAbs before (A) and after (B) treatment with PNGaseF. The inset shows the mass spectrum acquired under denaturing conditions. Maxent deconvolution resulted in the masses of each component. The mass differences between species in (A) are a mass of 162 Da corresponding to the addition of multiple hexose units. The deglycosylated antibody (B) results in only a single peak indicating that the heterogeneity in the glycosylated version stems solely from the heterogeneity of the glycan chain. Adapted from ref. 21. Copyright 2008 Bentham Science Publishers Ltd.

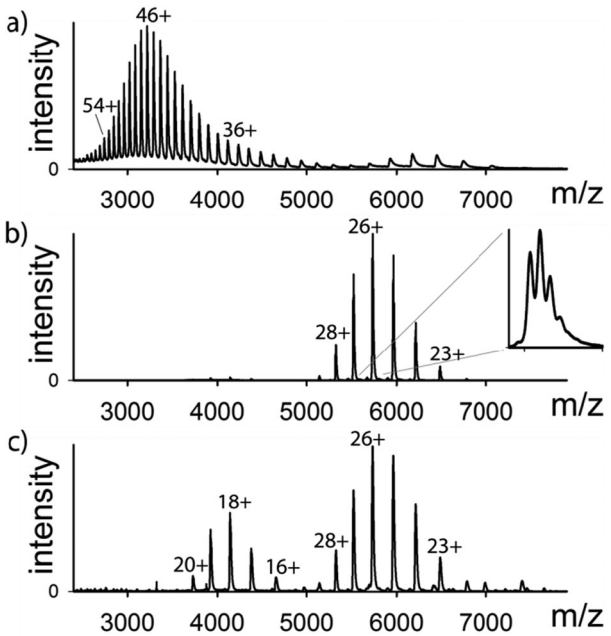

Fig. 4 ESI-mass spectra of intact antibodies. (a) An intact antibody spectrum acquired under denaturing conditions showing highly charged ion signals at relatively low *m*/*z*. (b) The same antibody sprayed under native conditions with the inset showing the various glycoforms present. (c) The native mass spectrum of a non-covalently bound hinge-deleted IgG4 molecule, revealing both half (low *m*/*z*, *ca.* 75 kDa) and whole (high *m*/*z*, *ca.* 150 kDa) antibodies present. Charge states are indicated above the peaks.

used to monitor, amongst other properties, different glycoforms of mAbs,[9,19–21] modifications and isoforms of IgG molecules,[22–30] covalent dimerisation,[31] and stability.[32–34] SEC-MS and LC-MS have also been combined to determine the number of drug molecules bound in antibody–drug conjugates.[11,35–37] Glycoprofile analysis of intact antibodies generally utilizes ESI-TOF platforms with mAbs reduced and electrosprayed under denaturing conditions. A variety of deconvolution software packages is available to calculate the masses and (relative and/or differential) abundances of the various glycoforms present (Fig. 3). The masses can be directly verified using the often known amino acid sequence of the antibody. In this example, the mass differences between glycoforms correspond to the mass of sequential hexoses. This sort of analysis has been used to measure glycoforms heterogeneity between batches[19] yet is unable to provide details of the exact glycan structure. For finer details, it is common to use tandem mass spectrometry (MS/MS) on the released glycans rather than the intact antibody.[21]

3. MALDI-TOF analysis of intact antibodies

MALDI provides an alternative ionisation method that gently generates ions of biomolecules. As mentioned previously, early analyses of intact mAbs by mass spectrometry used primarily MALDI to transfer the proteins to the gas phase. MALDI-TOF is still used for the characterisation of intact antibodies, including the analysis of batch-to-batch heterogeneity,[25] effects of aging on antibody structure,[34] and for the determination of the average drug-to-antibody ratio (DAR) of antibody–drug conjugates (ADCs).[38,39] As MALDI-TOF generally allows less mass resolution and accuracy compared to ESI-Q-TOF methods the latter methods seem to become the preferred choice, at least for intact mAb analysis.

4. Mass spectrometry of intact antibodies under native conditions

Native MS has emerged as a powerful, complementary approach in structural biology when studying individual proteins and protein complexes, yielding information about their stoichiometry, topology, stability, binding properties, and structure.[15,40] In native MS, the samples are typically kept in a volatile aqueous buffer at neutral pH prior to ionisation, such that samples retain their native-like structures and binding properties. Native ESI-MS of antibodies produces ions carrying significantly fewer charges than observed under denaturing conditions (Fig. 4b). The mAb signal is also collapsed into fewer peaks which can potentially increase sensitivity. However, the mass analysers need adjustments to be able to detect species in an *m*/*z* range well over 5000. Technological advancements,[41,42] in particular

the use of ESI-TOF technologies, have ensured that even protein complexes in the mega Dalton molecular weight range, such as intact viruses, are now accessible.[43–46] Maintaining folded structures, and therefore binding interactions, allows in-depth knowledge regarding biophysical and conformational properties to be gained. Native MS has also been proven to be applicable to the characterisation of antibodies, opening up additional avenues for antibody characterisation, including analysis of the status of disulphide bridges under non-reducing conditions, the non-covalent assembly of antibody heavy chains (Fig. 4c), antibody–antigen complexes, oligomerisation and aggregation status, and even conformational changes in mAbs. Below, we describe some of these applications in more detail.

4.1 Non-covalently assembled IgG4 antibodies

To illustrate the unique capabilities of native MS in the analysis of biopharmaceuticals, we first describe work by Rose *et al.* who looked at hinge-deleted IgG4 constructs engineered to form half-molecules, or 'unibodies'.[47] IgG4 antibodies exist both as covalently and non-covalently assembled molecules, *i.e.*, a fraction does not have inter-heavy chain disulphide bonds but rather intra-chain bridges. To study the non-covalent interactions involved between the heavy chains, a native MS study was carried out, providing a means to distinguish and measure simultaneously the abundance of the IgG4 half-molecules (mass of approximately 75 kDa) and intact IgG antibodies (mass of approximately 150 kDa). The methodology was used to determine the solution phase K_D to be 50 nM for wild-type hinge-deleted IgG4 (Fig. 4c). Next, a series of single-point mutations made in the CH3 domain were evaluated revealing the sensitivity of this dimerisation to the CH3–CH3 interaction, with the measured K_D varying from sub-nM to 100s of μM for individual mutants.[47] The strength of the non-covalent interaction within IgG4 was found to correlate well with the Fab-arm exchange phenomenon, *i.e.*, the mixing of IgG4 half-molecules to form bispecific antibodies.[48] Understanding this exchange is important due to its implications for the use of different IgG4 antibodies as therapeutics, as they may undergo Fab-arm exchange *in vivo* with endogenous antibodies.[49] Native MS was also used to measure the kinetics of formation of mixed antibodies for different mutants in real time; since data can be acquired in just a few minutes using this technique, such reactions can be monitored directly.[47]

4.2 Antibody–antigen complexes

Native MS has also been explored by several groups as a technique to measure the non-covalent binding of mAbs to their cognate antigens.[50–53] Since antibody–antigen complexes of different stoichiometries can be distinguished by mass, native MS can help determine the relative amount of unbound, singly-bound, and doubly-bound antigens. Most alternative techniques, *e.g.*, surface plasmon resonance (SPR), isothermal calorimetry (ITC), and SEC, only measure such populations as a weighted average. Already in 2001, Tito *et al.* measured the stoichiometry of antibody–antigen complexes using a recombinant V antigen from *Yersinia pestis*. As expected, a 1 : 2 antibody to antigen complex, *i.e.*, antibody with doubly-bound antigen, was detected and was established to be specific, since

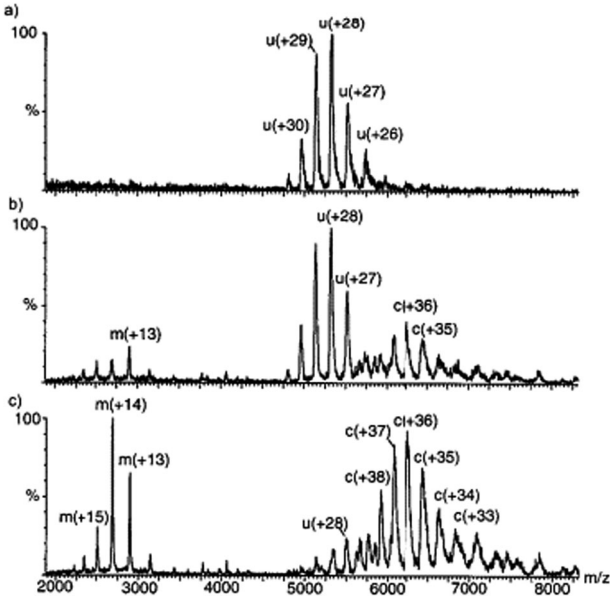

Fig. 5 Antibody–antigen binding monitored by native MS using the rV antigen from *Y. pestis* at various Ab : Ag ratios. Addition of the antigen in substoichiometric amounts converts the unbound antibody (u) to the antibody–antigen complex (c). Upon the addition of an equimolar amount of antigen, the entire antibody population is converted to the complex, and excess monomeric antigen (m) is observed. (a) 1 : 0.1 molar ratio Ab : Ag; (b) 1 : 0.2 molar ratio of Ab : Ag; (c) 1 : 1 molar ratio of Ab : Ag. Reprinted from ref. 50, Copyright 2001 with permission from Elsevier.

no binding was detected between the non-cognate antigen F1 and the antibody (Fig. 5).[50] In another example, Atmanene *et al.* showed that two distinct mAbs bind the junctional adhesion molecule A (JAM-A) with ratios from 1 : 2 to 1 : 4, attributed to either monomeric or dimeric JAM-A binding to each Fab arm.[53] Comparing antibody–antigen binding measured by various techniques, including native MS, analytical ultracentrifugation (AUC), and SPR, has revealed that the binding stoichiometries of antibodies free in solution or immobilised on a surface can differ, suggesting that the interaction depends on Fc flexibility.[51,52] Thus, the application of native MS for studying these non-covalent interactions can clearly provide important information that is not readily evident using other techniques.

4.3 Antibody–drug conjugates

Antibody–drug conjugates (ADCs) are another exciting type of molecule emerging within the biopharmaceutical field, with the ability to deliver a drug to a specific cell-type, *e.g.*, to tumour cells, potentially reducing side-effects. One popular option of ADC formation is the conjugation of the drug molecule to cysteines in the natural sequence of antibodies, which become exposed following partial reduction. When the inter-chain disulphides become reduced, it is possible that the heavy chains and light chains are only non-covalently bound together, a structure that can be probed by native MS. Moreover, MS is an obvious choice for determining the stoichiometry of drug molecules conjugated, which can vary from zero to eight for an IgG1 depending on the number of free cysteines and completeness of the conjugation reaction. LC-MS methods have

been utilised effectively for ADC analysis,[11,35–37] however in the case of drug binding to exposed cysteines, due to the potential non-covalent nature of the product, approaches using denaturing conditions are not ideal. Valliere-Douglass *et al.* have demonstrated how native MS can be applied to distinguish between and define antibodies with varying amounts of conjugation, including covalently and non-covalently bound molecules.[54]

4.4 Composite mixtures of antibodies

A current movement in the biopharmaceutical industry is towards the development of stable bispecific mAbs, further increasing the necessity for analyses of more complex mixtures ideally using simple, reproducible, fast, and accurate techniques. It has recently been demonstrated that native MS may be a very efficient method for quantifying oligoclonic mixtures of antibodies, in which the constructs are expressed in different quantities within a single cell line, and the heavy chains can pair up in different combinations.[55] Combining native MS with peak fitting and integration algorithms, mixtures of up to 10 IgG1 antibodies, differing in molecular weight by between 20 and 493 Da, could be characterised qualitatively and quantitatively (Fig. 6). The data generated are in quality and detail at least as good as data generated by more conventional methods that use separation by cation exchange chromatography (CEX) for such analyses.[56]

4.5 Antibody aggregation

An important consideration during antibody production and storage is the undesired formation of inactive high-order oligomers and/or nonspecific aggregates. Several techniques, such as AUC and SEC, are routinely used to check for aggregation, however, it remains challenging to define the actual identity of the aggregation products. Because native MS combines the retention of non-covalent structures with mass-based identification, it is ideally suited for detection of higher-order oligomers. In this context, native ESI-MS has been applied to characterise aggregates formed when IgG1 is subjected to pH stress. Combined with SEC, oligomers up to and including tetramers could be detected.[57]

5. Hydrogen–deuterium exchange mass spectrometry

Changes in conformation, including misfolding, can significantly impact activity and stability of antibodies, and thus must be monitored in the development and production of biopharmaceutical products. Due to the flexible linkers between the Ig domains in mAbs, structural characterisation of these proteins tends to be challenging. mAbs are still too large for standard NMR procedures and also tend to be difficult to crystallise. Therefore, alternative techniques for efficiently gathering structural information on mAbs are needed. Hydrogen–deuterium exchange MS (HDX-MS) is a rapidly developing technique employed to investigate protein dynamics, conformational changes, and interaction interfaces.[58–61] HDX-MS exploits the different rates of exchange of hydrogens between protein backbone amide groups and the aqueous solvent, which depend on solvent exposure and secondary structure elements, thus allowing information about structure and dynamics to be deduced.

When HDX-MS is applied to the structural study of mAbs, continuous labelling conditions are typically used whereby the antibody is diluted into a deuterated buffer for a defined time. The rate of deuteration of the amide hydrogens is highly dependent on localised solvent exposure and hydrogen bonding

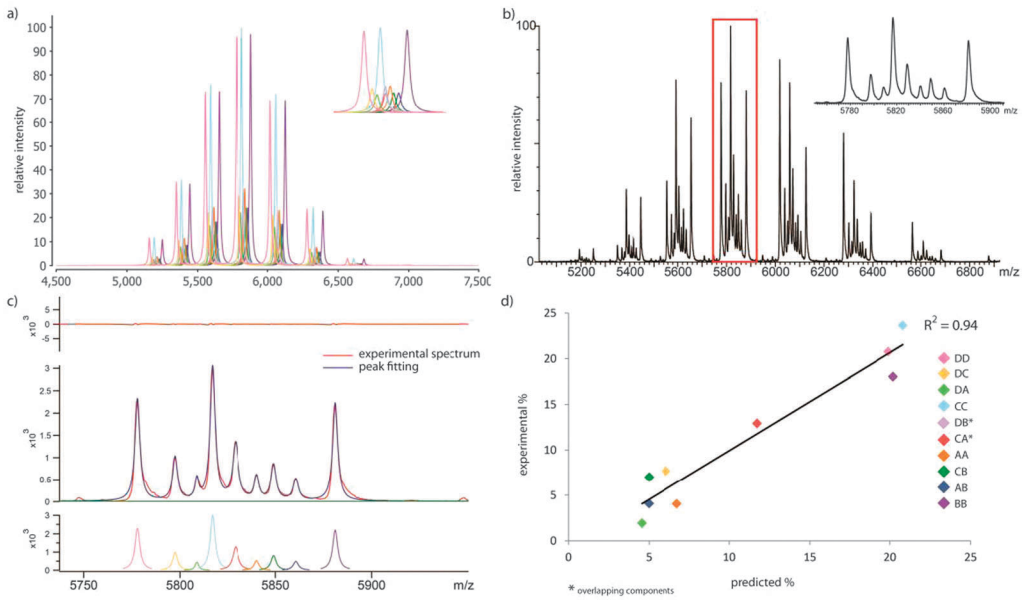

Fig. 6 Theoretical and experimental mass spectra highlighting the resolution of native MS and the ability to identify and quantify multiple components in an antibody mixture. The theoretical spectrum (a) was generated using the SOMMS program with prior knowledge of the antibody masses and expected abundances. The experimental spectrum (b) revealed a similar pattern. Peak fitting for quantitation (c) was performed using Igor Pro v6.22A and the relative percentage of each component was plotted against the predicted amount (d) resulting in an approximate unit correlation.

kinetics, factors that can affect the rate of deuteration up to eight orders of magnitude. Decreasing the pH and temperature quenches the exchange reaction with optimal quenching conditions of pH 2.7 and 0 °C, decreasing the exchange reaction by five orders of magnitude[62–64] and resulting in the minimisation of back-exchange during sample analysis. The whole workflow of the HDX experiments has been subjected to progressive improvements during recent years. Initially, the labelling and proteolytic digestion steps were conducted manually off-line. Nowadays, the automation of both the labelling and digestion steps[65] has improved both speed and reproducibility.[61,66] Of great importance is automation at the level of data processing and analysis which significantly saves manpower and time.[67,68] With these recent improvements, HDX-MS is becoming popular and holds further potential, not only for studies on protein conformation and dynamics, but also in the analysis and quality control assays of biopharmaceuticals. Below we describe further how global HDX (whereby exchange is measured in the intact proteins) and more in-depth peptide-centric HDX has been used in such analyses.

5.1 Structural details revealed by HDX-MS

In a global HDX experiment, the mass of the intact protein is directly interrogated by MS, revealing the uptake of deuterium over time. Due to its simplicity and speed, this kind of experiment is preferable when extensive conformational changes are expected and spatial resolution of the exchange along the protein backbone is not required. Moreover, it represents a quick way to investigate the overall reactivity of the protein for D_2O uptake and is commonly used to optimise the labelling timescale prior to an HDX experiment involving proteolytic digestion.

Houde *et al.* described global HDX measurements comparing the structure of the Fab and Fc regions individually, as opposed to within the intact IgG1 molecule.[69] No significant difference in deuterium uptake was observed, and furthermore, only one labelled population was observed, suggesting little conformational heterogeneity in the IgG1. Incomplete deuteration after four hours of labelling indicated that some highly protected regions were still present, and the overall increases in the extent of deuteration for the entire time frame studied suggested slow dynamic motions of these protected regions.

To achieve a more detailed picture of protein structural changes using HDX-MS, an enzymatic digestion step is incorporated to increase the spatial HDX resolution to the peptide level. Digestion must be performed under optimal quench conditions, meaning that a protease active at acidic pH is necessary. Pepsin is the most commonly used protease for this purpose, as it is active at pH 2.5 and cleaves nonspecifically. Pepsin-induced proteolytic peptides are then analysed by LC-MS, and the uptake of deuterium is measured for each individual peptide. With this type of experiment, conformational changes can be localised to small regions of the protein by comparing the deuterium uptake of a peptide produced from the protein under two or more different conditions. Several studies illustrate this, reporting on the use of HDX-MS to investigate conformational changes induced by various glycosylation profiles on mAbs.[69,70]

The nature of the glycan chains on mAbs is highly dependent on the expression system, and glycosylation is known to influence the binding to the Fc receptor.[70,71] Houde *et al.* used HDX-MS to show that complete deglycosylation cause significant changes in the Fc region, while the rest of the protein did not show any changes in deuterium uptake.[69] Subsequently, this group also performed HDX-MS on a number of IgG1 variants differing in the nature of the glycosylation, which generally showed changes in the structural dynamics of the CH2 domain.

The efforts in optimisation of therapeutic antibodies to improve antibody properties, such as pharmacokinetics, pharmacodynamics, stability, and effector response, often lead to the development of products with single-point amino acid substitutions.[72–75] However, single mutations can bring dramatic conformational changes that can either positively or negatively affect the potential drug candidate. Rose *et al.*[76] used HDX-MS to show how single-point mutations in the CH3 domain not only affect the conformation of the domain where the mutation is located but also induce allosteric changes in the CH2 domains. These allosteric changes occurred in parallel with significant changes in the attached glycan chain structure.

Besides glycosylation, other post-translational modifications can affect the overall molecular structure; methionine oxidation provides such an example affecting mAb stability. HDX-MS has been used to measure the overall conformational stability upon methionine oxidation,[70,77] known to be significant when antibodies are stored for a long time, as in the case of therapeutic products. It was shown that methionine oxidation can not only structurally affect residues immediately surrounding the site of oxidation but also adjacent domains.

Aggregation is also often observed in antibody preparations, especially when mAbs are stored at high concentrations. HDX-MS can be used to detect regions involved in aggregation. Zhang *et al.*[78] used HDX-MS to monitor antibody aggregation under both thermal and freeze-thaw stress conditions. Aggregation resulted in destabilisation of the interface between the light chain and heavy chain as indicated by an increase in deuterium uptake (Fig. 7). Additionally, the small regions which show decreased deuterium uptake after stress likely represent the aggregation interface.

One application of HDX-MS that is rapidly becoming popular is mapping the antigen epitope.[79–81] Characterisation of the molecular determinants of the interaction between antibodies and their targets is essential when antibodies are used as therapeutic agents and also required for intellectual property reasons. An interesting strategy to carry out this type of experiment is to perform the labelling on an immobilised antibody in order to selectively analyse the antigen. The successful application of this strategy has been demonstrated by Baerga-Ortiz *et al.* and by Coales *et al.*[82,83] Also worthwhile mentioning is the application of a HDX dilution strategy to determine antibody–antigen binding affinities and residues involved in the epitope, as developed by Tu *et al.*[84]

6. Chemical labelling and cross-linking

6.1 Chemical labelling

Highly complementary to HDX-MS, covalent chemical labelling of specific residues on the protein can be used to study both conformational changes and interaction surfaces. The advantage

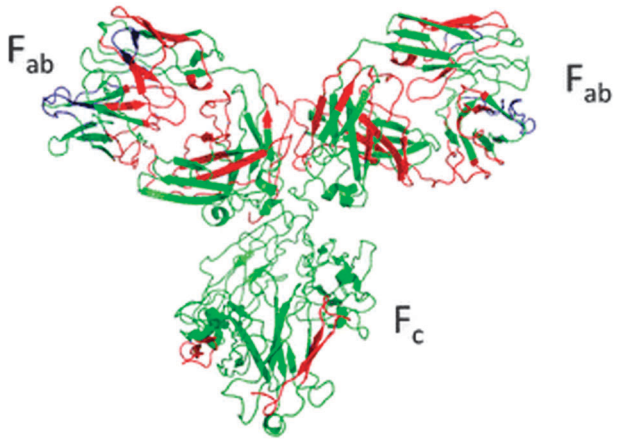

Fig. 7 Conformational changes upon thermally-induced aggregation of Bevacizumab determined by HDX-MS mapped onto a three-dimensional antibody structure created *via* homology modelling using 1IGY as a template. Differences in deuterium uptake were measured using reporter peptides produced by proteolytic digestion using an online immobilized pepsin column. Red regions indicate increases in deuterium uptake, *i.e.*, the region is less protected after aggregation, and blue regions indicate decreases in deuterium uptake, *i.e.*, the region is more protected after aggregation. Reprinted from ref. 78, Copyright 2012, with permission from Springer Science and Business Media.

of a chemical labelling strategy over HDX-MS is the irreversibility of the reaction, which circumvents the back-exchange issue often involved in HDX-MS.[85] Numerous types of chemical labels can be used, though smaller labels and more efficient reactions are beneficial as they should affect the native conformation of the protein to a lesser extent. There are presently only a few examples of chemical labelling for the structural analysis of mAbs. One of the more elegant methods uses fast photochemical oxidation[86,87] for epitope mapping. HDX-MS results previously obtained on the same system agreed with the conclusions obtained from photochemical oxidation.[88] In a second example, the positive charge patch on antibodies that can affect the separation profile in CEX was characterised. Two different chemical labels for lysines and arginines were used, allowing the location of positive charges on the antibody surfaces to be mapped.

6.2 Chemical cross-linking

Chemical cross-linking combined with MS is a technique that makes use of very reactive small molecules (cross-linkers) able to react with functional groups of the side chains of the amino acids. Their role is to link two functional groups of the same protein or of two interacting proteins that are close in space in the native conformation. Digestion of the linked protein and MS analysis of the linked peptides allow the investigation of the three-dimensional structure of proteins, and characterisation of protein interactions.[89–91] Chemical cross-linking with MS-based detection represents a useful alternative method for obtaining structural restraints for antibodies. For example, Bich *et al.* have demonstrated how newly developed cross-linkers can successfully be used in this manner to study structurally antibody–antigen interactions.[92]

7. Top-down structural analysis of intact antibodies

7.1. Ion mobility mass spectrometry

With the advance of native MS and the ability to preserve non-covalent interactions, the gas-phase structure of the studied species can be probed. Combining ion mobility spectrometry with MS (IMMS)[93] yields not only mass-based identification but also gas-phase collision cross-sections, which may provide insight into the gas-phase structure, albeit at low resolution. IMMS has recently been applied in the determination of the structural heterogeneity of both mAbs (IgG2) and antigen targets. Differences in disulphide linkages can affect not only the structure of an mAb but also the function. IMMS was used to successfully observe multiple gas-phase conformers, which were then confirmed to be the result of different disulphide bond formation *via* point mutation of a cysteine in the hinge region.[94] IMMS also revealed disulphide bond heterogeneity of an antigen under native conditions; whereas a single population was observed by IMMS under reducing conditions, multiple conformers were observed under non-reducing conditions.[53] These studies illustrate how IMMS can rapidly assess structural variability of antibodies and their antigens. Flexibility between the Ig domains in the antibody molecule makes IMMS a challenging technique for absolute deduction of structure, however, it can easily be incorporated into MS workflows for comparability studies.

7.2 Electron transfer dissociation

Electron transfer dissociation (ETD) has facilitated top-down structural analysis of peptides but also intact proteins,[95] as it proceeds largely by extensive backbone fragmentation retaining labile post-translational modifications.[96,97] These two aspects of ETD and other radical-driven dissociation techniques represent unique advantages over the traditional dissociation method of collision induced dissociation (CID). However, the application of ETD to large protein structures has been hampered by the presence of non-covalent interactions which remain undisrupted in radical-driven dissociation techniques.[98] Thus, there are presently still only a few examples of the use of ETD or the related electron capture dissociation (ECD) on large proteins (>50 kDa). Recently, ETD was used to probe the structure of intact mAbs using a high-resolution quadrupole-TOF instrument.[99] The presence of intact disulphide bonds limited the sequence coverage of the intact mAb, but this sequence coverage was increased when accounting for regions protected by disulphide bonds. Beyond pushing the upper mass limit of top-down ETD MS to 150 kDa (75 kDa of unique sequence), this study also showed that ETD produced more product ions than CID of IgGs and that the ETD product ions were complementary to those formed by CID. After these encouraging results, it can be expected that ETD will have a prevalent future role in the top-down analysis of intact mAbs.

8. Future developments and perspectives

In this review, we have described an array of MS-based technologies as used in the analysis of intact antibodies. While current

MS-based techniques provide a wealth of information, there are still quite a few limitations as to what can be achieved in a single experiment. Further advances in technology will be pushing this limit and increasing the applicability of MS for detailed molecular, biophysical, and structural characterisation of antibodies. At the end of this review, we highlight a few such emerging and expected advances.

8.1 Native MS for batch-to-batch quality control

While native MS is gaining interest as a possible technique to characterise therapeutic mAbs qualitatively, it has yet to be implemented in a high throughput manner. The success of native MS analysis is still too dependent on expert operators and is time consuming. Automated sampling in native MS has been demonstrated within the pharmaceutical field for monitoring protein–ligand interactions,[53,100,101] and software is currently being developed to increase the ease of data analysis.[102,103] Combination of these two innovations may enable native MS to become a competitive alternative to current therapeutic mAb quality control assays.

8.2 Increasing mass resolution for intact mAb analysis

MS analysis of intact mAbs is still hampered by limits in mass resolution, meaning that small mass differences due to post-translational modifications remain undetected.[4] Until recently, MS of proteins and protein complexes under non-denaturing conditions has been the realm of TOF-based mass analysers, due to their high m/z capabilities, as well as the possibility of instrument modification to allow collisional cooling and improved transmission. Improvements in instrumentation for the high resolution analysis of large mass ions have recently reached a point where the isotopic analysis of intact mAbs is possible. This has been demonstrated using expensive high-field Fourier Transform ion cyclotron resonance (FTICR) mass spectrometers, albeit that the analysis of large mass ions is still seriously limited by both adduct formation during the ESI process and Coulombic interactions in the ICR cell decreasing the coherence of the ion packet.[104] Increased voltages and capillary temperature prior to injection to the ICR cell increased the desolvation and removal of adducts from the mAb ions, and the incorporation of an automated phase correction resulted in the increased mass resolution. In contrast to the analysis of mAbs on FTICRs, performed under denaturing conditions, we recently showed that Orbitrap analysers can be successfully modified and used for the analysis of native proteins and non-covalent complexes, with m/z values up to 22 000 Th.[105] The advantage of using this technology is the very high mass resolution, enhanced desolvation, and sensitivity. As such, small mass differences such as post-translational modifications, glycosylation or sequence variations can be clearly resolved. This level of resolution (up to 20 000 FWHM at m/z > 5000 Th) facilitates accurate characterisation of antibodies with complex glycosylation patterns from a native mass spectrum (Fig. 8). Analysis of an IgG antibody by static nanoESI is possible with attomolar amounts of material; at concentrations well below 1 μM (0.1 mg mL^{-1}), a high quality, high signal to noise spectrum is obtainable from a single scan of

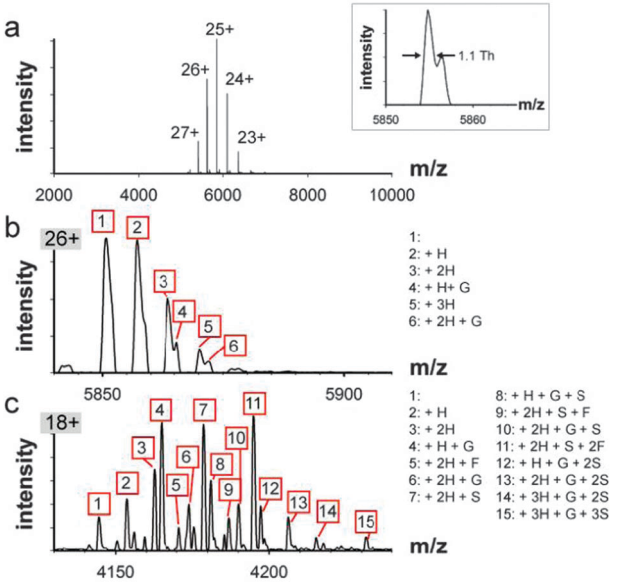

Fig. 8 Orbitrap-based analysis of intact, native, glycosylated mAb. (a) The complete mass spectrum of an intact mAb acquired under native conditions. The inset shows the FWHM to be approximately 1.1 Th. (b) Glycosylation pattern of an intact antibody with baseline-resolved glycan peaks. (c) Complex glycosylation patterns of a half-antibody, also with baseline-resolved glycan peaks. Individual glycoforms were assigned based on the differences in m/z between peaks, corresponding to 162 Da (hexose/galactose, H), 203 Da (GlcNAc, G), 146 Da (fucose, F) or 291 Da (sialic acid, S), as indicated by the lists on the right of each spectrum.

1 second. This instrumentation can further enhance the analysis of antibody mixtures and antibody–antigen interactions.[106] Increases in mass resolution not only improve identification of mAbs but also facilitate relative quantitation of various components, whether they be different mAbs, attached glycans, or other structural variations within a single mAb population.

8.3 Reducing deuterium scrambling for improved HDX-MS resolution

Hydrogen–deuterium scrambling during MS/MS of deuterated peptides has remained one of the key limitations of HDX-MS using proteolytic digestion. Recently, several groups have started to employ radical-driven dissociation techniques, such as ETD and ECD, as methods to fragment the deuterated peptides without inducing deuterium migration. It has been shown that these electron dissociation techniques do afford efficient backbone dissociation with little to no movement of deuterium atoms.[107–109] The incorporation of ETD and ECD in HDX-MS workflows has resulted in increased HDX resolution to the single amino acid level for over 50% of the protein sequence[107,110] improving accuracy of deuterated sites in the intact protein structure. With improvements in technology, HDX-MS using radical-driven dissociation methods has reached a point where it can be fully automated.[111] Also, ETD and ECD can be applied on a top-down level to yield extensive sequence and deuterium uptake information while removing the chromatographic separation step which often comes at the expense of increased back-exchange.[108,109]

9. Conclusions

The role of MS in the characterisation of intact proteins and protein complexes, including intact therapeutic mAbs, has expanded significantly over the past 10 years. Here, we have covered multiple MS-based methods that yield varying levels of detail, ranging from the presence of mutations and post-translational modifications to global conformational changes due to scrambled disulphide bonds to the residues involved in antibody–antigen interactions. The analysis of mAbs by MS under denaturing conditions, in combination with chromatographic techniques, has become routine in the pharmaceutical industry as a rapid way to confirm molecular weight and monitor small changes within an antibody population. Native MS is as rapid and efficient experimentally as denaturing MS and also can yield information on the non-covalent interactions that are crucial for antibody structure and function. Recent advances in the instrumentation and automation of HDX-MS have increased its capability for the study of antibody interactions and conformational changes. With so many MS-based techniques enabling the analysis of a wide range issues related to protein structure function, MS has taken an essential role in the structural and functional characterisation of therapeutic mAbs.

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