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Insights from native mass spectrometry and ion mobility-mass spectrometry for antibody and antibody-based product characterization

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ABSTRACT

Over the past 15 years, monoclonal antibodies (mAbs) have emerged as the most successful class of therapeutics. Their specific structural and functional properties make them highly effective treatments for various diseases. Most therapeutic mAbs are based on chimeric, humanized or human G immunoglobulins (IgGs) selected from three isotypes (1, 2 and 4). IgGs are large and highly complex multimeric glycoproteins. They are constituted of a mixture of isoforms including macro and micro-variants that must be extensively characterized prior to their investigation as a drug candidate in clinical trials. The IgG backbone is also used to design more potent but also more complex biopharmaceuticals such as antibody-drug conjugates, bispecific antibodies, Fc-fusion proteins, and antibody mixtures to name a few. Mass spectrometric approaches in combination with electrophoretic and chromatographic separation methods play a central role in the analytical and structural multi-level (top, middle and bottom) characterization of these compounds. Importantly, techniques allowing the characterization of intact mAbs and related products under non-denaturing conditions are attracting increasing interest. Here, we review the current state of the art in native mass spectrometry and ion mobility methods for the characterization of mAbs and mAb-based products.

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1. Introduction

The number of protein biopharmaceuticals approved for the treatment of diseases ranging from cancer, cardiovascular diseases, diabetes, infection, inflammatory, and autoimmune disorders continues to increase [1]. Of these drugs, monoclonal antibodies (mAbs) and their related compounds make up the largest class in human therapeutics [2,3]. The success of mAbs in this context stems notably from their high specificity and affinity (in the nM to pM range), their long circulating half-lives (up to 20 days), their ability to induce immune cell effector response, and their structural versatility. More than 70 mAb-based pharmaceuticals have already been approved in different forms, such as antibody-drug-conjugates (ADCs), radio-immunoconjugates, bispecific mAbs (bsAbs), antibody mixtures, scFv fragments, Fab fragments and Fc-fusion proteins or peptides. More than 50 others are under phase III clinical trials [4], with an expected approval rate of ~20%.

Although the engineered formats differ, these protein therapeutics mostly share a common immunoglobulin G (IgG) scaffold. Canonical mAbs are 150 kDa tetrameric glycoproteins, the covalent assembly of two light chains and two heavy chains. Depending on the isotype, the number of disulfide bridges in the hinge region that links the two heavy chains varies from two for IgG1 and IgG4 to four for IgG2, the other 12 disulfide bridges being intramolecular [5].

In many cases however, canonical mAbs show a limited efficacy or face resistance, so several families of either armed antibodies (such as ADCs) or bsAbs have been developed to overcome these limitations. ADCs are tripartite molecules consisting of a mAb onto which highly cytotoxic small molecules are conjugated by cleavable or non-cleavable linkers. The specificity of the mAb helps to deliver the highly cytotoxic drug to the targeted cancer cells. To date, two ADCs have been approved by the US Food and Drug Administration (FDA) and European Medicine Agency (EMA) for use in humans [6]. Brentuximab vedotin (marketed as Adcetris® by

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Seattle Genetics/Takeda) is indicated for the treatment of hematological malignancies (Hodgkin's lymphoma and systemic anaplastic large-cell lymphoma) [7], while trastuzumab emtansine (T-DM1, marketed as Kadcyla® by Genentech/Roche) has been licensed as a single agent for the treatment of patients with human epidermal growth factor receptor 2 (HER2)-positive metastatic breast cancer who previously received trastuzumab and a taxane, separately or in combination. The indications are that patients should have either received prior therapy for metastatic disease or developed disease recurrence during or within six months of completing adjuvant therapy [8]. In addition, gemtuzumab ozogamicin has been approved in Japan [9].

The other class of compounds being developed to overcome the inefficiency of single-target mAbs in immunotherapy, bsAbs, are designed to target two different antigens [10,11]. The advantages of bsAbs are as follows [12,13]: (i) they can redirect specific immune cells to the tumor cells to kill more of the latter; (ii) they allow two different mediators/pathways that exert unique or overlapping functions in pathogenesis to be blocked simultaneously; and (iii) their ability to interact with two different cell-surface antigens (instead of one) may confer an increased binding specificity. Hampered for a long time by production issues, the availability of bsAbs has improved through the development of new, more stable, and easier to produce formats. More than 30 bsAbs are currently in clinical development, with two of these catumaxomab (anti-EpCAM × anti-CD3, Removab®, Fresenius Biotech, Trion Pharma,) [14] and blinatumomab (anti-CD19 × anti-CD3, Blincyto®, Amgen) [15]) having already been approved for marketing.

Last but not least, with the first licensed mAbs coming off-patent, new generic versions of mAbs called biosimilars should spread across the market [16]. Obtaining sufficiently close copies of 150 kDa, highly heterogeneous (glycoforms, deamidations, disulfide bridge pairing, etc.), multimeric glycoproteins is very challenging. This is why regulatory agencies are strict in evaluating biosimilars based on their level of similarity with the originator [17]. To date nonetheless, more than 20 biosimilars have been approved in Europe, including two mAb biosimilars of infliximab (anti-TNFalpha, Remsima®, Celltrion and Inflectra, Hospira) which have recently received marketing authorization [18] as well etanercept as an Fc-fusion protein (anti-TNFalpha, Benepali®, Biogen).

The analytical characterization of mAbs, ADCs, bsAbs and related products is challenging and requires the use of a plethora of orthogonal techniques, typically chromatography, electrophoresis and mass spectrometry. Several recent papers have extensively reviewed the advantages, drawbacks and complementarity of such approaches [16,19–22]. The analytical characterization of mAbs (ADCs, bsAbs) usually follows a multi-level workflow, where biopharmaceuticals are studied at the intact protein (top) level, after enzymatic digestion into smaller mAb subdomains (the middle level), or after proteolytic digestion using a combination of enzymes to generate peptides (the bottom level). The middle and bottom levels require preliminary sample treatment in order to reduce the micro-heterogeneity of the mAbs, which can affect the quality of the final product (increased deamidation, oxidation, generation of aggregates, etc.). Top level methods that operate on intact mAbs are thus of utmost interest, as they require less sample handling, but are still in their infancy. We will focus here on native mass spectrometry (MS) and ion mobility (IM-MS), as emerging techniques for the analysis of intact mAbs.

2. Native mass spectrometry

Native mass spectrometry has been developed since the early 90s [23,24] and involves analyzing non-covalent complexes directly in the gas phase of the mass spectrometer. Initially applied to the study of protein/ligand systems [25–30], native MS is now widely used to investigate protein/protein [31–33] and protein/nucleic acid complexes [34,35] ranging in size from a few hundreds to thousands of kDa [36–38]. Native MS is of particular interest to the biopharmaceutical industry for the characterization of recombinant proteins and for the analysis of monoclonal antibodies [39].

2.1. Native MS workflow for mAb analysis

The native MS workflow for the analysis of mAbs and related products (ADCs, bsAbs, etc.) is quite straightforward and rapid (Fig. 1) [40,41]. The observation of intact mAbs in native MS is the most direct approach available to characterize an antibody. The only specific requirement for native MS analysis is buffer exchange (also called the "desalting" step), whereby the storage mAb buffer is exchanged against volatile ammonium salts (mostly ammonium acetate) amenable to electrospray analysis under native conditions [42]. Prior to the desalting step, deglycosylation (using either PNGase-F of IgGZero) can be performed in order to reduce mAb heterogeneity, a step that is particularly valuable for the characterization of ADCs. The mAb sample is next infused into an electrospray ionization (ESI) mass spectrometer for native MS analysis. Infusion can be achieved either through direct syringe pump injection, through a chip-based automated device or, as is becoming increasingly popular, by coupling size exclusion chromatography (SEC) to native MS.

Even though mAbs are built around covalent interactions between light and heavy chains, the charge state reduction and subsequent ease of data interpretation afforded by native conditions make native MS preferable over classical denaturing MS (or LC–MS). Indeed, ESI under native conditions generates mass spectra with a narrower charge state distribution shifted towards higher m/z values (Fig. 2a and b). This charge state reduction intrinsically reduces peak overlap in the high m/z range. While native MS has historically been performed on time-of-flight (TOF) and quadrupole (Q)-TOF instruments, one can now conduct these experiments on higher-resolution Fourier-transform ion cyclotron resonance (FT-ICR) [43] and Orbitrap spectrometers [44,45], which also yield higher mass accuracies.

2.2. Bispecific mAbs and ADCs

Native MS is even more beneficial for the analysis of bsAbs and ADCs, which potentially comprise noncovalent interactions. For example, the bsAb formed by Fab-arm exchange of two IgG4 antibodies consists of mixtures of covalent and noncovalent species, a result of flexibility in the hinge region [46]. Instead of the usual disulfide bonds connecting two heavy chains (covalent bounds maintain heavy chains), intra-heavy chain disulfide bonds may form, resulting in bsAbs maintained by noncovalent interactions between heavy chains. These noncovalent bonds can break under the harsh solvent conditions used for LC–MS analysis, leading to the detection of half-mAb moieties (Fig. 2c). In contrast, native MS analysis, during which noncovalent interactions are preserved, allows the detection of a unique and homogeneous population of the intact bsAb (Fig. 2d). Native MS also appears to be able to detect intact bsAbs resulting from IgG4 recombination [47,48].

Similarly, native MS is mandatory for the characterization of cysteine-linked ADCs, which consist of a broad population of noncovalent assemblies. Native MS can provide accurate estimates of the three main quality attributes of ADCs, namely, the drug load profile and distribution, the average drug-to-antibody ratio (DAR), and the proportion of unconjugated mAbs [49]. The analysis of cysteine-ADCs by native MS was pioneered by Valliere-Douglass et al., first in vitro [50] and more recently in vivo for DAR mea-

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