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Performing native mass spectrometry analysis on therapeutic antibodies

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ABSTRACT

Since the introduction of "soft" ionization techniques, the role of mass spectrometry (MS) in the field of structural biology has increasingly expanded. With the incorporation of volatile buffers as electrospray ionization (ESI) solvents, non-covalent protein complexes could be efficiently transferred to the gas phase for mass analysis. While native MS has not become a technique used for standard characterization of therapeutic proteins in an industrial setting, it is increasingly used to probe the structural heterogeneity of these complex biomolecules. Here, we describe a detailed sample protocol for the analysis of monoclonal antibodies (mAbs) by native MS and highlight some recent applications of native MS in the analysis of intact mAbs and mAb-based therapeutics.

1. Introduction

Mass spectrometry (MS) is a technique with a broad range of applications, in part due to the universal nature of its ability to separate and identify multiple components by differences in mass. MS was initially limited to the study of small molecules due to the inability to transfer large biomolecules to the gas phase efficiently. However, with the introduction of so-called "soft" ionization techniques, i.e., matrix-assisted laser desorption ionization (MALDI) [1] and electrospray ionization (ESI) [2], MS became capable of studying much larger biomolecules. The application of ESI was further extended via the incorporation of volatile buffers at neutral pH as solvents, e.g., aqueous ammonium acetate, to achieve the retention of non-covalent complexes in the gas phase, a technique termed native MS [3–5]. Native MS has evolved to utilize nanoflow ESI for even more gentle ionization conditions and quadrupole-time-of-flight (Q-TOF) analyzers, with a theoretically infinite mass range, and now has the ability to characterize very large, intact biomolecules and biomolecular complexes such as ribosomes and whole viruses [6,7].

One such class of biomolecules that has recently been probed by native MS is monoclonal antibodies (mAbs) and mAb-based therapeutics. The development and application of recombinant mAbs as biotherapeutics has expanded rapidly in the past decade. mAbs have been developed for treatment for a variety of diseases, ranging from many types of cancers to neurodegenerative diseases [8–10]. These biotherapeutics possess some unique advantages due to their high target specificity resulting in general decreases of adverse side effects. mAbs have also been shown to have slow clearance after administration generally but run the risk of inducing immunogenic response [11]. To this end, mAb-based therapeutics, which started as murine and chimeric-based mAbs, are now dominated by humanized and fully human mAbs [12]. With over 30 different mAb-based therapeutics approved for clinical use as well as many more in research, development, and pre-clinical stages [13,14], the necessity for thorough characterization is evident. However, mAbs are large, complex biomolecules with the potential for a wide range of heterogeneity stemming from not only the means of production but also the processing, storage, and administration of these therapeutics.

Most therapeutic mAbs are based on the immunoglobulin G (IgG) class, which is comprised of four polypeptide chains forming a homoheterodimer, i.e., two light chain-heavy chain pairs that are dimerized through disulfide bridges in the flexible hinge region (Fig. 1). While the mAb structure is stabilized by these disulfide bonds, initial interactions that pair the two light chain-heavy chain heterodimers are partly governed by the C-terminal constant region of the heavy chain (CH3) [15,16]. The specificity of the mAb is determined through the complementarity determining regions (CDRs) that define the antigen binding region in the variable domains of both the heavy and light chains. The variable domains of the light and heavy chains (VL and VH, respectively) with the light chain constant domain (CL) and the first heavy chain constant domain (CH1) comprise the antigen-binding fragment (Fab). The remaining two heavy chain constant domains (CH2 and CH3) form the crystallizable fragment (Fc) and typically display less sequence heterogeneity than the Fab region.

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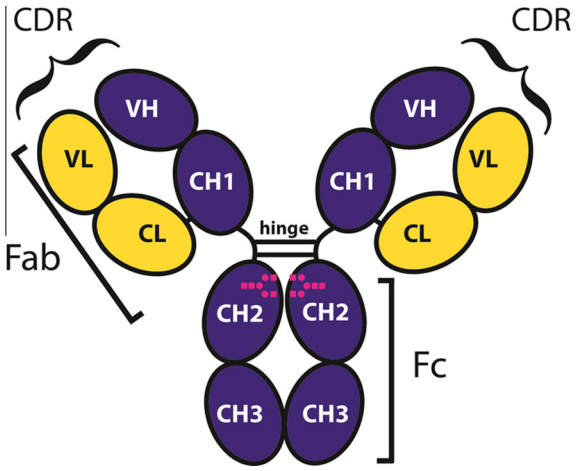

Fig. 1. Cartoon of an IgG1 antibody highlighting the various domains and fragments. The light chains are shown in yellow, and the heavy chains in blue, with two disulfide bonds linking the CL and CH1 domains and two disulfide bonds linking the heavy chains in the hinge region. Antigen binding occurs via complementarity determining regions (CDRs). The glycan chains attached to the CH2 domain are represented by the pink structures. The CH3 domains on the heavy chain interact strongly, albeit non-covalently, with each other.

IgG-based therapeutics possess a common global structure, yet there are several sources of heterogeneity that make detailed structural characterization of these compounds challenging. Variations in post-translational modifications (PTMs), such as glycosylation, oxidation, and deamidation, as well changes in primary sequence can result in a complex mixture within a single sample [17]. Other modifications are engineered into the mAb structure, such as the covalent linking of cytotoxic drugs to the antibody. Many of these modifications are quite small in mass compared to that of the intact antibody, e.g., glycosylations add approximately 1–3 kDa compared to the mAb back-bone mass of 150 kDa. Some variations, such as disulfide bond scrambling, result in no change in mass but may induce a drastic change in structure. Whether mAb heterogeneity is engineered or a result of the production process, it is crucial that these mixtures are accurately and precisely characterized as differences can result in adverse effects of mAb-based therapeutics [18,19].

While the analysis of mAb-based therapeutics by MS is often used in conjunction with enzymatic digestions and chromatographic separations [18–20], we will focus on the application of native MS. Native MS is a relatively new technique that has only recently been applied to the challenges of characterizing mAb heterogeneities, but much has been accomplished in this short time [21]. Native MS has been shown to be capable of tackling both the heterogeneity stemming from PTMs as well as non-covalent structural analysis of mAb-based therapeutics. Below we describe the features of native MS that must be taken into account when analyzing mAb-based therapeutics and then highlight a selection of accomplishments utilizing this technique.

2. Experimental features

2.1. Sample preparation

One of the strengths of native MS is the relative straightforward ease of sample preparation required for the analysis (Fig. 2). Here, we describe the details of the sample preparation necessary prior to native MS analysis of mAbs. The two main steps are: enzymatic deglycosylation, which is optional depending on the goal of the experiment, and buffer exchange, which is essential prior to native MS analysis.

2.1.1. Deglycosylation (optional)

As mentioned above, glycosylation is an important PTM in mAbs. In IgGs, these glycan chains are mostly found linked to Asn-297 of each heavy chain contributing to an increase in the total molecular weight roughly between 1000 and 3000 Da. Because of the branched and very heterogeneous nature of these PTMs, a single mAb can exist in different isoforms, each one with a slight difference in molecular weight. As a consequence, in the MS spectrum of an intact glycosylated mAb, the signal arising from a single antibody results in a spread of many different peaks corresponding to the different isoforms.

When the glycans do not represent the object of the study, an enzymatic deglycosylation performed prior to MS analysis can be beneficial, as it reduces mass heterogeneity due to the glycan chains. Besides the simplicity of the resulting mass spectrum, there are two main advantages of analyzing deglycosylated mAbs: (i) the increase of the signal intensity as many peaks are now combined into a single peak; and (ii) a higher confidence in mAb identification.

An enzyme commonly used for mAb deglycosylation is the Peptide-N-glycosidase F (PNGaseF). Generally, 2 units of enzyme are sufficient to deglycosylate 50 μg of antibody. The procedure is extremely straightforward; it is enough to add the enzyme to the antibody sample, and incubate at 37 °C for at least 4 h (overnight is preferable). In our experience, phosphate buffered saline (PBS) is a good incubation buffer, though different enzyme vendors suggest their own optimal incubation buffer. It is worth bearing in mind that the incubation buffer can severely affect the efficacy of deglycosylation.

2.1.2. Buffer-exchange

Analysis by MS is generally hampered by the use of buffers, due to intense signals from salts and the formation of adducts to the analyte proteins of interest. The main requirement for a buffer or a solvent to be used in ESI is volatility, so as to ensure a complete transfer of the analyte into the gas phase, reducing competition for ionization with background buffer molecules. When performing native MS, the requirements for the buffer become extremely stringent. In fact, the buffer has to be not only volatile, but it also has to preserve the native conformations of the proteins. An aqueous solution of ammonium acetate at a neutral pH, (but also ammonium bicarbonate or triethylammonium bicarbonate) has all these characteristics, and, therefore, is one of the most commonly used buffers for native MS. Concentrations between 10 and 500 mM are generally used. Optimal conditions of ionic strength and pH need to be investigated empirically for each protein. According to our experience, 150 mM ammonium acetate buffer at pH 7.5 is an excellent buffer for mAbs. Nevertheless, slightly different conditions would not significantly influence the analysis for most mAbs.

As most of the mAb samples are not purified using ammonium acetate buffer, a buffer-exchange step is required. Small spin-column filters are optimal for this purpose. These devices are commercially available in different sizes, and with different molecular weight cut-offs (MWCO). Our recommendation is to buffer-exchange your samples until the concentration of the original buffer is below nM range.

The buffer-exchange is an essential step. When the buffer is not efficiently exchanged, high concentration of salts, containing cations such as sodium or potassium, can induce the formation of adducts causing the broadening of the detected ion signals, thereby negatively affecting accurate mass assignments and quantitation.

2.2. Instrumentation

In general, native MS is a technique that not only allows the measurement of intact protein and protein complexes in their