Analysis of bispecific and multispecific antibody therapeutics using native mass spectrometry on a modified hybrid Orbitrap mass spectrometer

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Abstract

Purpose: Bispecific antibodies (BsAb) have gained considerable attention recently. In contrast to monospecific monoclonal antibodies that recognize an antigen and then elicit an immune response through the adaptive immune system, bispecific antibodies function by recognizing different antigens on different cells or different antigens on the same cell. This enables recruitment of T-cells for direct cell killing or by blocking proliferation of signaling pathways. Bispecific antibodies contain different heavy and light chain mixes and various formats consist of additional domains present in either the antigen binding or Fc region of the antibody incorporated by engineered hinges or additional disulfide bonds. These engineered scaffolds make bispecific antibodies analytically challenging and demand the use of analytical methods that preserve molecular structure such as native mass spectrometry.

Methods: Bispecific and multispecific antibodies expressed in mammalian cells were obtained in purified form from commercial suppliers. As many of these materials are still in an investigational stage, the materials analyzed are considered as such and not as commercial drug product. Online buffer exchange into ammonium acetate was performed using a Thermo Scientific™ NativePac™ OBE-1 SEC column coupled to the Thermo Scientific™ Orbitrap™ Excedion™ Pro BioPharma hybrid mass spectrometer which enabled measurement up to 12k *m/z*. Optimization of source parameters such as the use of in-source CID to promote desolvation was performed to ensure efficient transfer into the gas phase. For antigen complexes, incubation was performed offline prior to injection into the NativePac column.

Results: In this study, bispecific antibodies with mass range between 127 and 240 kDa were confidently analyzed on the Orbitrap Excedion Pro BioPharma mass spectrometer. Several proteoforms were identified with mass accuracy below 10 ppm. Moreover, the study investigated the possibility to track the interaction between bispecific antibodies and their antigens and the presence of aggregates species when bispecific antibodies are undergoing stress conditions.

Introduction

BsAbs are broadly categorized into Fc based (IgG-like and IgG-appended) or fragment based (IgG-less or IgG-free) based on the presence or absence of the Fc region in their structure. Fc-based bsAbs have some of the advantages of the endogenous IgG molecules, which have a favorable plasma distribution profile and serum stability compared with other Ig isotypes. Fragment-based bsAbs usually represent a simpler structure with a smaller size and at least two variable domains [1]. Generally, bsAbs are synthesized by fusing two different heavy chains, and two different light chains. The two variable binding domains allow simultaneous targeting of two epitopes that are leveraged for therapeutic effects [1]. Although engineering efforts have led to increase in the yield of the products with the correct assembly, when compared to standard IgG formats, the presence of mispaired variants still constitutes a major problem for the design, the development and the production of bsAb [2]. Online buffer exchange coupled with native MS (OBE-nMS) brings unique advantages for bsAb analysis [3] although mass range of most instruments can constitute a limitation, especially for more complex, multi-specific formats that can have a molecular mass as high as 250 kDa for the monomer. In this study 5 bsAbs were analyzed, having different constructs and different molecular weight. In particular, we investigated (Figure 1):

- Bafisontamab, a Fab in tandem bsAb (MW ~240 kDa) targeting EGFR and cMET
- Cadonilimab, a scFv IgG bsAb (MW ~ 200 kDa) targeting PD-1 and CTLA-4
- Glofitamab, a 2+1 crossmAb T-cell engager (MW ~200 kDa) having bivalency for CD20 and monovalency for CD3
- Zanidantamab, a scFv Fab Fc bsAb (MW ~125 kDa) targeting two different epitopes of HER2
- Tebotelimab, a DART (Dual Affinity ReTargeting) bsAb (MW ~170 kDa) targeting PD-1 and LAG-3

After screening the performance of the extended mass range available on the Orbitrap Excedion Pro BioPharma mass spectrometer for the analysis of all the molecules in their native state we focused our attention on tebotelimab.

Figure 1. Schematic representation of the bispecific antibodies investigated in the present study.

The DART bsAb was analyzed after heat stress to investigate the presence of higher order structures. Moreover, tebotelimab was incubated with both targeted antigens to evaluate the formation of complexes between the bsAb molecule and each or both antigens. All analysis were performed on a single platform using an online buffer exchange column for introduction of the samples in the mass spectrometer.

Materials and methods

Sample Preparation

Research grade cadonilimab, glofitamab, bafisontamab, zanidantamab and tebotelimab were purchased by MedChem Express. All the products were diluted to 0.5 mg/mL with MS grade water and directly analyzed. For heat stress, 50 µg of diluted tebotelimab were incubated at 37 °C for 72h. To study the interaction with the antigens, PD-1 and LAG-3 were incubated with tebotelimab in a 1:1:1 molar ratio or individually in a 1:1 ratio and then injected using the same workflow.

LC-MS Settings

LC-MS analysis was performed on a NativePac OBE-1 SEC column (2.1 x 50 mm, 3 µm) using an isocratic gradient of 50 mM ammonium acetate in MS grade water. Flow was kept at 0.2 mL/min, column temperature was set to 25 °C and the analysis was carried for 5 minutes. MS source and scan settings were as follows: spray voltage 3.8 kV, sheath gas 28 arbitrary units (au), auxiliary gas 10 au, ion transfer tune temperature 275 °C, vaporizer temperature 250 °C, Intact Protein application mode, Trapping gas set to High, resolution 30,000 (at *m/z* 200), scan range between 2,500-10,000 *m/z*, RF lens 200%, source CID 130 eV, max injection time 250 ms, 10 microscans, acquisition gain control 100%,

Data Analysis

Data acquisition was performed using Thermo Scientific™ Xcalibur™ software v. 4.7. Raw data were deconvoluted using Thermo Scientific™ BioPharma Finder™ software v. 5.3. Average over selected time range option was selected to deconvolute desalted peak from each analysis using ReSpect™ algorithm. Deconvolution settings were as follows: output and model mass range were adapted for each molecule based on their theoretical mass, deconvolution mass tolerance for the charge states was set to 10 ppm, charge states range between 5 and 50, while the minimum number of adjacent charge states was set to 4. For component identification, sequence mass tolerance was set to 20 ppm.

Results

BsAb native MS analysis

The NativePac OBE-1 SEC column allowed rapid analysis of all bispecific antibodies in their native state. Upon deconvolution most of the proteoforms detected were identified with a mass accuracy below 10 ppm (Table 1). Figure 2 shows two examples of cadonilimab (top) and bafisonatamab nMS analysis. Cadonilimab presents an increased heterogeneity derived from the additional N-glycosylation site present on the scFv subunit. Nonetheless, it was possible to achieve baseline resolution of the glycoforms present. Bafisontamab is the biggest molecule present in this study. Upon deconvolution, the variants present were ranging from 236 to 241 kDa; the analysis allowed the confident identification of 5 main N-glycoforms, including an immature N-glycan (A1G0F) and a glycoform lacking one N-glycan on the heavy chain.

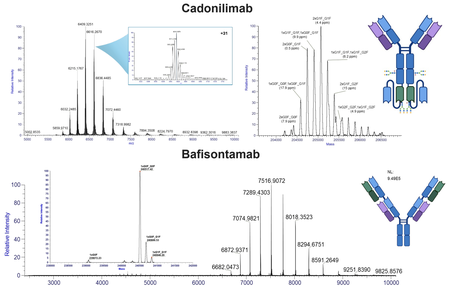


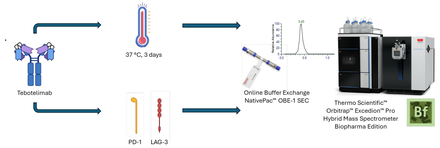
Figure 2. Cadonilimab (top panel) and bafisontamab (bottom panel) native MS analysis. In the top panel, charge envelope for cadonilimab is presented showing excellent resolution of the glycoforms generated by the N-glycans present, with a zoom on the +31 charge state. On the right, the deconvoluted spectrum obtained after data processing on BioPharma Finder software v 5.3. In the bottom panel, raw and deconvoluted spectra for bafisontamab are presented.

Table 1. Experimental and theoretical masses of the most abundant proteoforms for the 5 bispecific molecules analyzed in this study.

	Experimental Mass (Da)	Theoretical Mass (Da)
Glofitamab G0F_G0F, 2pQ	196,940.83	196,942.96
Tebotelimab G0F_G0F	169,592.09	169,591.98
Bafisontamab G0F_G0F	240,517.42	240,515.19
Cadonilimab 2x G1F_G1F	205,067.25	205,066.34
Zanidantamab G0F_G0F	127,540.23	127,538.52

Tebotelimab Heat Stress and antigens binding

Tebotelimab was chosen to perform further studies and investigate the performance of the same workflow for the analysis of higher order structures and complex formation



First, the sample was analysed after incubation at 37 °C for 3 days. Control and stressed samples were injected on the NativePac OBE-1 SEC column and analysed similarly to the previous samples.

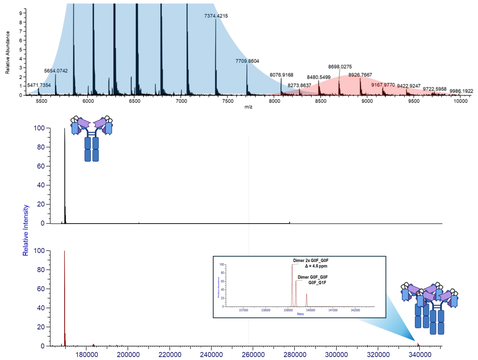


Figure 3. Native MS analysis of control and stressed tebotelimab. Top panel shows the mass spectrum of heat stressed tebotelimab highlight the charge envelopes of the monomer (blue) and dimer (red). Bottom panel shows the comparison of the deconvoluted spectra of control and stressed tebotelimab.

Analysis of heat stressed tebotelimab showed the presence of the dimer not present in the control material, with relative abundance of 1.4 %. Two glycoforms were identified with high confidence showing mass accuracy below 5 ppm (Figure 3).

To investigate the formation of complexes of the bsAb with its antigen, PD-1 and LAG-3 were incubated in 1:1 molar ratio individually and combined (Figure 4).

Several source CID values were tested to optimize the complex transmission into the mass spectrometer. However, lower source CID values did not allow a good ionization of the analyte and did not provide clear data.

With source CID set to 130 eV, it was possible to observe good ionization although the data deconvolution presented only signals derived from tebotelimab. Nonetheless, the raw data indicate the presence of a bi/tri-modal distribution of the charge states when tebotelimab interacts with PD-1 antigen, indicating a possible loss of the native configuration following the interaction with the variable domain.

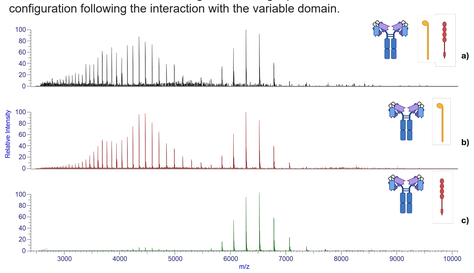


Figure 4. Native MS analysis of tebotelimab incubated with a) PD-1 and LAG-3, b) PD-1 only and c) LAG-3 only.

Conclusions

- Online buffer exchange was used in combination with Orbitrap Excedion Pro Biopharma mass spectrometer to analyze several bispecific products. All molecules investigated were confidently identified with mass accuracy below 10 ppm for most proteoforms.
- Heat stressed tebotelimab was analyzed after online buffer exchange to monitor the formation of dimers. A charge envelope corresponding to dimer was identified with excellent accuracy and quantified with 1.4% relative abundance.
- Interaction of tebotelimab with PD-1 and LAG-3 antigens did not result in the presence of complexes although it was possible to monitor changes in the conformation of the bispecific antibody from the charge distribution of the native spectra.

References

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Conflict of interest disclosure

CB, LF, SC, and JB are employees of NIBRT. CW, HK, KS, and KB are employees of Thermo Fisher Scientific, the manufacturer of instrumentation, consumables and certain software used in the generation of data presented here. JB received funding as part of a collaborative research agreement between NIBRT and Thermo Fisher Scientific. SC, LF and CB are funded through that collaborative research agreement. The authors know of no other information that may affect the impartiality or subjectivity of the scientific study.

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