Application Note LCMS-94

Native and Subunit Analysis of an Antibody Drug Conjugate Model with Ultra-High Resolution Quadrupole Time-of-Flight Mass Spectrometry

Abstract

Advances in biotech drug development such as Antibody Drug Conjugates constantly raise new analytical challenges. This case study describes the utilization of the maXis HD UHR-QTOF to characterize key attributes of an ADC model. The analysis is performed on the intact molecule by native MS for drug to antibody ratio determination and at the subunit level for linker degradation monitoring.

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Keywords

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<th>Instrumentation and Software</th>
<th>Keywords</th>
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<td>maXis HD</td>
<td>Biopharma</td>
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**Introduction**

Monoclonal antibodies (mAbs) are a proven class of therapeutic proteins that can be used to target cell receptors with great selectivity. This selectivity is a valuable tool to treat a number of indications allowing, for example, the ability to interfere with tumor growth in oncology. In some instances mAbs can be internalized by the cell and be recycled or processed by the proteasome.

The concept of harnessing this internalization capability to deliver a cytotoxic payload to diseased cells has been researched for many years. Some highly toxic drugs have systemic effects and are too unspecific to be delivered as a free drug. However, combining them with a mAb minimizes side effects by focusing the delivery to the targeted cells. The processing of the combined molecules after internalization in the cell frees up the drug allowing targeted killing of, for example, cancer cells. This sub-class of therapeutic molecules, Antibody Drug Conjugates (ADCs), has recently gained more visibility after the FDA and EMA approval of Adcetris™ (Seattle Genetics) and Kadcyla™ (Genentech), which validates the concept.

ADC treatments typically permit much lower doses than regular antibody therapies. This can result in a greater safety profile and increased efficacy. In addition, lesser quantities of mAb material are required. This makes ADCs an attractive class of therapeutic molecules and almost 40 such molecules are currently undergoing clinical trials.

The added complexity of combining a cleavable linker and a cytotoxic drug to an already complex recombinant protein product requires improved analytical methods. It is not only necessary to control heterogeneities but also the drug loading, the Drug-to-Antibody Ratio (DAR), the amount of free antibody and drug, and the linker chemistry. This case study presents how an Ultra-High Resolution Quadrupole Time-of-Flight instrument (UHR-QTOF), such as the maXis HD, can be used to determine these important quality attributes in a cysteine-linked ADC.

**Experimental**

**Sample**

This study focuses on an Antibody Fluorophore Conjugate (AFC), which is an ADC non-toxic model. Having a non-toxic payload, these AFCs are valuable molecular tools for mechanistic studies and PK evaluation. The AFC evaluated here is based on the conjugation of dansyl-sulfonamide ethyl amine (DSEA) with a maleimide linker on inter-chain cysteines of trastuzumab. The DSEA-linker payload mimics the chemistry of many ADCs currently in clinical trials.

**Buffer exchange for infusion**

The sample was analyzed in its native form after three consecutive steps of buffer exchange on a molecular weight cut-off filter (Amicon Ultra 0.5 mL, 30 kDa membrane) with 150 mM ammonium acetate and removal of the heavy chain glycosylation with IgGZero™ (Genovis).

**Subunits preparation**

In order to facilitate the study of the AFC at the subunit level, 100 μg of protein were digested with 100 units of FabRICATOR™ enzyme (Genovis) in 150 mM ammonium acetate at room temperature for 30 minutes (Wagner-Rousset et al. 2014). After cleavage, the fragments were reduced with 6 M guanidine-HCl and 50 mM TCEP for 30 minutes. Finally, 10% trifluoracetic acid (TFA) were added to obtain a pool of 3 AFC fragments Fc/2, Fd and light chain (Figure 1, Wagner-Rousset et al. 2014). The precise cleavage at the hinge facilitates the observation of variants such as linker degradation in this instance.

**Native mass spectrometry**

Native-spray of larger proteins at neutral pH conditions results in molecular ions with relatively low numbers of charges. In addition, the absence of organic solvent in native MS requires enhanced desolvation condition. A Bruker maXis HD instrument capable of 75,000 resolving power and equipped with High Mass Option was used. This added capability enables the increase of the pressure in the first ion transfer stage resulting in an enhanced protein desolvation and higher ion transfer efficiency. This tuning improves the results when analyzing high molecular weight proteins and non-covalently bound complexes. The pressure can be set back to standard conditions in a few seconds.

This capability is highly beneficial for the analysis of molecules such as ADCs because the inter-chain disulfide bonds are reduced and so the protein remains in its tetrameric conformation due only to electrostatic and hydrophobic interactions. Exposure to organic solvent would cause unfolding and subsequent separation of the light and heavy chains, precluding the analysis of the intact protein.

**Table 1: Elution conditions for AFC subunits**

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<thead>
<tr>
<th>Time (minutes)</th>
<th>Solvent A (%)</th>
<th>Solvent B (%)</th>
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<tbody>
<tr>
<td></td>
<td>0.1% formic acid</td>
<td>60% isopropanol + 40% acetonitrile + 0.1% formic acid</td>
</tr>
<tr>
<td>0</td>
<td>95</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>76</td>
<td>24</td>
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<td>33</td>
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<td>34</td>
<td>20</td>
<td>80</td>
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<tr>
<td>39</td>
<td>20</td>
<td>80</td>
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</table>
The desalted sample was infused with a NanoMate (Advion) at a concentration of 1 μg/μL. Source voltage was set at 1.65 kV, isCID voltage at 200 eV, and funnel pressure at 3.7 mbar for optimal desolvation.

LCMS of subunits

For IdeS-generated AFC subunits, 1.5 μg of the IdeS digest were loaded on the column and desalted online for 23 min using a flow rate of 0.2 mL/min at 5% solvent B. This was followed by an elution step described in Table 1 and reconditioning of the column at 5% B. The ESI source was set at 4.5 kV, 190° C, 1.6 bar nebulization gas and 9 L/min dry gas. The whole LC-MS system and data analysis were automated under BioPharma Compass™ 1.1 control (Bruker).

**Figure 1: Expected fragments after digestion of the AFC by FabRICATOR followed by reduction (Wagner-Rousset et al. 2014)**

**Figure 2: maXis HD high mass option, tunable funnel pressure and collision gas for optimal desolvation**

1. Vacuum tuning valve to optimize funnel 1 pressure.
2. In source activation is applied by a DC voltage between the funnel 1 and funnel 2 to facilitate desolvation and transmission of supermolecular complexes.
3. Software controlled tunable back pressure in the collision cell further enhances ion desolvation and thermalization.
4. Access to the vacuum tuning valve.
Data analysis

The subunit data were processed automatically using Biopharma Compass™. After integration of the peaks detected in the total ion chromatogram, average spectra were calculated for each compound. Following this step MaxEnt™ was used to reduce the multiply charged envelopes to neutral spectra. With the maXis HD (Bruker) ultra-high resolution capabilities (75,000 at full sensitivity) baseline resolved isotopic envelopes were easily obtained for all the digestion products. Finally, the SNAP (Bruker) algorithm was applied to determine the monoisotopic mass of the detected AFC subunits.

Results

Intact analysis in native conditions

The deconvoluted spectrum for the intact AFC (Figure 3) reveals that all possible forms of the AFC are present (From 0 up to 8 conjugated drugs). The main forms detected have an even DAR of 2, 4, and 6. Figure 4 shows the charge state cluster (23+ to 26+) for those forms prior to deconvolution.

Using the peak area of each conjugated species, a DAR of 3.9 can be calculated. This is in agreement with the targeted DAR. This AFC contains a significant amount of odd species with about 10% contribution from DAR3 and DAR5 to the total peak area. This most likely derives from the conjugation reaction conditions. Also of interest is the shoulder visible on some of the peaks in the intact spectrum. It indicates some level of heterogeneity that is more easily investigated at the subunit level.

Analysis of subunits by reverse phase HPLC

All major species detected in the chromatogram (Figure 5) match the theoretical monoisotopic masses expected from the trastuzumab-AFC digested with a mass accuracy better than 1ppm (Table 2). Major glycoforms of the Fc are also easily observed, including one containing a high mannose glycan.

The conjugation level of the observed fragments correlates well with the expected conjugation mechanism. Only the cysteines responsible for the inter-chain disulfide bridges are reduced and conjugated. A maximum of one drug on the light chain and three on the heavy chain fragment containing the hinge domain (Fd) are measured; this is as expected based on the position of the cysteines involved in inter-chain bridges. A DAR of 3.8 is derived from this subunit data, which is in agreement with the intact protein data.

The high resolution and sensitivity obtained at the subunit level allows detection of heterogeneities closely related in mass with high confidence. The spectra of conjugated fragments confirm the presence of additional peaks that were hypothesized after the intact mass analysis.

The maleimide-based linkers have the potential to hydrolyze as described by Shen et al. (2012). This hydrolysis is dependent on the reaction conditions and the storage of the AFC. The charge environment of the conjugation site affects the speed of this hydrolysis reaction, which is accelerated by the presence of basic amino acids. The ratio of intact-to-hydrolyzed linker is an attribute that is important to understand and control for the optimization of the conjugation process and degradation studies.

The number of additional peaks is linked to the amount of conjugated fluorophore and corresponds to the gain of a water molecule. For example, the spectrum for the Fd conjugated with a single fluorophore (Figure 6) exhibits a peak at the expected mass for this compound, but also a more intense peak at +18Da. Looking at the primary sequence, this can be explained by the presence of basic amino acids surrounding the CH1 domain cysteine, which will accelerate
Summary

This approach demonstrates the ability of the maXis HD with high mass option to characterize antibody conjugation processes.

The analysis of the intact protein in native conditions offers a global view of the molecule. Ionization conditions are soft enough to keep the AFC integrity despite its lack of inter-chain disulfide bridge. The DAR can be determined with good precision through a simple infusion experiment.

Further analysis of the subunits offers deeper insights into the molecule’s attributes. The maXis HD resolution permits the observation of the isotopic patterns of the subunits, enabling the measurement of monoisotopic masses with sub-ppm accuracy; thus allowing confirmation of the primary sequence and detection of variants closely related in mass. Some observations can be made about the localization of the conjugates on the AFC fragments and the hydrolysis status of the linker can be followed.

The maXis HD, combined with the reporting and automation capabilities of BioPharma Compass, is a prime platform for ADC development, enabling fast DAR evaluation and greater insights into the attributes of the ADC through improved subunit analysis workflows.
Publication bibliography

Shen, Ben-Quan; Xu, Keyang; Liu, Luna; Raab, Helga; Bhakta, Sunil; Kenrick, Margaret et al. (2012): Conjugation site modulates the in vivo stability and therapeutic activity of antibody-drug conjugates. In Nat. Biotechnol. 30 (2), pp. 184 – 189. DOI: 10.1038/nbt.2108.