Glycosylated and Deglycosylated Subunit Analysis of Antibody Drug Conjugates

Trastuzumab Emtansine Analysis using Benchtop X500B QTOF Mass Spectrometer

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Introduction

Antibody-drug conjugates (ADCs) are highly potent biotherapeutics which consist of a monoclonal antibody coupled to a linker and a drug payload. The antibody targets specific cell by binding to target receptors. After binding, the ADC is internalized and the cytotoxic payload is released. Safety and efficacy are important for ADCs and hence characterization of such complex molecules and their payloads is extremely important. As part of the development and commercialization process it is critical to ensure that the payload levels do not varyso drug-antibody ratios (DAR) need to be calculated in order to ensure that the safety and efficacy are maintained. [1-3]

The analysis of the subunits which correspond to the light and heavy chain of the antibody backbone allows for more specific analysis of the abundance of drug molecules on the light chain and heavy chain separately. Here we present a robust and efficient method for subunit analysis using the X500B benchtop QTOF with BioPharmaView™ software for the calculation of multiplicity and for the calculation of the drug-antibody ratio (DAR).

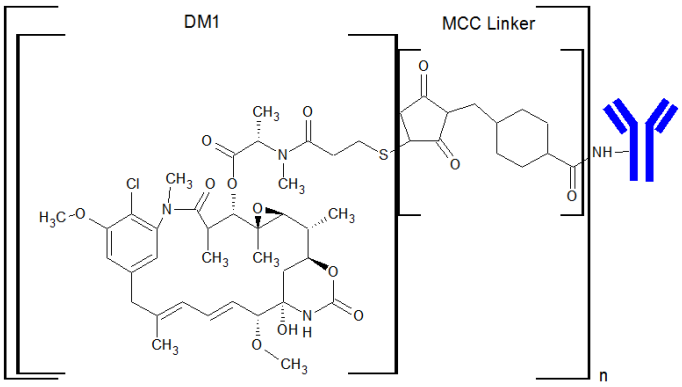

Figure 1: Trastuzumab emtasine (T-DM1) Trastuzumab antibody back bone is conjugated through the lysine residues to the MCC linker and the cytotoxic drug DM1)

Experimental

PNGase F was purchased from New England BioLabs (Ipswich, MA, USA). Tris-(2-carboxyethyl) phosphine (TECP) was purchased from Sigma.

Samples were aliquoted and a portion was deglycosylated using the standard protocol for PNGase F.

Reduction was carried out with the addition of TCEP to the samples and heating the samples for 56°C for 30 minutes.

LCMS analyses were conducted using a benchtop X500B QTOF instrument equipped with SCIEX OS and connected to an ExionLC™ system. Table 1 and 2 list the LCMS conditions used in these analyses.

Table 1 Shimadzu LC conditions

Column	*Agilent Poroshell 300SB-C8 1.0 x 75mm 5 µm*
Mobile phase A	*0.1 % Formic acid in water*
Mobile Phase B	*0.1% Formic acid in acetonitrile*
Flow rate	*0.2 mL/min*
Column Temperature	*75 °C*
Run time	*10 min*

Table 2 X500B mass spectrometry conditions

Source parameters:	
CUR	*30*
GS1	*50*
GS2	*50*
Ion Spray Voltage	*5000 V*
Source Temperature	*400°C*
TOFMS mass range	*900 – 4000* m/z
DP	*250 V*
Accumulation time	*0.5 s*
Time bins to sum	*80*
Intact Protein Mode (IPM)	*On*
Large Proteins (>70kDa)	*On*
Decrease Detector Voltage	*On*
DP	*250 V*

Results and Discussion

Glycosylated Subunit Analysis

Trastuzumab emantasine (T-DM1) was used for the subunit analysis. This ADC is composed of a trastuzumab antibody back bone which is lysine conjugated to a linker (MCC) and drug (DM1). To generate the subunits, the ADC was treated to reduce the disulfides. Analysis of the reduced ADC resulted in spectra

for the light and heavy chain. Reconstruction of both the light chain and the heavy chain in BioPharmaView indicates that there are up to two drugs bound to the light chain (Figure 2A) and up to three drugs bound to the heavy chain (Figure 2B).

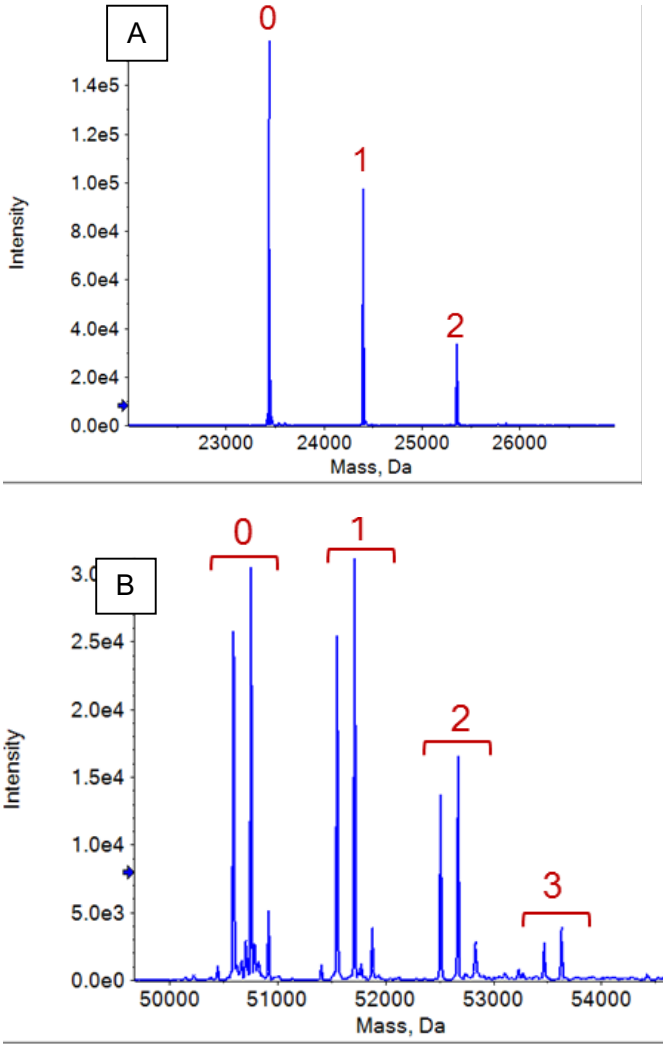

Figure 2: Reconstruction of the light (A) and heavy (B) chain showing up to 2 and 3 drugs conjugated respectively.

Previously published data also suggested the presence of a +221 Da mass shift corresponding to a modified linker with no DM1 present. A mass corresponding to this shift is seen in the subunit data but at a very low intensity, likely due to the spread across the various forms of the subunits. [2]

BioPharmaView was used to calculate the DAR for both the light and the heavy chain respectively. This calculation is automatically generated when a DAR or modification is added into the system and can be selected away from the rest of the modifications as seen in Figure 3. The combined data correlates well to previously published results. [1-3]

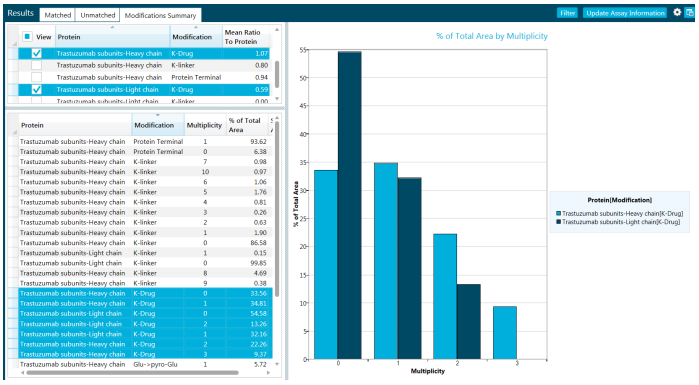

Figure 3 DAR calculation for the light and the heavy chain. Top left highlighted forms are the DAR ratios for the light (0.59) and heavy (1.07) chain.

BioPharmaView allows for relative ratios of drug bound to each subunit to be easily viewed as seen in Figure 3. In this case, the light chain shows that most of it is unmodified with 55% of the total area for the light chain corresponding to 0 drugs conjugated. The area percent with one drug bound becomes 32% and only 13% of the light chain corresponds to two drugs bound. In contrast, the heavy chain has a slightly higher area with one drug bound (35%) vs 0 drug bound (34%). Up to three drugs (9%) were found on the heavy chain whereas a maximum of two (22%) drugs were found on the light chain.

Deglycosylated Subunit Analysis

The deglycosylated reduced ADC was also analyzed. Reconstruction of the deglycosylated light and heavy chain (Figure 4 A and B respectively) agrees with the data collected on their glycosylated forms (Figure 2A and 2B), with the light chain having up to two drugs conjugated and the heavy chain with up to three drugs conjugated.

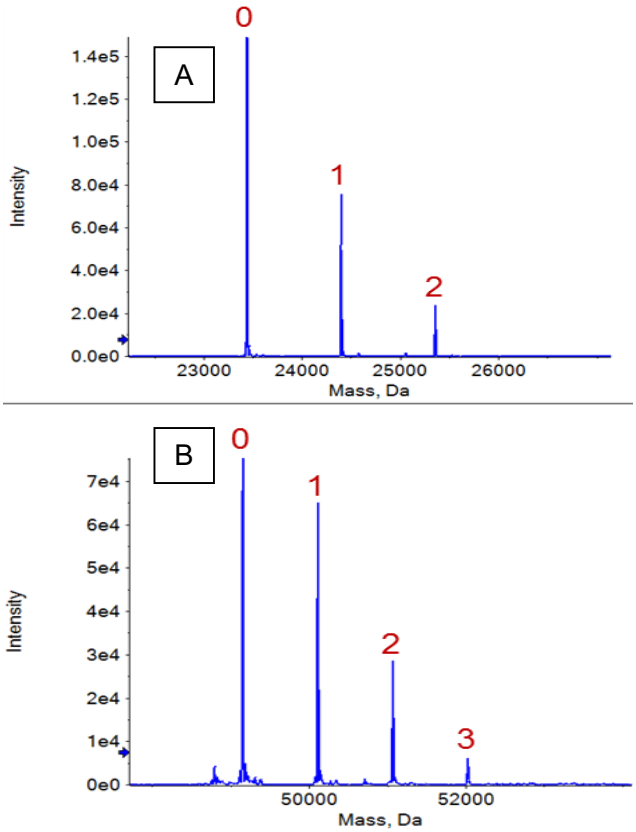

Figure 8: Deglycosylated reconstructed light (A) and heavy (B) chain. The light chain shows up to 2 drugs conjugated while the heavy chain shows up to 3 drugs attached.

With the deglycosylated data, a +221 Da is present at very low intensity, but agrees with both the glycosylated subunit data and with previously published intact data. [2]

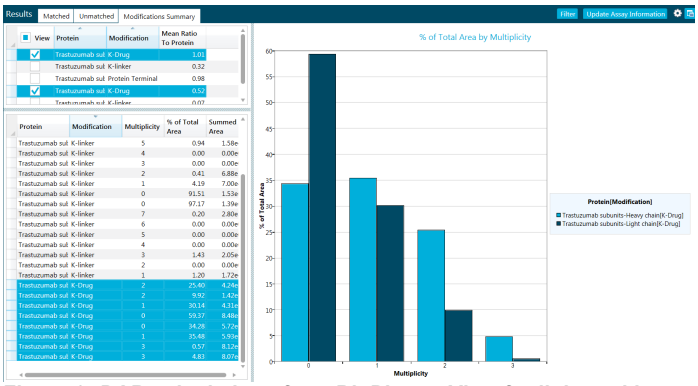

Figure 9: DAR calculations from BioPharmaView for light and heavy chain deglycosylated. Light chain shows a DAR ratio of 0.52 and the heavy chain shows a DAR of 1.01.

Conclusion

Complex ADCs can be quickly reduced into the subunit form and analyzed without the requirement of any sample preparation such as deglycosylation to obtain the information. Here we show a rapid, simple and effective workflow for the analysis of both glycosylated and deglycosylated subunit forms of ADC. The reconstruction in BioPharmaView allows for the glycosylation pattern to be used and monitored and allows for the multiplicity and DAR to be calculated for the overall subunits. Batch processing allows for monitoring of such critical quality attributes across a large number of samples and a visual result allowing for a definitive identification of significant differences between samples analyzed.

References

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[3] Liuxi Chen, Lan Wang, Henry Shion, Chuanfei Yu, Ying Qing Yu, Lei Zhu, Meng Li, Weibin Chen, and Kai Gao. In-depth structural characterization of Kadcyla (ado-trastuzumab emtansine) and its biosimilar candidate. MABS, 2016, VOL. 8, NO. 7, 1210–1223

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