A streamlined workflow for the determination of the drug-to-antibody ratio (DAR) of antibody-drug conjugates (ADCs)

Featuring the intact protein analysis workflow using the ZenoTOF 7600 system and Biologics Explorer software from SCIEX

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This technical note highlights an efficient intact protein analysis workflow for determining drug-to-antibody ratios (DARs) of antibody-drug conjugates (ADCs)[1]. This workflow leverages high-resolution mass spectrometry (MS) for accurate intact mass measurement in addition to automated protein deconvolution and DAR calculation provided by Biologics Explorer software. Average DAR values of 3.6 and 3.5 were obtained for the glycosylated (native) and deglycosylated forms of trastuzumab emtansine (T-DM1), respectively.

ADCs—a new generation of biotherapeutics—combine potent cytotoxic drugs with monoclonal antibodies (mAbs) that target specific cells.[1] The drug and mAb are connected through a cysteine-based, lysine-based or site-specific linker. Depending on the type of linkage used in an ADC, DAR values as high as 8 might be observed.[2] This variability underscores the need for accurate and reliable DAR measurements to optimize ADC performance and efficacy.[3] The mean quantity of drugs conjugated to antibodies is a critical parameter for ADCs. Therefore, DAR measurement is pivotal in determining essential properties, such as drug clearance, pharmacokinetics and biodistribution, which are vital considerations for developing targeted therapies.

In this work, the glycosylated and deglycosylated forms of T-DM1 were characterized using the ZenoTOF 7600 system, followed by automated data analysis using Biologics Explorer software (Figure 1), leading to accurate DAR measurements in a streamlined workflow.

Key features of the intact protein analysis workflow for ADC characterization

- **Efficient:** The streamlined workflow leverages high-resolution MS for accurate mass measurement of intact ADCs and powerful software tools for automated DAR calculation
- **Accurate DAR measurement:** Biologics Explorer software offers powerful algorithms for confident protein deconvolution and accurate DAR calculation
- **Easy to implement:** The integrated workflow and intuitive software interface make ADC analysis accessible to various researchers, regardless of their expertise level
- **Routine ADC characterization:** This workflow can be rapidly implemented for routine ADC characterization to ensure product quality

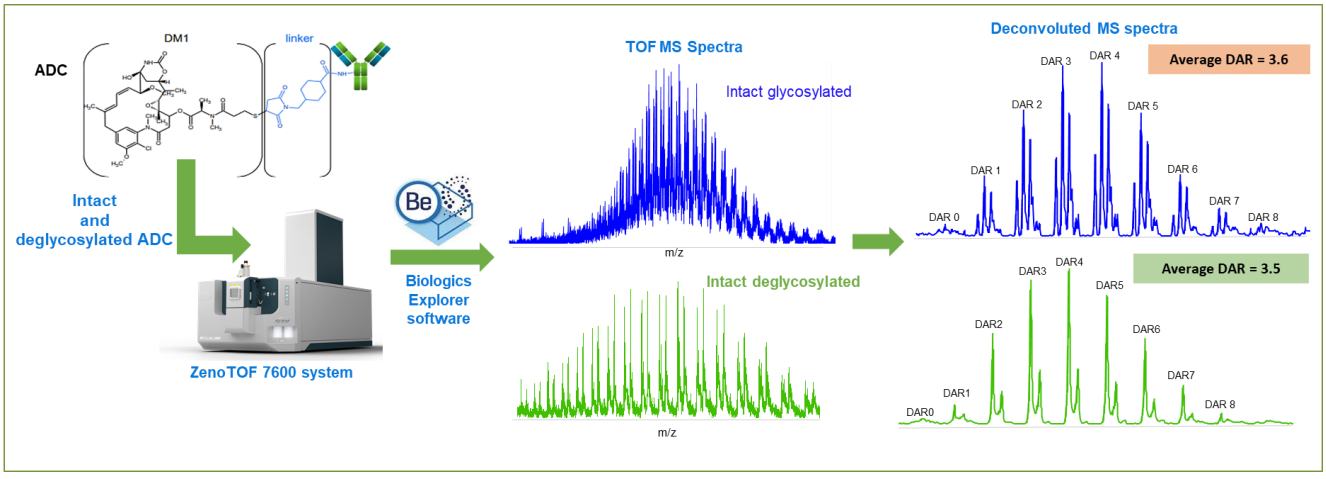

Figure 1. A streamlined intact mass analysis workflow for determining the average DAR values of the glycosylated and deglycosylated forms of T-DM1 using the ZenoTOF 7600 system. Intact T-DM1 MS data were analyzed by Biologics Explorer software using an optimized intact mass analysis workflow template for automated DAR calculation. Average DAR values of 3.6 and 3.5 were obtained for the glycosylated and deglycosylated forms of T-DM1, respectively.

Methods

Sample preparation: Lyophilized T-DM1 was reconstituted in deionized (DI) water to a final concentration of 5 mg/mL. For the intact ADC analysis, the reconstituted T-DM1 was diluted with 0.1% formic acid (FA) in water to 1 mg/mL prior to LC/MS analysis. Deglycosylation of T-DM1 was performed by adding 1 µL of 50 units/µL PNGase F (Sigma-Aldrich) in 20mM Tris-HCl buffer (pH = 8.2) to 100 µL of 1 µg/µL T-DM1 (100 µg). The mixture was incubated overnight at 37°C. Finally, 5 µL samples of T-DM1 were injected for LC-MS analysis.

Chromatography: Intact glycosylated and deglycosylated ADC samples were separated using the LC gradient displayed in Table 1, using a Waters ACQUITY BEH C4 column (2.1 mm x 50 mm, 1.7 µm, 300 Å, Waters). The flow rate was set to 0.3 mL/min for all LC runs. The column was kept at 60°C in the column oven of an ExionLC AD system (SCIEX). Mobile phases A and B consisted of 0.1 % FA in water and 0.1% FA in acetonitrile, respectively.

Table 1. Chromatographic gradient for T-DM1 separation.

Time (min)	Mobile phase A (%)	Mobile phase B (%)
0.0	*90*	*10*
2	*90*	*10*
6	*10*	*90*
7	*10*	*90*
7.10	*95*	*5*
10	*95*	*5*

Mass spectrometry: Intact LC-MS data of glycosylated and deglycosylated ADCs were acquired using the ZenoTOF 7600 system (SCIEX). The source parameter and TOF MS settings are listed in Tables 2 and 3.

Data processing: Intact T-DM1 LC-MS data were analyzed using the intact protein analysis workflow template in Biologics Explorer software. The DM1 and linker were added to the conjugate table.

Table 2. Source and gas parameters.

Parameter	Value
Polarity	*Positive*
Ion source gas 1	*50 psi*
Ion source gas 2	*50 psi*
Curtain gas	*30 psi*
Source temperature	*400°C*
CAD gas	*7*

Table 3. TOF MS parameters.

Parameter	Value
Spray voltage	*5500 V*
TOF start mass	*900 m/z*
TOF stop mass	*4000 m/z*
Accumulation time	*0.25 s*
Declustering potential	*250 V*
Collision energy	*10 V*
Time bins to sum	*80*

Intact protein deconvolution and DAR measurement for T-DM1

To determine the DAR of T-DM1, intact mass measurements of the glycosylated and deglycosylated forms of T-DM1 were performed using the ZenoTOF 7600 system. Complex charge state distributions were observed for the 2 forms of T-DM1 in the high-resolution TOF MS spectra (Figure 2A and 2D). The results of intact protein deconvolution from Biologics Explorer software revealed that the complex MS profile of the glycosylated T-DM1 consists of different glycoforms—mainly G0F, G1F and G2F—conjugated with as many as 8 molecules of the payload DM1 (Figure 2B and 2C). By comparison, the removal of N-linked glycosylation led to a simpler MS profile (Figure 2D–F), where the deglycosylated T-DM1 carrying up to 8 DM1 was detected. The 2 forms of T-DM1 were identified with a <10 ppm mass accuracy and were automatically integrated through Biologics Explorer software. Figure 3 shows the DAR distributions of the glycosylated and deglycosylated forms of T-DM1. In both cases, the major T-DM1 species had a DAR value of 2–4 (Figure 3).

The average DARs of the glycosylated and deglycosylated forms of T-DM1 can be calculated from the DAR distributions within Biologics Explorer software. Average DAR values of 3.6 and 3.5 were measured for the glycosylated and deglycosylated forms of T-DM1, respectively. The measured DAR values agree with those reported for T-DM1 in the literature.[5, 6]

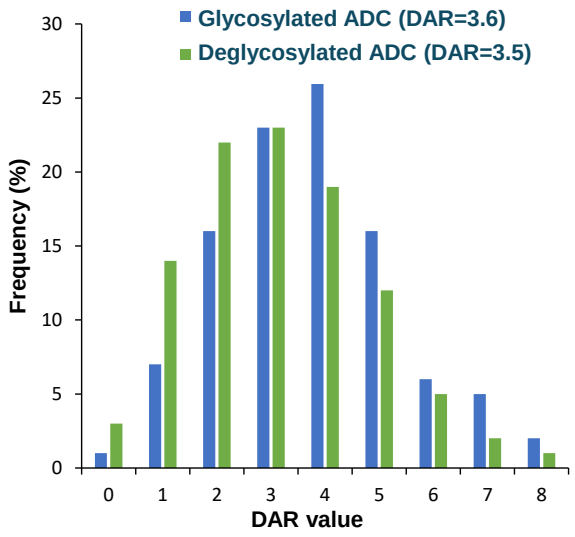

Figure 3. DAR distributions of the glycosylated and deglycosylated forms of T-DM1. Average DAR values of 3.6 and 3.5 were determined for the glycosylated and deglycosylated forms of T-DM1, respectively.

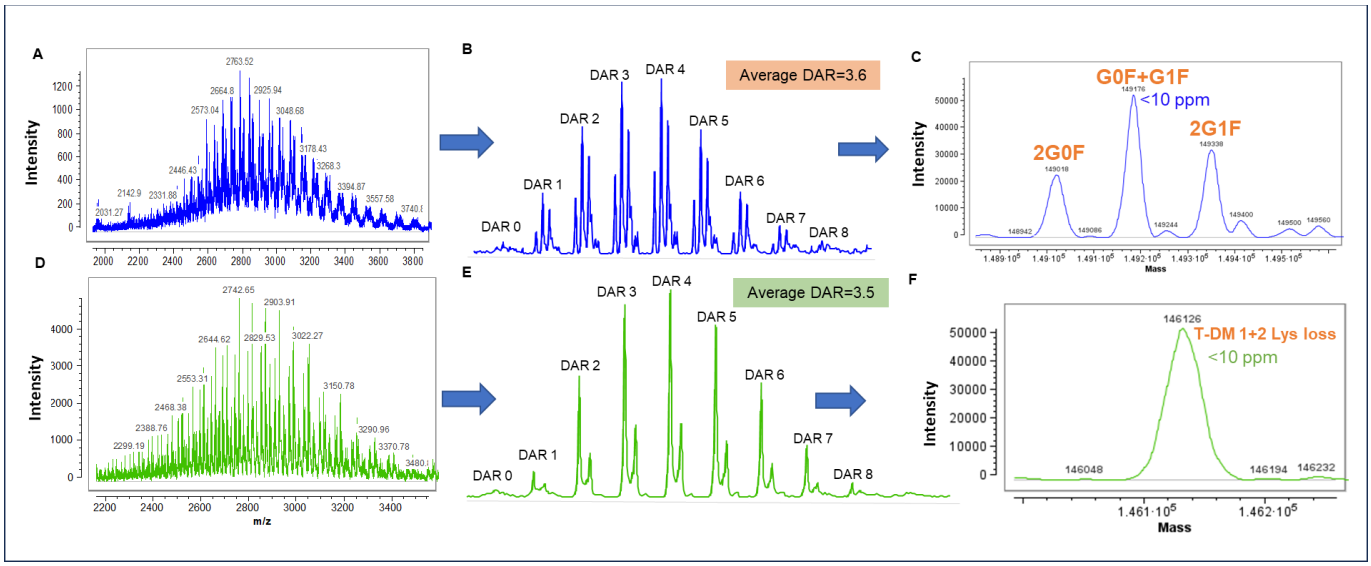

Figure 2. Intact protein deconvolution and DAR measurement of the glycosylated and deglycosylated forms of T-DM1 using Biologics Explorer software. (A and D) Charge state distributions of the glycosylated and deglycosylated forms of T-DM1. (B and E) Deconvoluted mass spectra of the glycosylated and deglycosylated forms of T-DM1 that reveal the DAR distribution of the detected peaks. (C) Zoomed view of the deconvoluted spectrum of the glycosylated form of T-DM1 with 3 major glycoforms annotated. (F) Zoomed view of the deconvoluted spectrum of the deglycosylated form of T-DM1.

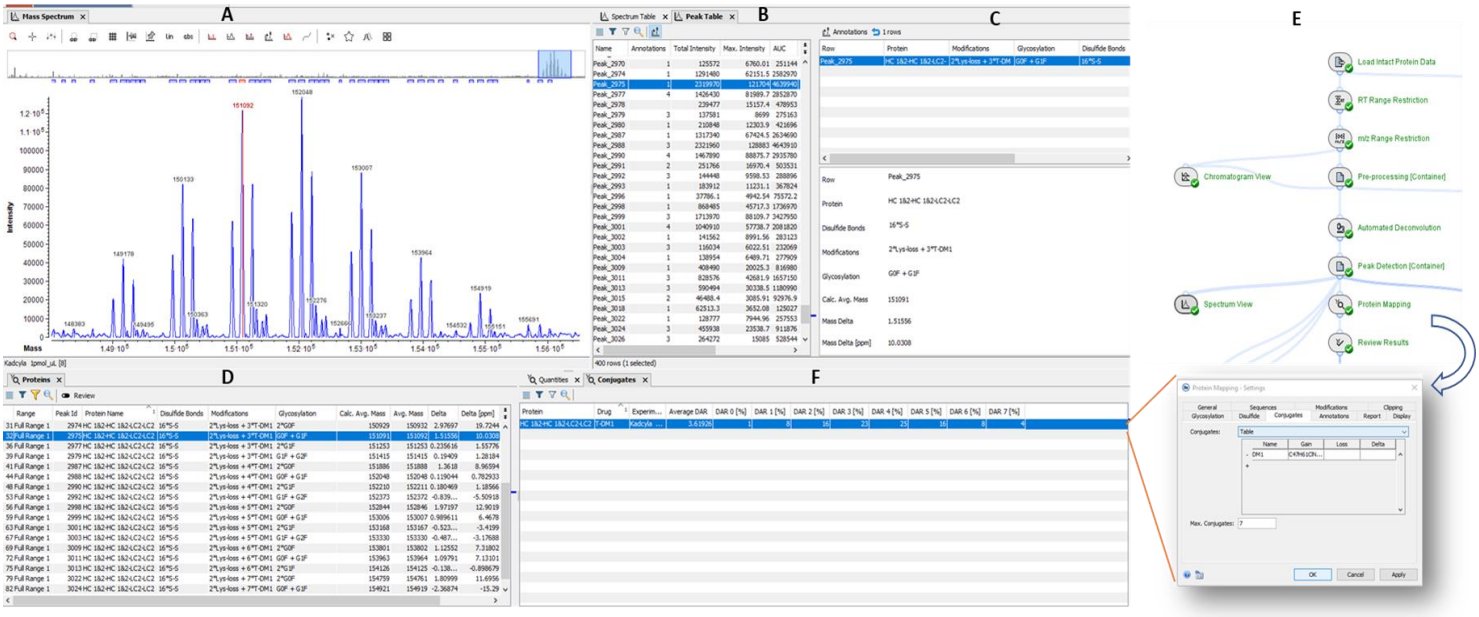

Figure 4. A snapshot of Biologics Explorer software for analyzing T-DM1 data and calculating DAR values. The intact protein analysis workflow within Biologics Explorer software offers streamlined and automated interpretation of intact ADC data. In addition, the software provides powerful tools for reviewing the deconvolution spectrum (A), annotated peaks (B–D) and the results of DAR measurement (F) in the same page. The payload and linker composition can be conveniently defined in the conjugate table within the protein mapping activity of the intact protein analysis workflow (F).

Streamlined ADC analysis using Biologics Explorer software

The analysis of intact ADC data was streamlined in 1 workflow using Biologics Explorer software, as shown in Figure 4. This compelling software displays the deconvoluted spectrum (Figure 4A) and detailed information about the annotated peaks (Figure 4B–D) on a single page for easy results review. Moreover, the payload and linker composition can be conveniently defined in the conjugate table within the protein mapping activity (Figure 4E), which provides an automated average DAR measurement of the glycosylated T-DM1 (Figure 4F). This intuitive template highlights the ability of Biologics Explorer software to seamlessly guide users through accurate measurements and informative data analysis.

Conclusions

- The streamlined, intact protein analysis workflow leverages the power of the ZenoTOF 7600 system for accurate intact mass measurement and automated data interpretation offered by Biologics Explorer software for rapid protein deconvolution and DAR determination
- Accurate mass measurement led to high-confidence annotation of T-DM1 glycoforms conjugated with as many as 8 molecules of the payload
- Average DAR values of 3.6 and 3.5 were measured for the glycosylated and deglycosylated forms of T-DM1, respectively
- Biologics Explorer software provides intuitive workflows and powerful tools for confident protein deconvolution, automated DAR calculation and rapid results review

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