Comprehensive characterization of a multi-specific antibody therapeutic

Intact and subunit mass analysis with the SCIEX X500B QTOF LC-MS/MS System and Protein Metrics software for accurate data interrogation of a trispecific antibody therapeutic

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Here, the comprehensive analysis of a trispecific monoclonal antibody (mAb) with various post-translational modifications (PTMs) using intact analysis and different subunit approaches in a routine manner is showcased.

Antibody-based derivatives have received increased attention as therapeutics in biopharmaceutical development because of their unique capacity to engage multiple molecular targets[1], engage immune effector cells[2] or even penetrate tissues previously inaccessible to conventional mAbs[3]. In order to address complex disease pathways more specifically (by involving multiple targets, for example), new modalities such as multi-specific antibodies have become a promising therapeutic platform. These therapeutics can be more complex and their development requires increased attention to ensure safety and efficacy. Liquid chromatography coupled to mass spectrometry (LC-MS) is frequently used as a characterization tool. However, it can be challenged by the additional structural complexity of multi-specific antibodies, due to different domains and PTMs such as glycosylation. To address this analytical challenge, a comprehensive intact and subunit mass analysis has been evaluated to confirm intact integrity and achieve domain specific results. This method is suitable as a generic assay for complex biotherapeutics characterization as well as further attribute monitoring in both upstream and downstream development.

Key features for the characterization of multi-specific antibodies

- Excellent data quality for increased confidence in the results for a wide range of biopharmaceutical applications with a benchtop, high resolution TOF MS instrument
- An easy to use, yet powerful newly designed accurate mass system for expert and non-MS trained scientists
- Direct compatibility of raw data files with state-of-the-art data processing tools from Protein Metrics especially suited for complex biotherapeutics characterization
- A generic platform-based method suitable for characterizing biopharmaceuticals from standard proteins to new modalities with increasing complexity

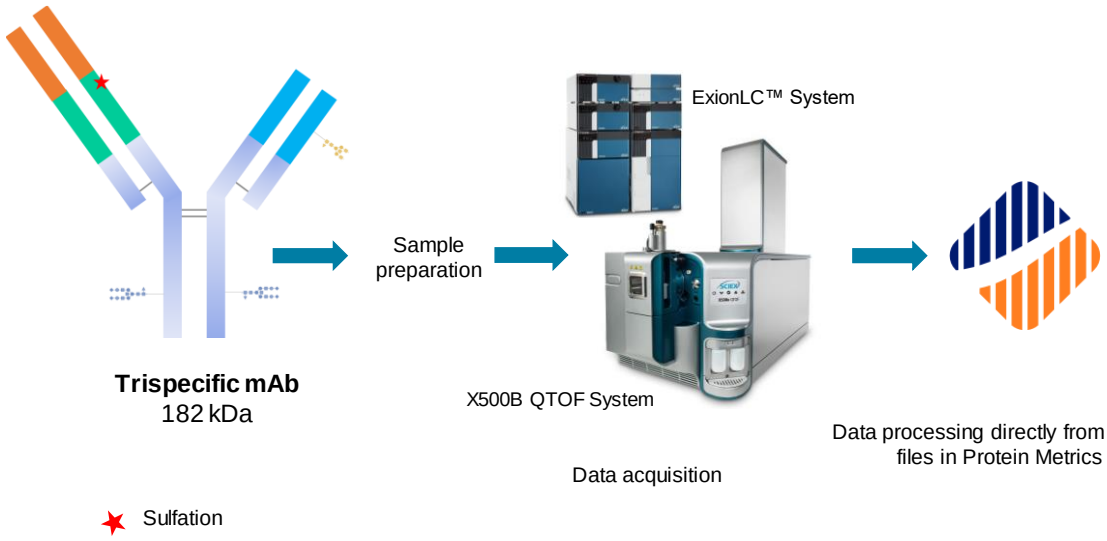

Figure 1. Overview of workflow for characterization of the multi-specific mAb. Different sample preparations were performed. The prepared samples were analyzed using an ExionLC™ system coupled to an X500B QTOF System. Raw data were analyzed using Byos software from Protein Metrics.

Methods

Intact analysis: The trispecific mAb sample was diluted to 1 g/L and subjected to LC-MS analysis. For a subset of the sample PNGase F (New England Biolabs) was utilized to remove N-linked glycosylation enzymatically.

Subunit analysis: Subunit analysis was performed via site-specific digestion above the hinge region with IgdE (FabALACTICA, GenovisAB). For digestion, 1 unit of enzyme per µg of mAb was added and incubated at 37 °C overnight.

Deglycosylation: Rapid PNGase F (New England Biolabs) was used to remove the N-linked glycans on the multi-specific antibody. Both non-reducing and reducing formats were used. In detail, the mAb was diluted to 0.8 g/L in Rapid PNGase F buffer and denatured at 75°C for 10 min. After cooling down to room temperature, 1 µL of Rapid PNGase F was added and incubated at 50°C for 15 min.

Reduction: The intact or IgdE-digested antibody was denatured and reduced with 300 mM DTT/5 M Guanidine hydrochloride for 30 min at 37°C.

Chromatography: Separation was accomplished using an ExionLC System fitted with a Waters BEH SEC column (4.6 mm × 300 mm, 1.7 µm) at 60°C using the gradient shown in Table 1. Mobile phase A was 0.1% formic acid, 25 mM ammonium formate in water and mobile phase B was 100% acetonitrile. The flow rate was set to 300 µL/min.

Table 1. LC conditions for intact and subunit analysis.

Time (min)	%A	%B
Initial	*30*	*70*
10.0	*30*	*70*
10.1	*25*	*75*
20.0	*25*	*75*
20.1	*30*	*70*
35.0	*30*	*70*

Mass spectrometry: A SCIEX X500B QTOF System was used for data acquisition. Data were acquired using TOF MS mode with the intact protein mode turned on. Detailed MS parameters are listed in Table 2.

Table 2. MS parameters for intact and subunit analysis.

Parameter	Setting
Scan mode	*TOF MS positive*
Ionspray voltage	*5000 V*
Gas 1	*50 psi*
Gas 2	*50 psi*
Curtain gas	*30 psi / 35psi (IgdE reduced)*
Ion source temperature	*300 °C / 450 °C (IgdE reduced)*
Time bins to sum	*80*
Accumulation time	*0.5 sec*
*Mass range**	*600 – 5,000 m/z*
Declustering potential	*300 V(intact)/ 150 V (subunit)*
CAD gas	*7*
Collision energy	*10 V*

**adjusted for different sized mAb fragments*

Data processing: Data were processed using Byos v3.9 software from Protein Metrics which is directly compatible with the raw data format .wiff2 derived from SCIEX X500 QTOF Systems.

Intact analysis of the trispecific mAb

The multi-specific antibody consists of four different chains, LC1, LC2, HC1 and HC2 (Figure 2). Both LC1 and HC1 were bioengineered with an additional antigen binding domain, which increases the protein's heterogeneity. The multi-specific antibody contains three consensus sequences with N-linked glycosylations (two on the heavy chains and one on the LC2), which increases the complexity further. In addition, the HC1 contains a sulfation site (indicated by the asterisk in Figure 2).

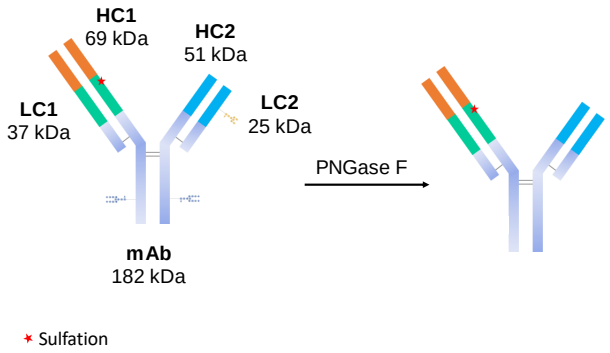

Figure 2. Sample preparation for intact mass analysis. The trispecific mAb was analyzed with glycosylations and without glycosylations (PNGase F treated).

To fully evaluate molecular integrity and composition of the multi-specific antibody, a comprehensive characterization was performed. As a first step, the multi-specific antibody was analyzed as in both its glycosylated intact state and its deglycosylated intact state.

The raw data (Figure 3 A, B) were used for reconstruction (Figure 3 C, D) in Protein Metrics software and results were compared against the theoretical information (Table 3).

The observed mass of de-glycosylated antibody was 80 Da higher than the expected molecular weight based on the sequence, which suggests either a phosphorylation or a sulfation. Additional subunit (see next section), peptide mapping experiments[4] and binding-assays revealed that the antibody contains a sulfation[4] on the HC1, which is positively affecting the binding to the target.

Glycoforms could be assigned to the sample, which highlighted the increased complexity of the trispecific mAb (Figure 3D). Because different glycan combinations can result in the same mass shift observed on intact level, the assignment needs to be further verified with subunit analysis.

Table 3. Summary of (de)glycosylated intact mass analysis.

Proteoform	Experimental mass [Da]	Theoretical mass [Da]	Δ mass [Da]
Deglycosylated, sulfation	*176979.1*	*176979.1*	*0.0*
G0F/G0F/G2S, sulfation	*181780.6*	*181784.5*	*3.9*
G0F/G0F/G2S2, sulfation	*182072.6*	*182075.7*	*3.1*
G0F/G1F/G2S2, sulfation	*182234.6*	*182237.3*	*2.7*
G1F/G1F/G2S2, sulfation	*182396.6*	*182394.5*	*-2.1*

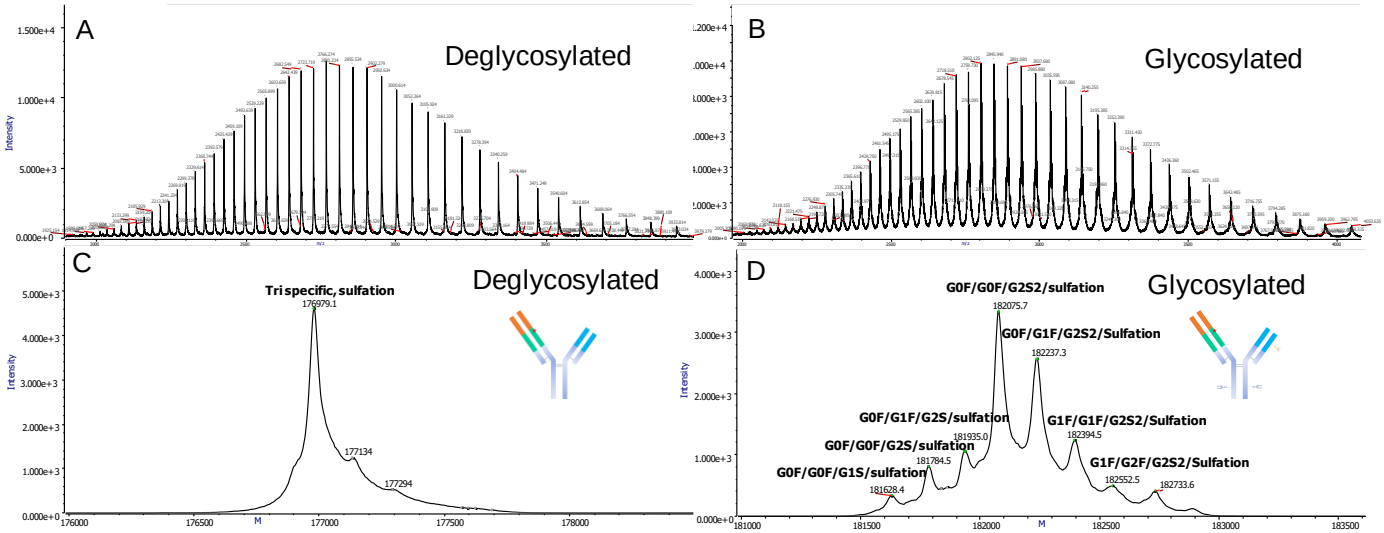

Figure 3. Raw and reconstructed spectra of the multi-specific antibody. Antibody treated with PNGase F for deglycosylation (A)(C); glycosylated antibody (B)(D).

Subunit analysis of the trispecific mAb using reduction

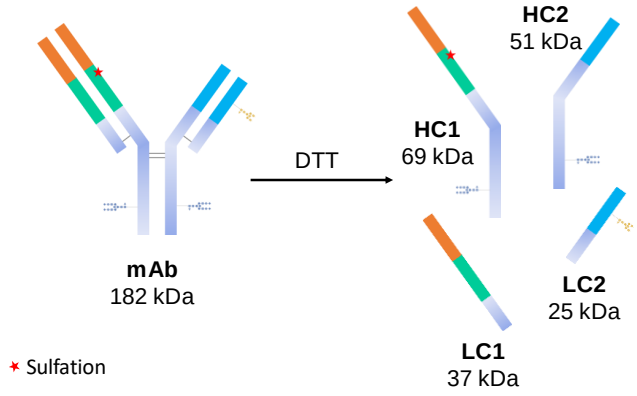

Figure 4. Sample preparation for subunit mass analysis via reduction. The trispecific mAb was analyzed with glycosylations and without glycosylations (PNGase F treated).

In order to obtain domain specific information, the mAb was reduced, resulting in four different chains of various molecular weights (Figure 4). The reconstructed data of the reduced sample with and without glycosylations are demonstrated in Figure 5. The observed masses of the deglycosylated sample were consistent with theoretical molecular weight for LC1, LC2 and HC2, while HC1 showed a mass shift of +80 Da, which is consistent with the observation during the intact protein analysis (Table 4). These results confirm that the modification is located at the HC1 chain. The most abundant glycoforms on both HC1 and HC2 were found to be G0F, G1F and G2F. Trace levels of G0 and G0F-GlcNAc were detected in HC2 (Figure 5F). The analysis of the third N-linked glycosylation site located on LC2 revealed G2S2, G2S1 and G2F+2NeuAc as the major glycan species. The high mass accuracy for all of the assigned glycoforms suggests high confidence in the identification (Table 4).

The trace levels of certain glycoforms on the HC2 could not be identified directly on intact level due to the overall size and complexity of the molecule, but could be matched with excellent mass accuracy applying a subunit approach (Figure 5F, Table 4). Peptide mapping analysis and/or released glycan analysis via orthogonal methods can provide further details, however, the information about which part of the molecule contains certain glycoforms might be lost. Subunit analysis can close this gap with minimal sample preparation and fast turn-around times.

Table 4. Summary of (de)glycosylated, reduced subunit analysis.

Proteoform	Experimental mass [Da]	Theoretical mass [Da]	Δ mass [Da]
Deglycosylated			
LC2	*23082.0*	*23082.5*	*0.5*
LC1	*36832.0*	*36832.6*	*0.6*
HC2	*49503.0*	*49502.8*	*-0.2*
HC2, sulfation	*67601.0*	*67600.4*	*-0.6*
Glycosylated			
LC1	*36832.0*	*36832.6*	*0.6*
LC2, G2S	*24995.5*	*24996.2*	*0.7*
LC2, G2S2	*25287.0*	*25287.5*	*0.5*
HC2, G0	*50801.0*	*50801.1*	*0.1*
HC2, G0F	*50947.0*	*50947.2*	*0.2*
HC2, G1F	*51108.4*	*51109.4*	*1.0*
HC2, G2F	*51271.1*	*51271.5*	*0.4*
HC1, G0F, sulfation	*69046.9*	*69045.4*	*-1.5*
HC1, G1F, sulfation	*69208.0*	*69207.4*	*-0.6*

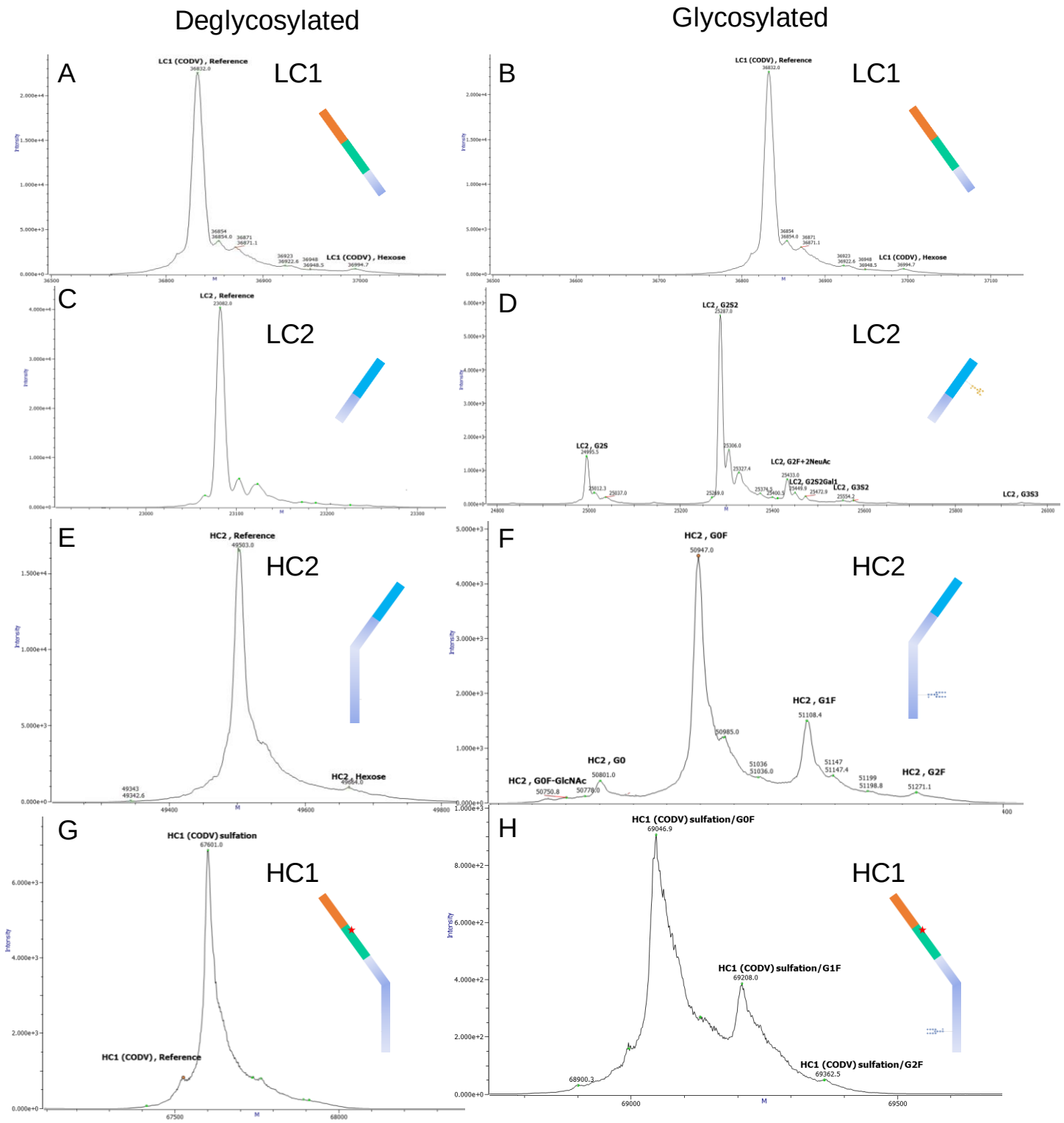

Figure 5. Reconstructed spectra of the reduced multi-specific antibody. Deglycosylated subunit data shown on the left hand side. A: deglycosylated LC1, C: deglycosylated LC2, E: deglycosylated HC2, G: deglycosylated HC1. Glycosylated subunit data shown on the right hand side: B: glycosylated LC1, D: glycosylated LC2, F: glycosylated HC2, H: glycosylated HC1.

Subunit analysis of the trispecific mAb using site-specific enzymatic digestion

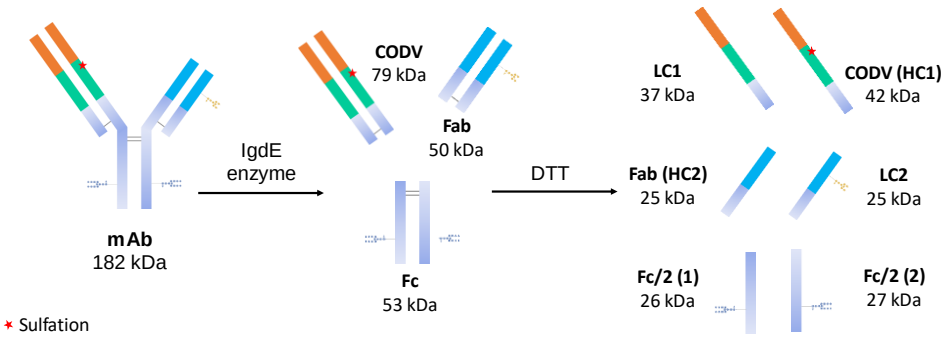

Figure 6. Sample preparation for subunit mass analysis using site-specific enzymatic cleavage and reduction. The trispecific mAb was analyzed with glycosylations attached.

Besides reduction, IgdE enzyme was used to cleave above the hinge region, resulting in three subunits Fab, CODV and Fc (Figure 6, middle). For the Fab subunit, multiple bi-, tri-antennary sialylated glycans were observed including, G1S, G2S, G2S2, G2F+NeuAc G3S2 and G3S3 being in alignment with the reduced subunit analysis (Figure 7A, Figure 5D).

As expected, the sulfated form was the dominant species on Fab (CODV) part, while G0F/G0F and G0F/G1F were the dominant glycoforms of the Fc region, which is consistent with the results from reduced subunit analysis (Figure 5F).

Further, the IgdE digested sample was reduced, resulting in six different subunits (Figure 6, right). Since chromatographic baseline separation of the complex mixture of subunits could not be achieved (Figure 8A), the data were processed using custom slices in the Byos software (Figure 8B). With this approach user-defined chromatographic peak integration can be performed, allowing for accurate identification of each subunit while minimizing interferences (Figure 9). For the LC2 eight N-linked glycans were detected and all of them are sialylated, which is consistent with previous light chain analysis after reduction. LC1, Fab (HC2) and CODV (HC1) were detected at MW being consistent with the theoretical mass, assuming a sulfation in case of the CODV (HC1) (Figure 9C, Table 5). As observed previously, the Fc/2 (1) and Fc/2 (2) contained G0F and G1F as the predominant glycoforms.

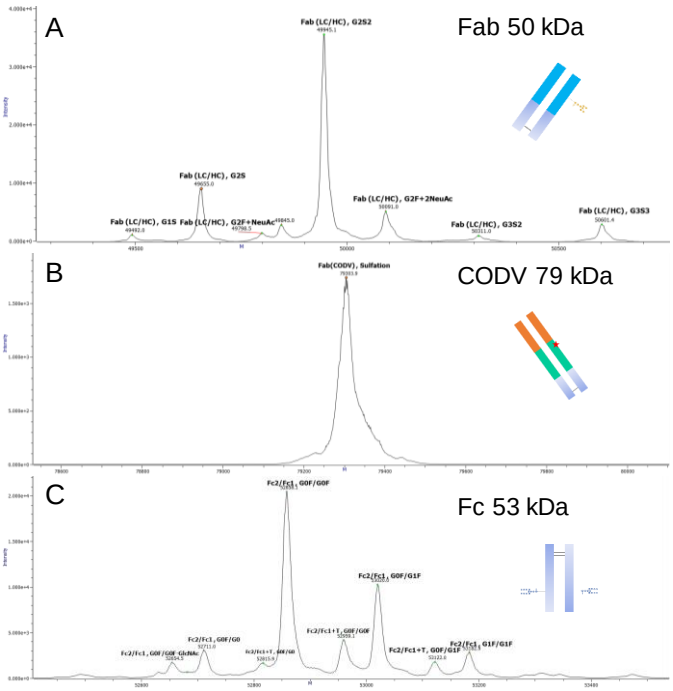

Figure 7. Reconstructed spectra of the IgdE digested multi-specific antibody. (A) Fab (B) Fab (CODV) (C) Fc

In summary, this assay provided information on the integrity of the trispecific mAb and furthermore revealed domain-specific information including information on different modifications using a combination of intact and different subunit approaches. These different approaches provided rich glycosylation data on top of the tertiary confirmation. Furthermore, the sequence can be confirmed by the reconstructed masses matching the theoretically calculated masses. However, a peptide mapping analysis is recommended for full confidence in the correct amino acid sequence via MS/MS data.[4] It is important to note that intact and subunit experiments in combination provided information on the tertiary assembly that otherwise would have been lost in a peptide-level analysis. In addition, a tremendous amount of time was saved by applying intact and subunit analysis while reducing potential artifacts due to limited sample preparation needed by working exclusively at the intact or subunit level.

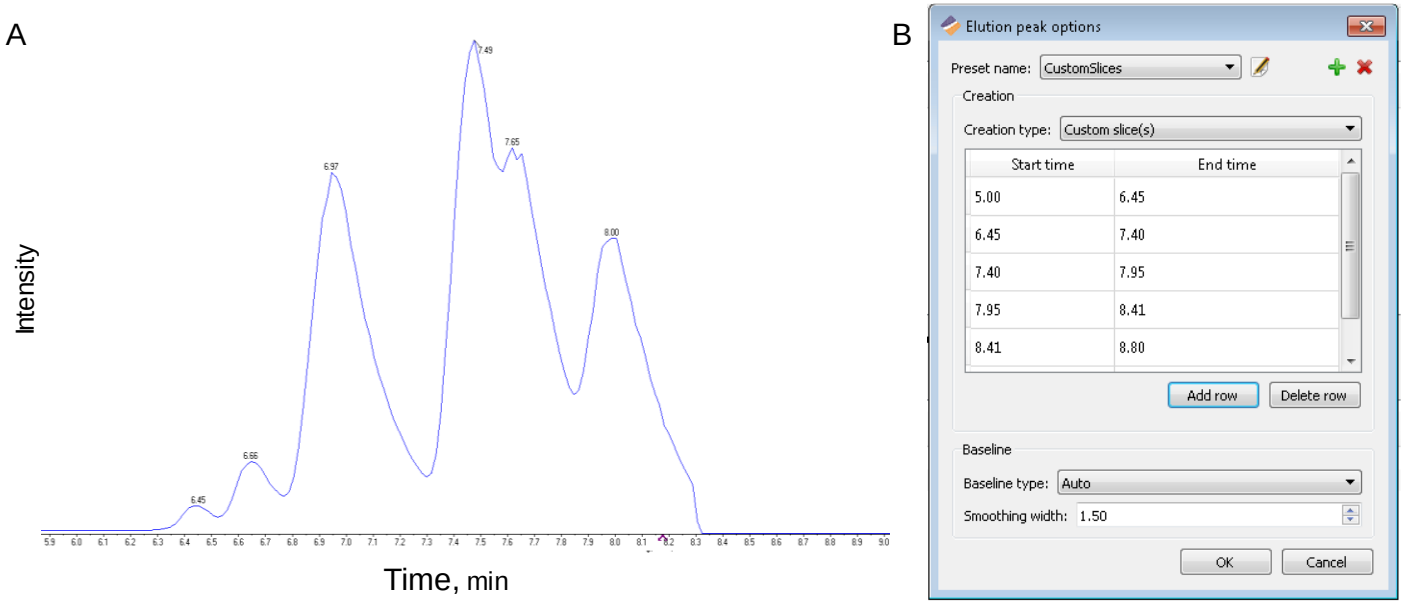

Figure 8. Reduced, IgdE digested mAb. (A) Total ion chromatogram. (B) Setup for using customized slices in Byos software. The retention time can be entered manually to allow for raw data visualization and reconstruction for each subunit individually without the need for chromatographic baseline separation.

Table 5. Summary of IgdE digested subunit analysis.

Proteoform	Experimental mass [Da]	Theoretical mass [Da]	Δ mass [Da]
CODV, sulfation	*79303.9*	*79304.7*	*0.8*
Fab, G1S	*49492.0*	*49491.8*	*-0.2*
Fab, G2S	*49655.0*	*49653.9*	*-1.1*
Fab, G2F+NeuAc	*49798.5*	*49800.2*	*1.8*
Fab, G2S2	*49945.1*	*49945.2*	*0.2*
Fab, G2F+2NeuAc	*50091.0*	*50091.4*	*0.4*
Fab, G3S2	*50311.0*	*50310.3*	*-0.7*
Fab, G3S3	*50601.4*	*50601.4*	*0.0*
Fc2, G0F/G0F-GlcNac	*52654.5*	*52655.0*	*0.5*
Fc2, G0F/G0	*52711.0*	*52712.0*	*1.0*
Fc2, G0F/G0F	*52858.1*	*52858.1*	*0.0*
Fc2, G0F/G0	*52815.9*	*52814.1*	*-1.8*
Fc2, G0F/G0F	*52959.1*	*52960.2*	*1.1*
Fc2, G0F/G1F	*53020.1*	*53020.3*	*0.2*
Fc2, G0F/G1F	*53122.0*	*53122.4*	*0.4*
Fc2, G1F/G1F	*53182.9*	*53182.4*	*-0.6*

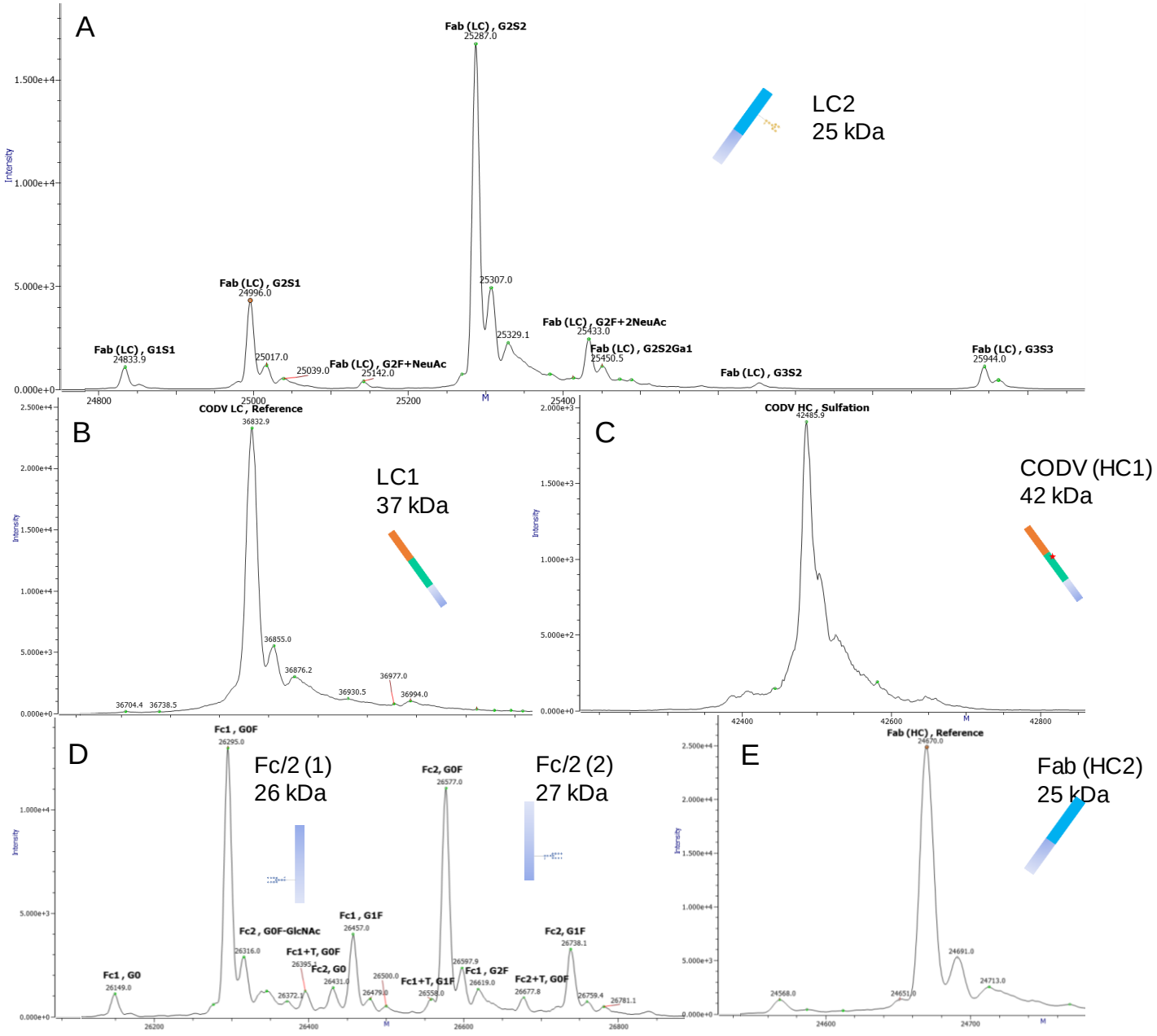

Figure 9. Reconstructed spectra of the reduced IgdE digested multi-specific antibody. (A) Fab LC (B) CODV LC (C) CODV HC (D) Fc/2 (1) and Fc/2 (2) (E) Fab (HC).

Table 6. Summary of IgdE digested and reduced subunit analysis.

Proteoform	Experimental mass [Da]	Theoretical mass [Da]	Δ mass [Da]
LC1	*36832.9*	*36832.6*	*-0.3*
LC2, G1S1	*24834.0*	*24834.1*	*0.2*
LC2, G2S1	*24996.0*	*24996.2*	*0.2*
LC2, G2F+NeuAc	*25142.0*	*25142.4*	*0.4*
LC2, G2S2	*25287.1*	*25287.5*	*0.5*
LC2, G2F+2NeuAc	*25433.0*	*25433.5*	*0.5*
LC2, G2S2Ga1	*25450.5*	*25449.6*	*-0.9*
LC2, G3S3	*25944.0*	*25943.8*	*-0.2*
Fab (HC2)	*24670.0*	*24669.8*	*-0.2*
Fc/2 (1), G0	*26149.0*	*26149.2*	*0.2*
Fc/2 (1), G0F	*26295.0*	*26295.3*	*0.3*
Fc/2+T (1), G0F	*26395.1*	*26396.3*	*1.2*
Fc/2 (1), G1F	*26457.0*	*26457.5*	*0.5*
Fc/2+T (1), G1F	*26558.0*	*26558.5*	*0.5*
Fc/2 (1), G2F	*26619.0*	*26619.6*	*0.6*
Fc/2 (2), G0	*26431.0*	*26430.5*	*-0.5*
Fc/2 (2), G0F	*26577.0*	*26576.6*	*-0.4*
Fc/2+T (2), G0F	*26677.8*	*26678.7*	*0.9*
Fc/2 (2), G1F	*26738.1*	*26738.7*	*0.6*
CODV (HC1), sulfation	*42486.0*	*42486.0*	*0.0*

Conclusions

- A complete workflow for the characterization of a complex new modality in a streamlined manner using the X500B QTOF System with Protein Metrics software solutions was shown
- Ease-of-use combined with excellent data quality on the X500B QTOF System offers a generic workflow platform for the characterization of complex next generation biotherapeutics
- Accurate reconstruction and software flexibility within the Byos software facilitated the comprehensive and time-efficient analysis of these complex therapeutics

References

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