

*Data article***Title: Glycosylation characterization of therapeutic mAbs by top- and middle-down mass spectrometry**

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**Abstract**

A reference monoclonal antibody IgG1 and a fusion IgG protein were analyzed by top- and middle-down mass spectrometry with multiple fragmentation techniques including electron transfer dissociation (ETD) and matrix-assisted laser desorption ionization in-source decay (MALDI-ISD) to investigate heterogeneity of glycosylated protein species. Specifically, glycan structure, sites, relative abundance levels, and termini structural conformation were investigated by use of Fourier transform ion cyclotron resonance (FT-ICR) or high performance liquid chromatography electrospray ionization (HPLC-ESI) linked to an Orbitrap. Incorporating a limited enzymatic digestion by immunoglobulin G-degrading enzyme *Streptococcus pyogenes* (IdeS) with MALDI-ISD analysis extended sequence coverage of the internal region of the proteins without pre-fractionation. The data in this article is associated with the research article published in *Journal of Proteomics* [1].

**Specifications Table**

Subject area	Biology, Chemistry
More specific subject area	Top- and middle-down mass spectrometric analysis of protein species and proteoforms
Type of data	Table, Figure
How data was acquired	Mass spectrometry data were collected on a 12T FT-ICR XR (Bruker) and a LTQ-Orbitrap Elite (Thermo Scientific).

Data format	MS spectra
Experimental factors	Top- and middle-down analyses with multiple fragmentation techniques including electron transfer dissociation (ETD) and matrix-assisted laser desorption ionization in-source decay (MALDI-ISD) for characterization of a reference monoclonal antibody IgG1 and a fusion IgG protein.
Experimental features	Glycan profiling including structure, sites, relative abundance levels, and termini structural conformation were investigated
Data source location	Baltimore and Gaithersburg, Maryland, USA
Data accessibility	Data is provided within this article.

### Value of the data

- Application of top-down and middle-down mass spectrometry for characterization of comprehensive glycosylated protein species from recombinant IgG and IgG-fusion proteins.
- Detection of glycan structure, sites, and relative abundance levels.
- Investigation of termini structural conformation; N-terminal glutamine (Q) to pyroglutamate (pyroGlu, aka. pE) conversion and C-terminal lysine truncation.
- Improvement on protein sequencing by MALDI ISD, ETD in combination with middle-down analysis by use of an enzyme, IdeS.

### Data

Glycosylation profiling of recombinant IgG and IgG-fusion proteins and sequence coverage information.

### Experimental Design, Materials and Methods

The recombinant human mAb (IgG1) and IgG1-fusion protein were manufactured using standard cell culture, purification and formulation processes [2]. The IgG1 has two identical light chains and two identical heavy chains with molecular weight of 150KDa and N-glycosylation site at N297. The IgG fusion protein, approximately 90kDa, was comprised of two chains each with a human protein domain fused onto the N-terminus of a human IgG1 constant domain (Fc). The protein has three N-glycosylation sites at N76, N108 and N207.

#### 1. Analysis of the IgG protein

The IgG protein was analyzed 1) in reduced form by MALDI-ISD in a 12 Tesla Fourier Transform Ion Cyclotron Resonance (FT-ICR XR) mass spectrometer (Bruker, Bremen, Germany), and 2) after IdeS digestion and dithiothreitol (DTT) reduction to generate three

smaller fragments; light chain, Fab, and Fc/2 after which analyzed by LC-MS/MS on an Orbitrap Elite (Thermo Scientific, Saint Jose, CA, USA).

### **1.1. MALDI-MS analysis of reduced protein**

The IgG protein in 1 µg/µL in 50mM ammonium bicarbonate buffer was reduced to light and heavy chain by 20mM dithiothreitol (DTT, product #43815, Sigma-Aldrich, St. Louis, MO, USA) at 80°C for 15min. The reduced protein sample was buffer exchanged to 5% acetonitrile (ACN) /0.1% formic acid (FA) using a 10kDa molecular weight cut off (MWCO) centrifugal filter (product # UFC201024, Merck Millipore, Tullagreen, Carrigtwohill, Ireland) and then diluted to 17 µg/µL in 50% ACN/0.1% FA. One µL sample and 1 µL of 1,5-diaminonaphthalene (1,5-DAN, product # 56451, Fluka, St. Louis, MO, USA) saturated in ACN were spotted on a stainless steel MALDI plate. MALDI-MS experiment was performed with a transient domain of 1 mega-word data points (estimated resolving power of 270,000 at m/z 400) covering m/z range 400-10,000. MALDI-MS mass spectra after phase correction [3] were processed to identify c- and z+2 ions from light chain and heavy chain with mass tolerance 50 ppm using DataAnalysis 4.2 and BiTools software 3.2 (Bruker) (Figure 1).

### **1.2. LC-MS/MS analysis of IdeS-digested protein**

The IgG protein was digested with IdeS (product # A0-FR1-020, Genovis AB, Lund, Sweden) at a ratio of 1 unit of enzyme per 1 µg protein for 2 hours at 37°C, followed by DTT treatment. Approximately 14 µg of the IdeS/DTT treated sample was separated on a 100 µm x 20 mm column packed with Jupiter 5 µm C5 particles (product # 04A-4052, Phenomenex, Torrance, CA, USA) by a fast mobile gradient of 5% to 50% ACN/0.1% FA in 15 min at a flow rate of 0.3 µL/min. Light chain and Fd domain were detected in a range of 350-3500 m/z at resolving power of 120,000 in positive ion mode on the Orbitrap Elite (Figure 2). Precursor masses were selected for ETD fragmentation. ETD spectra were acquired with 5 microscans at resolving power of 120,000 and processed by MASH Suite version 1.0 software [4]. The fragment ions and corresponding sequence coverage are shown in Figure 3A for the light chain and Figure 3B for the Fd domain. Glutamine (Q) to pyroglutamate (pyroGlu) conversion was confirmed on the N-terminal of heavy chain (Figure 3B).

## **2. Analysis of the IgG fusion protein**

The fusion protein was analyzed 1) by accurate mass measured in FT-ICR for protein species detection 2) by MALDI-MS following DTT reduction in FT-ICR, and 3) by accurate mass measurement following IdeS cleavage in FT-ICR or ETD MS/MS in the Orbitrap Elite.

### **2.1 Accurate mass measurement of intact protein**

The fusion protein sample was buffer exchanged to 5% ACN and 0.1% TFA using Amicon 50kDa MWCO centrifugal filters (product # UFC505024, Merck Millipore). Since the fusion protein sample was stored in buffer with detergent, detergent removal was performed by Pierce detergent

removal kit (product # 87778, Thermo Scientific, Rockford, CA, USA). The detergent-free protein sample was diluted to 10  $\mu\text{g}/\mu\text{L}$  final concentration in 50% ACN/0.1% FA and introduced to electrospray ionization (ESI)-FT-ICR for intact protein mass measurement for detection of protein species. The experiment was carried out in positive ion mode on the 12 T FT-ICR at flow rate 2  $\mu\text{L}/\text{min}$ . Mass spectra were collected in magnitude mode with a transient domain of 128,000 word data points to cover  $m/z$  range 400 – 4000, with an accumulation of 1650 scans. Figure 4 shows the protein species detected in  $m/z$  region 2365 – 2450 with high heterogeneity of glycosylation. Corresponding glycan nomenclature and structures are shown in Figure 5.

## 2.2 MALDI-ISD analysis of reduced protein

The protein sample was diluted to 1  $\mu\text{g}/\mu\text{L}$  in 50mM ammonium bicarbonate buffer and reduced with 20mM DTT at 80°C for 15min. The reduced sample was then detergent removed. The detergent-free protein was diluted in 50% ACN/0.1% FA at a final concentration of 7  $\mu\text{g}/\mu\text{L}$ . One  $\mu\text{L}$  of sample and 1  $\mu\text{L}$  of DAN matrix were spotted on stainless steel MALDI plate. MALDI-ISD mass spectra were recorded for  $m/z$  range 400-10,000 with a transient domain of 1 mega-word data points and estimated resolving power of 270,000 (at  $m/z$  400). MALDI-ISD spectra were phased corrected and processed using DataAnalysis and Biotoools software. Fragment ions were identified with mass tolerance 50 ppm and sequence coverage of the fusion protein is shown in Figure 6.

## 2.3 ETD MS/MS analysis of IdeS-digested protein

The fusion protein sample was diluted to 1  $\mu\text{g}/\mu\text{L}$  in 50mM ammonium bicarbonate solution and digested with enzyme IdeS at ratio of 1 enzyme unit to 1  $\mu\text{g}$  protein for 2 hours at 37°C. 20mM DDT was added to cleave disulfide bonds at 80°C for 15min. The sample was further concentrated using 10kDa MWCO filter and subject to detergent removal. The sample buffer was exchanged to 5% ACN/0.1% FA and diluted to final concentration of approximately 7  $\mu\text{g}/\mu\text{L}$  in 50 %ACN/0.1% FA. IdeS digestion and DTT reduction cut the fusion protein to half the size producing N-terminal and C-terminal fragments.

Accurate mass measurement of the IdeS/DTT treated sample was performed by ESI FT-ICR. Mass spectra were collected in magnitude mode with a transient domain of one mega-word data points to cover  $m/z$  range 400 – 4000. Seven glycoforms were identified from the C-terminus at multiple charge states. Table 1 represents glycosylated protein species detected from 17+ charge peaks and their glycan nomenclature and structures are shown in Figure 5.

Potential glycosylated protein species	Observed $m/z$	Charge (z)	Observed monoisotopic mass $[M+H]^+$	Theoretical monoisotopic mass $[M+H]^+$	Mass error between observed and theoretical	
					(Da)	(ppm)
<b>G0</b>	1479.64124	17+	25137.77353	25139.397044	-1.62	-64.58

<b>G0f</b>	1488.28997	17+	25284.83767	25282.454953	2.38	94.24
<b>G1f</b>	1497.76750	17+	25445.88614	25444.507776	1.38	54.17
<b>G2f</b>	1507.29477	17+	25607.90766	25606.560599	1.35	52.61
G1f+NANA	1514.88175	17+	25736.94787	25735.063193	1.88	73.23
G2f+NANA	1524.41738	17+	25899.02107	25897.656016	1.37	52.71
G2f+NANA2	1541.54188	17+	26190.11349	26188.751433	1.36	52.01

Table 1. Glycosylated protein species detected by Fourier transform ion cyclotron resonance (FT-ICR) accurate mass measurement of DTT/IdeS treated fusion protein from the 17+ charge state. Highlighted in bold are the most abundance glycoforms detected on the C-terminal fragment of the protein. Additional information regarding the glycan nomenclature and structure are provided in the Figure 6.

The sample was also directly infused into the Orbitrap Elite. Data were collected in positive ion mode for 350-3500 m/z range at resolving power of 120,000 (Figure 7). Precursor masses of the glycoforms were isolated for ETD fragmentation. ETD mass spectra were acquired with 5 microscans at resolving power of 120,000 and processed by MASH Suite for sequence coverage analysis. Manual interpretation of the ETD spectra confirmed that glycosylation by G0f or G1f occurred on a site N207 and the protein has a C-terminal lysine truncation (Figure 8).

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