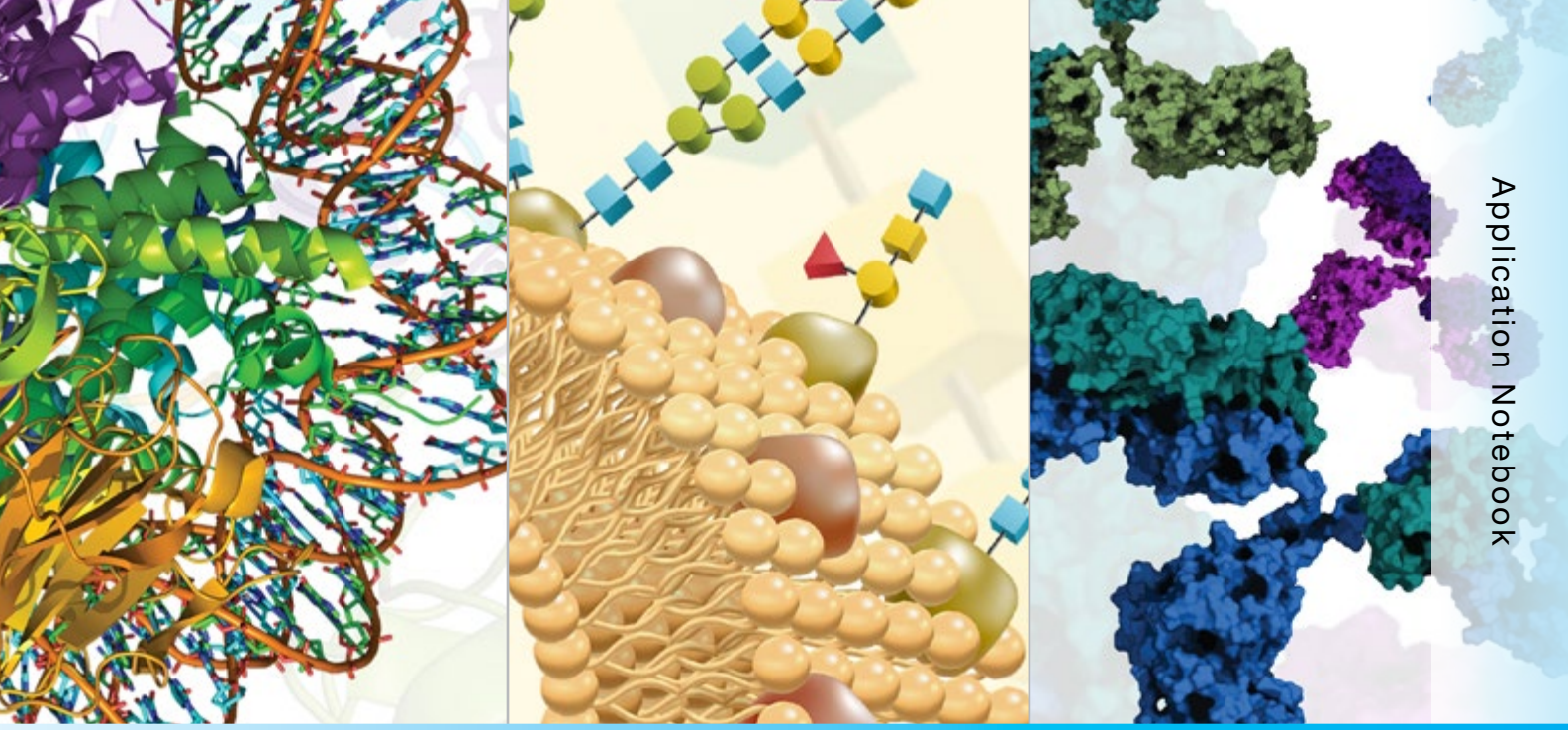

Application Notebook

Biopharmaceutical Characterization Application Compendium

- Native and Intact Protein Characterization
- Glycan and Glycopeptide Analysis
- Peptide Mapping and Analysis

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Biopharmaceutical Characterization Application Compendium

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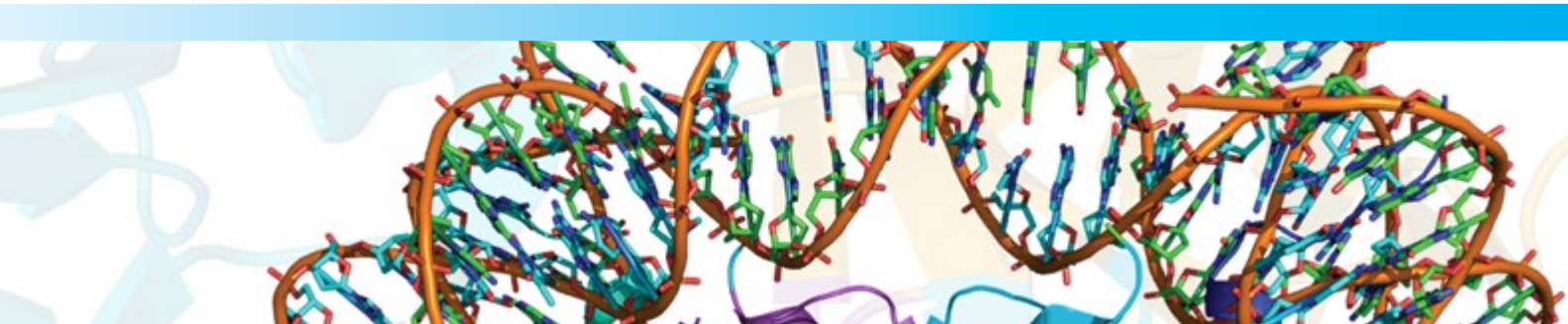
Native and Intact Protein Characterization

Biotherapeutic compounds, such as proteins and monoclonal antibodies (mAbs), as well as conjugates, complexes and assemblies based on them, make up the vast majority of the rapidly growing biologics drug market. Full characterization of these compounds by mass spectrometry includes determination of protein sequences, and identification and relative quantitation of protein isoforms, including identification and localization of one or multiple post-translational modifications (PTMs). Traditional workflows for such analyses use a "bottom-up" approach, where proteins are digested into their peptide counterparts. However, complete sequence coverage is rarely attainable, and qualitative and quantitative information about protein isoforms, including those resulting from post-translational modifications, is usually lost. These same research endeavors would, in many cases, also benefit from intact and native mass spectrometric analyses.

Intact and native protein characterization by mass spectrometry (MS) has emerged as a valuable technology that has numerous advantages over bottom up sequencing. Intact and native MS with high-resolution, accurate mass (HRAM) technologies provide accurate information on various protein properties, such as intact molecular mass, glycosylation forms, amino acid sequence, post-translational modifications, and minor impurities due to sample processing and storage, as well as higher-order structural information, such as protein conformational changes upon modifications, noncovalent interactions between protein drugs and receptor proteins, and protein aggregation caused by misfolding. Mass analysis at the intact protein level is usually the first step of the structural characterization.

High-resolution mass spectrometry is essential for resolving co-eluting intact proteins as well as isotopic peaks of highly charged proteins for charge state determination and accurate mass determination. Additionally, due to the number of product ions generated during fragmentation of intact proteins, high-resolution mass analysis is required for accurate detection and assignment of product ions in the resultant complex MS/MS spectra.

The high-resolution accurate mass of the Thermo Scientific™ Orbitrap™ family of mass spectrometers, including the extended m/z range capability of selected models, enables novel native and intact workflows. Orbitrap-based mass spectrometers have the potential to become essential tools for routine intact and native biopharmaceutical analysis as demonstrated by a variety of examples below.



LC/MS Analysis of the Monoclonal Antibody Rituximab Using the Q Exactive Benchtop Orbitrap Mass Spectrometer

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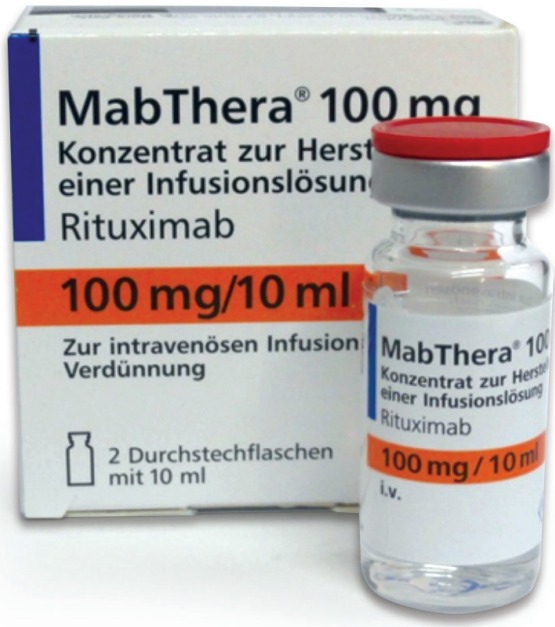
Key Words
Monoclonal antibody, intact protein mass measurement, sequence confirmation, protein deconvolution, top-down sequencing

Goal
Analysis and characterization of a monoclonal antibody using an optimized LC/MS workflow based on monolithic columns coupled online with the Thermo Scientific™ Q Exactive™ benchtop Orbitrap™ mass spectrometer.



Introduction
Monoclonal antibodies (mAbs) are one of the fastest growing classes of pharmaceutical products. They play a major role in the treatment of a variety of conditions such as cancer, infectious diseases, allergies, inflammation, and auto-immune diseases. Because mAbs can exhibit significant heterogeneity, extensive analytical characterization is required to obtain approval for a new mAb as a therapeutic product. Mass spectrometry has become an essential tool in the characterization of mAbs, providing molecular weight determinations of intact proteins as well as separated light and heavy chains, elucidation of glycosylation and glycan structures, confirmation of correct amino acid sequences, and identification of impurities such as host cell proteins (HCP) inherent to the production process.

Rituximab, which is known under the trade names Rituxan® (Biogen Idec/Genentech) in the United States and MabThera® (Roche) in Europe, is a recombinantly produced, monoclonal chimeric antibody against the protein CD20. It was one of the first new generation drugs in cancer immune therapy. Rituximab was approved by the U.S. Food and Drug Administration in 1997 and by the European Commission in 1998 for cancer therapy of malignant lymphomas. The variable domain of the antibody targets the cell surface molecule CD20, that can be found in some non-Hodgkin lymphomas.

In this application note, the capabilities and performance of the Q Exactive benchtop Orbitrap mass spectrometer in analyzing the intact and reduced forms of rituximab are demonstrated as well as sequence confirmation analyses using a combined top-down and bottom-up approach.

Furthermore, the sensitivity of two chromatographic setups using monolithic columns coupled online to the mass spectrometer is evaluated. The data obtained demonstrate superior resolution and mass accuracy of the Q Exactive mass spectrometer and present it as a high-confidence screening tool for accelerated and accurate biopharmaceutical product development and characterization.

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Experimental

Sample Preparation

The commercially available monoclonal antibody rituximab was used in all experiments. Rituximab is a sterile, clear, colorless, preservative-free, concentrated solution for intravenous infusion. It was supplied at a concentration of 10 mg/mL, formulated in 7.35 mg/mL sodium citrate buffer containing 0.7 mg/mL polysorbate 80, 9.0 mg/mL sodium chloride, and sterile water, and ready for injection. The pH was adjusted to 6.5 with sodium hydroxide or hydrochloric acid.

Prior to LC/MS analysis, rituximab was dialyzed due to polysorbate 80 in the sample. The dialysis was performed with a Thermo Scientific™ Slide-A-Lyzer™ dialysis cassette with a molecular weight cut off (MWCO) of 3.5 kDa. A 1 mL sample of rituximab was dialyzed for 48 h against 2 L of 20% aqueous acetonitrile (ACN) at 4 °C.

For analysis of the light and heavy chains of rituximab, disulfide bonds were reduced by incubation for 30 min at 60 °C with 5 mM tris(2 carboxyethyl)phosphine (TCEP).

For the bottom-up analysis of digested mAb, the sample was alkylated with 20 mM iodoacetamide (IAA) for 30 min at room temperature in the dark after the reduction step. The sample was purified with Thermo Scientific™ Pierce™ C18 tips dried in a Thermo Scientific™ SpeedVac™ concentrator and dissolved in 0.5 M triethylammonium bicarbonate buffer (TEAB). Sequencing grade modified trypsin (Promega) was added twice in a total ratio of 1:15 (w/w) at 0 h and 1.5 h and digestion was allowed to proceed for 2.5 h at 37 °C. The digest was stopped by addition of triflouroacetic acid (TFA) to approximately pH 3.

All samples were supplied in autosampler vials containing glass inserts (micro-inserts 0.1 ml, clear glass, VWR).

Liquid Chromatography

A monolithic 160 x 0.20 mm i.d. poly(styrene-divinylbenzene) copolymer (PS-DVB) capillary column, prepared according to a previously published protocol[1], and a Thermo Scientific™ PepSwift™ monolithic 250 x 0.20 mm i.d PS-DVB capillary column were used. Protein separations were performed with a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano system that included a detector equipped with a 3 nL z-shaped capillary detection cell.

Separations were accomplished at 55 °C with a gradient of 20–60% acetonitrile (ACN) in 0.050% aqueous triflouroacetic acid (TFA) in 10 min at a flow rate of 1 µL/min. For the proteolytic digest with trypsin, the gradient was adapted to run at 0–50% B in 30 min. For the reduced antibody samples, a gradient from 35–45% B in 15 min was selected.

Protein separation in a higher scale was performed using a Thermo Scientific™ ProSwift™ RP-10R monolithic 50 mm x 1.0 mm i.d. column with an UltiMate 3000 RSLCnano system that included a 45 nL detection cell. The column was run with a flow rate of 60 µL/min and a column temperature set to 55 °C. The gradient used was 26–80% B in 20 min. For the reduced antibody, a gradient of 26–56% B in 20 min was chosen to separate the heavy and the light chain.

The recorded back pressure of the monolithic columns for the gradients described above was in the range of 190 to 260 bar for the PepSwift 250 mm x 0.2 mm i.d. column and 120 to 180 bar for the ProSwift RP-10R 50 mm x 1 mm i.d. column.

For all experiments, the solvents used were water with 0.05% TFA (A) and acetonitrile with 0.05% TFA (B). The LC gradients are described in Tables 1 and 2.

Table 1. LC gradients used for experiments with the PepSwift 250 mm x 0.2 mm i.d. column, at a flow rate of 1 µL/min

Time [min]	Intact mAb [%B]	Time [min]	Reduced mAb [%B]	Time [min]	mAb Digest [%B]
0.0	20	0.0	35	0.0	0
10.0	60	15.0	45	30.0	50
10.1	85	15.1	85	30.1	85
16.0	85	21.0	85	40.0	85
16.1	20	21.1	35	40.1	0
30.0	20	30.0	35	50.0	0

Table 2. LC gradient used for experiments with the ProSwift RP-10R 50 mm x 1 mm i.d. column, at a flow rate of 60 µL/min

Time [min]	Intact mAb [%B]	Time [min]	Reduced mAb [%B]
0.0	26	0.0	26
15.0	80	15.0	56
20.0	80	15.1	80
20.1	26	20.0	80
30.0	26	20.1	26
		30.0	26

Mass Spectrometry

The Q Exactive benchtop Orbitrap mass spectrometer was used for all experiments in this study. Experiments using the ProSwift RP-10R 50 mm x 1 mm i.d. column were performed using the Thermo Scientific™ IonMax™ source with the heated electrospray ionization (HESI) sprayer, applying 4 kV spray voltage and sheath gas and auxiliary gas flow rates of 15 and 5 units, respectively.

All other experiments were performed using the Thermo Scientific™ NanoFlex™ ion source equipped with 15 cm PicoTip® emitter (New Objective, Woburn, USA; 20 µm i.d., 360 µm o.d., 10 µm tip), running with a flow rate of 1 µL/min. A source voltage of 1.5 kV was applied.

Method details are provided in Table 3.

	Intact Antibody	Reduced Antibody	Top Down AIF	5-plex MS/MS (Targeted MS^2)	Antibody Digest
Method type	Full MS	Full MS (2 segments)	Full MS-AIF	Targeted MS^2	Full MS-dd top 10 HCD
Total run time	30 min	0–15.8/15.8–30 min	25 min	25 min	40 min
Scan range *m/z*	1800–5000	800–3500/700–2500	300–2500	Fixed first mass 300	350–2000
Resolution (full MS/MS^2)	17,500/x	140,000/17,500	70,000	n.a./70,000	70,000/17,500
AGC Full MS	3.00×10^6	3.00×10^6	3.00×10^6	5.00×10^5	3.00×10^6 (MS)/1.00×10^5(MS^2)
Max inject time (Full MS/MS^2)	150 ms	150 ms/200 ms	150 ms	150 ms	100 ms/100 ms
Isolation window	n.a.	n.a.	n.a.	10 Th	2 Th
Microscans	10	5	5	5	1
Capillary temperature	275 °C	275 °C	275 °C	275 °C	275 °C
S-lens RF level	80	80	50	50	50
SID [eV]	80	0/60	n.a.	LC 0/HC 20	n.a.
NCE [%]	n.a.	n.a.	10 to 30	10 to 30	25

Source CID

The source CID (SID) parameter is a DC offset (0–100 eV) that is added to the source DC offset. The source DC offset consists of three voltages: capillary DC, S-lens DC, and S-lens exit lens. The application of this DC offset by setting the source CID parameter results in collisions of the analytes inside the injection flatapole with residual gas molecules present in the source region of the instrument.

All-Ion Fragmentation

All-ion fragmentation (AIF) is a fragmentation type in which all ions generated in the source are guided through the ion optics of the mass spectrometer, accumulated in the C-trap, and sent together to the higher-energy collisional dissociation (HCD) cell for fragmentation. In this case, the quadrupole is not set to select a particular precursor but operated in RF-only pass-through mode. For the analysis of intact proteins, this is a useful method since different charge states often show different fragmentation behavior and it is not easy to predict which one works best.

Data Analysis

Full MS spectra were deconvoluted using Thermo Scientific™ Protein Deconvolution™ software version 2.0. From the intact antibody and the intact heavy chain, the spectra acquired at a resolution setting of 17,500 were deconvoluted using the ReSpect™ algorithm. High resolution spectra from the intact light chain acquired at a resolution of 140,000 and top-down spectra acquired at 70,000 resolution were deconvoluted using the Xtract algorithm. To identify glycoforms of the intact antibody and the intact heavy chain obtained after reduction, the masses were compared to the expected masses with the various combinations of commonly found glycoforms.

The top-down HCD and AIF spectra were deconvoluted using the Xtract algorithm in the Thermo Scientific™ Qual Browser™ utility. Fragment ion assignment was performed using Thermo Scientific™ ProSightPC™ software version 3.0 in single protein mode with a fragment ion tolerance of 5 ppm.

The dataset obtained from the proteolytic digest was processed with Thermo Scientific™ Proteome Discoverer™ software version 1.4, using the SEQUEST® algorithm.

A three-protein-entry database was used consisting of the light chain, the heavy chain in two variants carrying either Ala or Val at position 219, and trypsin. Mass tolerances were set to 10 ppm for the precursor and 20 mmu for the fragment ions. Four variable modifications were considered: carbamidomethylation (Cys), oxidation (Met), deamidation (N, Q), Gln to pyro-Glu conversion, and N,N-dimethylation (Lys) (relevant for identification of trypsin autolysis products only).

Results and Discussion

Rituximab is an IgG1 class chimeric monoclonal antibody against the protein CD20, which consists of two light chains with 213 amino acids and two heavy chains with 451 amino acids each in length. The light and heavy chains are connected via 12 intrachain and 4 interchain disulfide linkages (Figure 1). The antibody is decorated with glycan structures attached to residue Asn^{301} of each of the two heavy chains. The composition and length of the attached glycans is quite diverse, resulting in a microheterogeneity of the molecule. The variety and relative abundance of the different glycostructures is essential for the efficiency of the antibody as a biological drug. The nomenclature of common glycans attached to antibodies are listed in Figure 2.

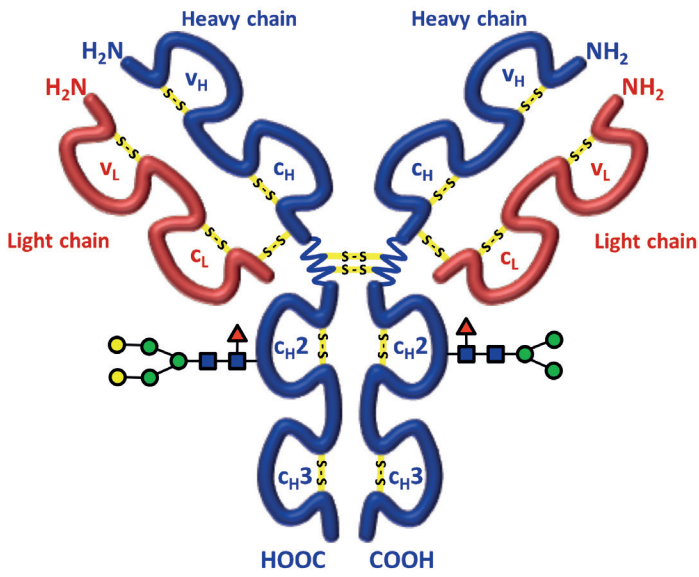


Figure 1. Schematic of molecular structure for the humanized IgG1 class monoclonal antibody rituximab

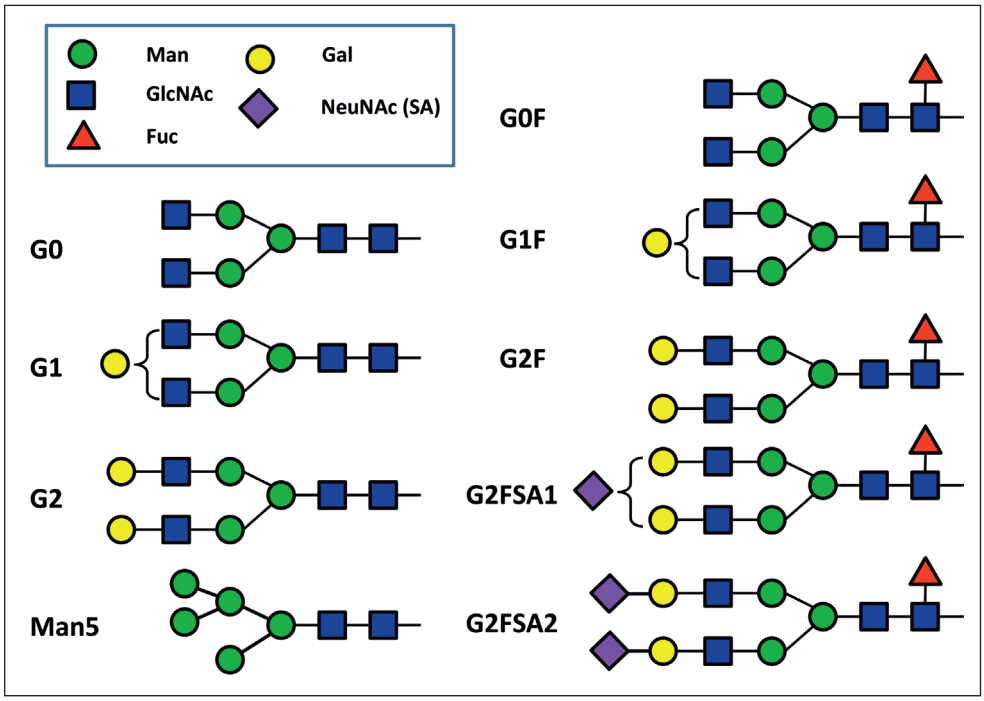


Figure 2. Nomenclature of carbohydrate structures commonly observed on antibodies

The full MS spectrum obtained from 20 ng rituximab applied to a 25 cm x 0.2 mm i.d. monolithic column is displayed in Figure 3. The mass spectrum, acquired over *m/z* 1800–5000 shows the typical charge distribution observed for large proteins. The most abundant charge state (z=+45) at *m/z* 3269, represented in the zoomed in insert, nicely pictures the four most abundant glycoforms of the intact antibody.

The intact mass of these four most abundant glycoforms and a series of less abundant glycoforms is obtained after the deconvolution of the full MS mass spectrum shown in Figure 4. The assignment of the peaks was based on the calculation of the proteins sequence, taking into account the various anticipated glycan structures shown in Figure 2.

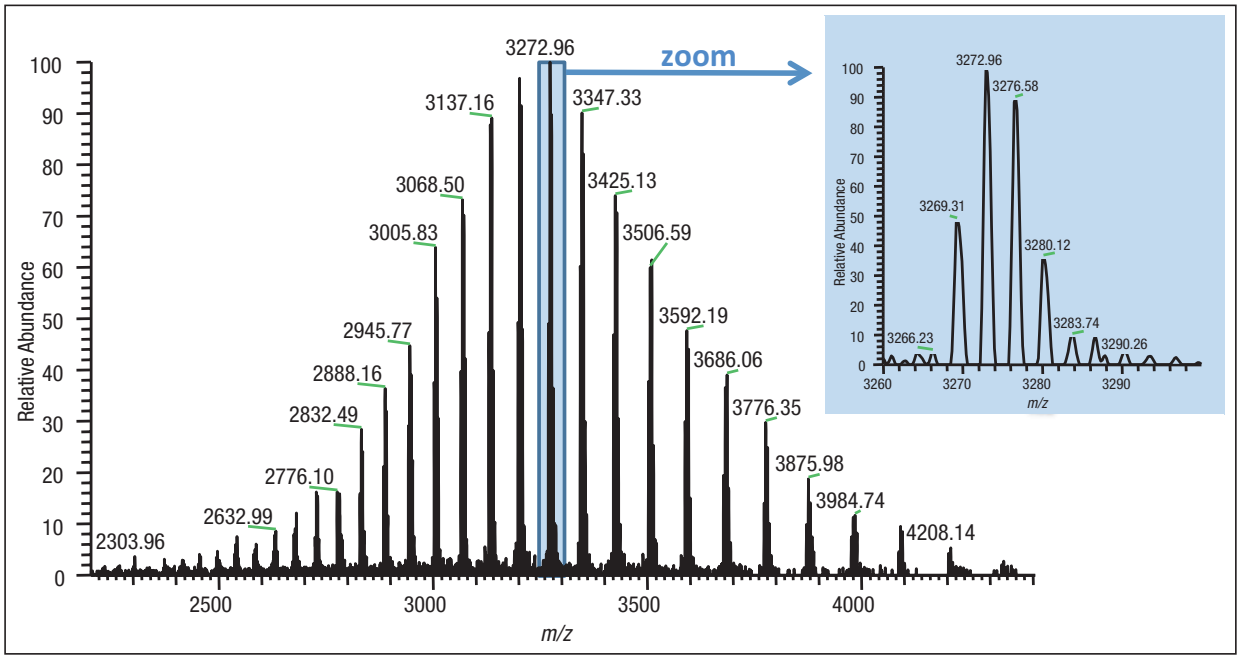
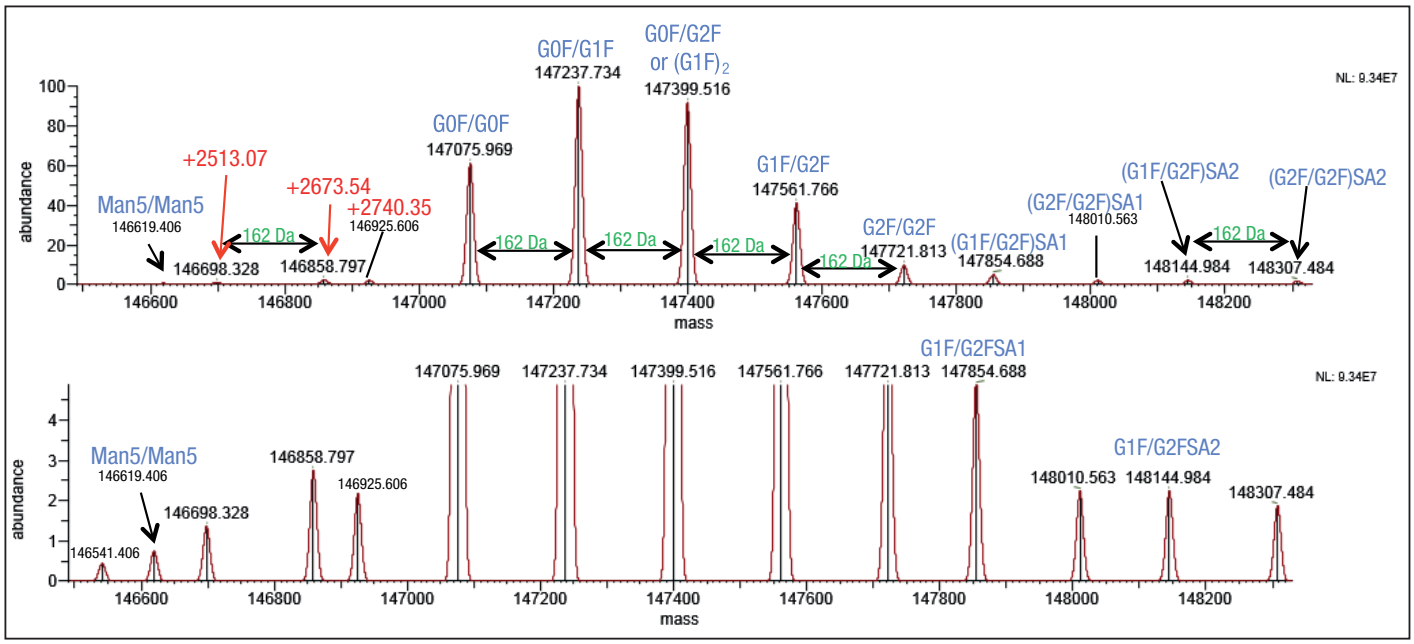


Figure 3. Single scan full MS spectrum (10 µscans) of rituximab, acquired from 10 ng sample loaded on a 250 x 0.2 mm i.d. column. The insert shows a zoomed in view of the most abundant charge state (z=+45). The observed peak pattern in the insert represents the different glycoforms of the molecule.



	G0F/G0F	G0F/G1F	G0F/G2F or $(G1F)_2$	G1F/G2F	G2F/G2F
Measured	147,075.969	147,237.734	147,399.516	147,561.766	147,721.813
Theoretical	147,074.985	147,237.126	147,399.267	147,561.408	147,723.549
Δ ppm	-6.7	-4.1	-1.7	-2.0	11.8

Figure 4. Deconvoluted mass spectrum of rituximab with annotated glycoforms (top) and comparison of theoretical and measured masses for the five most abundant glycoforms (table)

For the acquisition of the full MS spectrum of the intact antibody, the optimum setting of the source CID was evaluated (Figure 5). This setting was found to be crucial in obtaining a high quality spectrum. The application of 25–90% source CID is beneficial for most proteins. For this sample the optimum setting was 80% SID.

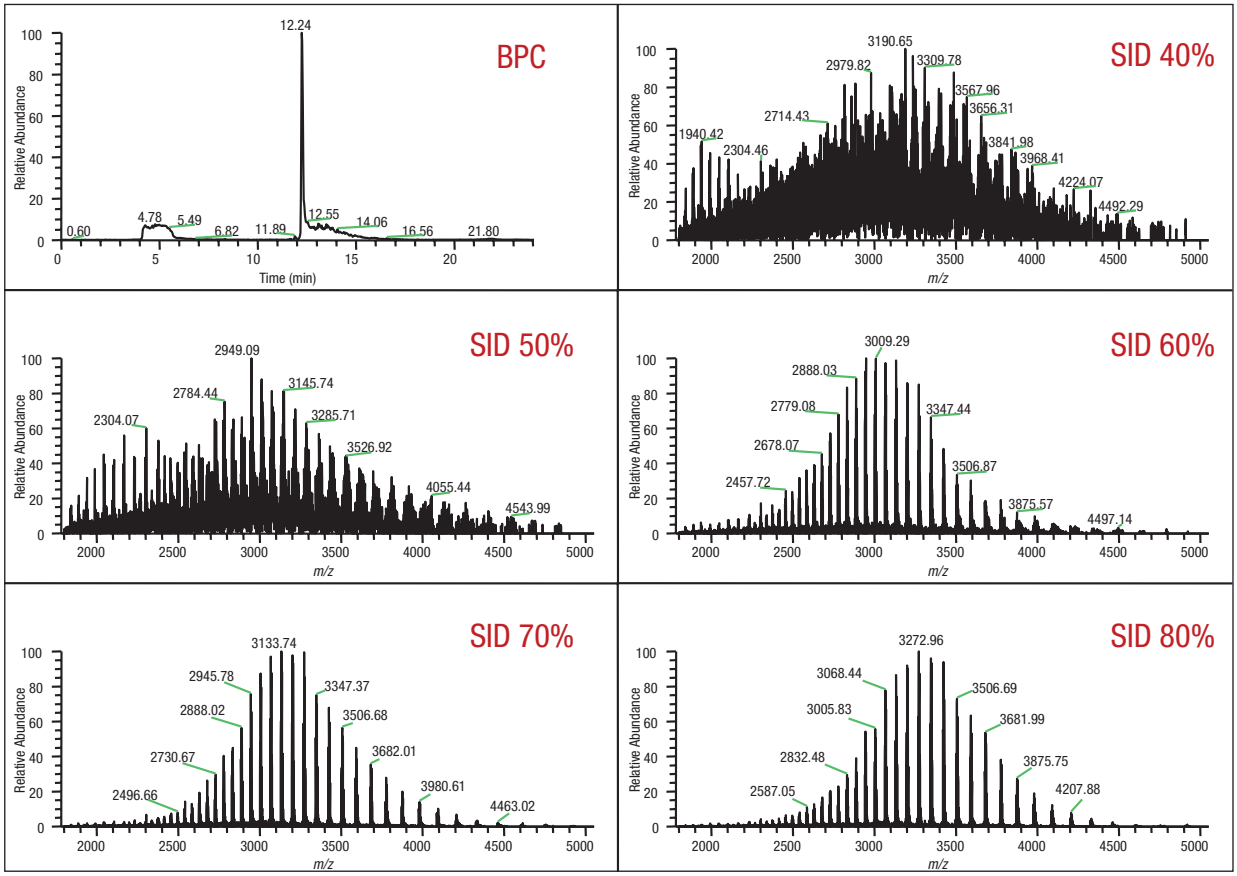


Figure 5. Full MS spectra acquired from 1 ng intact rituximab, applying increasing settings of source CID (SID)

The calculation of the masses for the light chain, unglycosylated heavy chain, and intact fully assembled antibody is presented in Table 4, showing the step-by-step calculation starting with the 213 respectively 451 amino acids of the light and heavy chain. Both protein sequences contain an N-terminal glutamine, which is anticipated to be modified to a pyro-glutamic acid, resulting in a deduction of mass of 17.0265 Da. Moreover, the C-terminal lysine present in the heavy chain is likely to be cleaved off, reducing the molecular weight by another 128.09497 Da. For assembling the intact antibody, a total of 16 disulfide linkages is considered by abstracting 32 protons. The glycan structures on each of the two heavy chains will add between 1217.1 and 2352.1 Da in mass. It has to be considered that the two chains can carry different glycans, resulting in a mixed composition, e.g. G01/G2F. Chemical composition and masses of individual carbohydrates are listed in Table 5. The monoisotopic and average atomic masses of the elements used to calculate molecular weights in Tables 4 and 5 are listed in Table 6.

Table 4. Chemical composition and step-by-step calculation of monoisotopic and average mass for the light and heavy chain, including their modifications as well as the intact antibody rituximab with various glycoforms. Detected masses shown in Figures 4, 6, and 7 are presented in the blue cells.

Elemental compositions	C	H	N	O	S	MW (monoisotopic)	MW (average)
Light chain (LC) full sequence aa 1-213	1016	1577	273	328	6	23,042.34369	23,056.5
N-terminal pyro Glutamic acid	1016	1574	272	328	6	23,025.31714	23,039.4
N-terminal pyro Glutamic acid, 2 intrachain S-S bonds	1016	1570	272	328	6	23,021.28584	23,035.4
2 x LC (N-term. pyroGlu)	2032	3148	544	656	12	46,050.63428	46,078.9
2 x LC (N-term. pyroGlu, 2 intrachain S-S bonds each)	2032	3140	544	656	12	46,042.57168	46,070.8
Heavy chain (HC) full sequence aa 1-451	2197	3389	577	676	16	49,183.40813	49,214.0
N-terminal pyro Glutamic acid	2197	3386	576	676	16	49,166.38158	49,197.0
minus C-term. K (aa 1-450)	2191	3374	574	675	16	49,038.28661	49,068.8
minus 4 intrachain S-S bonds	2191	3366	574	675	16	49,030.22401	49,060.8
HC-G0F (pyro-Glu, - K, fully reduced)	2247	3466	578	714	16	50,482.82048	50,514.2
HC-G1F (pyro-Glu, - K, fully reduced)	2253	3476	578	719	16	50,644.87330	50,676.3
HC-G2F (pyro-Glu, - K, fully reduced)	2259	3486	578	724	16	50,806.92613	50,838.5
HC minus 4 intrachain S-S bonds + G0F	2247	3458	578	714	16	50,474.75788	50,506.1
2 x HC (pyroGlu, - K)	4382	6748	1148	1350	32	98,076.57323	98,137.7
2 x HC (pyroGlu, - K, 4 intrachain S-S bonds each)	4382	6732	1148	1350	32	98,060.44803	98,121.6
Man5 (HexNAc)2 (Hex)5	46	76	2	35	0	1216.42286	1217.1
G0 (HexNAc)4 (Hex)3	50	82	4	35	0	1298.47596	1299.2
G0F (HexNAc)4 (Hex)3 Fuc	56	92	4	39	0	1444.53387	1445.3
G1 (HexNAc)4 (Hex)4	56	92	4	40	0	1460.52878	1461.3
G1F (HexNAc)4 (Hex)4 Fuc	62	102	4	44	0	1606.58669	1607.5
G2 (HexNAc)4 (Hex)5	62	102	4	45	0	1622.58161	1623.5
G2F (HexNAc)4 (Hex)5 Fuc	68	112	4	49	0	1768.63951	1769.6
G1FSA (HexNAc)4 (Hex)4 Fuc SA	73	119	5	52	0	1897.68211	1898.7
G1FSA2 (HexNAc)4 (Hex)4 Fuc (SA)2	84	136	6	60	0	2188.77752	2190.0
G2FSA (HexNAc)4 (Hex)5 Fuc SA	79	129	5	57	0	2059.73493	2060.9
G2FSA2 (HexNAc)4 (Hex)5 Fuc (SA)2	90	146	6	65	0	2350.83035	2352.1
Man5/Man5 (HexNAc)4 (Hex)10	92	152	4	70	0	2432.84572	2434.2
G0F/G0F (HexNAc)8 (Hex)6 (Fuc)2	112	184	8	78	0	2889.06774	2890.7
G0F/G1F (HexNAc)8 (Hex)7 (Fuc)2	118	194	8	83	0	3051.12056	3052.8
G1F/G1F (HexNAc)8 (Hex)8 (Fuc)2	124	204	8	88	0	3213.17338	3215.0
G1F/G2F (HexNAc)8 (Hex)9 (Fuc)2	130	214	8	93	0	3375.22621	3377.1
G2F/G2F (HexNAc)8 (Hex)10 (Fuc)2	136	224	8	98	0	3537.27903	3539.2
G1F/G2FSA (HexNAc)8 (Hex)9 (Fuc)2 SA	141	231	9	101	0	3666.32162	3668.3

Elemental compositions	C	H	N	O	S	MW (monoisotopic)	MW (average)
G1F/G2FSA2 (HexNAc)8 (Hex)9 (Fuc)2 (SA)2	152	248	10	109	0	3957.41704	3959.6
G2F/G2FSA (HexNAc)8 (Hex)10 (Fuc)2 SA	147	241	9	106	0	3828.37445	3830.5
G2F/G2FSA2 (HexNAc)8 (Hex)10 (Fuc)2 (SA)2	158	258	10	114	0	4119.46986	4121.7
Sum 2 x HC +2 x LC (4 x pyroGlu, -2K)	6414	9896	1692	2006	44	144,127.20750	144,216.6
minus 32 S-S bond protons	6414	9864	1692	2006	44	144,094.95710	144,184.3
2HC + 2LC - 16 S-S bonds + (Man5)2	6506	10016	1696	2076	44	146,527.80282	146,618.5
2HC + 2LC - 16 S-S bonds + (G0F)2	6526	10048	1700	2084	44	146,984.02484	147,075.0
2HC + 2LC - 16 S-S bonds + G0F/G1F	6532	10058	1700	2089	44	147,146.07766	147,237.1
2HC + 2LC - 16 S-S bonds + G0F/G2F or (G1F)2	6538	10068	1700	2094	44	147,308.13049	147,399.3
2HC + 2LC - 16 S-S bonds + G1F/G2F	6544	10078	1700	2099	44	147,470.18331	147,561.4
2HC + 2LC - 16 S-S bonds + G2F/G2F	6550	10088	1700	2104	44	147,632.23613	147,723.5
2HC + 2LC - 16 S-S bonds + G1F/G2F SA	6555	10095	1701	2107	44	147,761.27872	147,852.7
2HC + 2LC - 16 S-S bonds + G1F/G2F (SA)2	6566	10112	1702	2115	44	148,052.37414	148,143.9
2HC + 2LC - 16 S-S bonds + G2F/G2F SA	6561	10105	1701	2112	44	147,923.33155	148,014.8
2HC + 2LC - 16 S-S bonds + G2F/G2F (SA)2	6572	10122	1702	2120	44	148,214.42696	148,306.1

Table 5. Chemical composition and masses of monosaccharides

	Sum Formula	Monoisotopic Mass	Average Mass	C	O	N	H
Sialic Acid	$C_{11}O_8NH_{17}$	291.09542	291.3	11	8	1	17
Galactose	$C_6O_5H_{10}$	162.05282	162.1	6	5	0	10
N-Acetylglucosamine	$C_8O_5NH_{13}$	203.07937	203.2	8	5	1	13
Mannose	$C_6O_5H_{10}$	162.05282	162.1	6	5	0	10
Fucose	$C_6O_4H_{10}$	146.05791	146.1	6	4	0	10

Table 6. Monoisotopic and average atomic masses of the elements used to calculate the molecular masses in Tables 4 and 5

	Monoisotopic Mass	Average Mass
C	12.0000000	12.01074
H	1.00782503	1.00794
N	14.0030740	14.00674
O	15.9949146	15.99940
S	31.9720707	32.06608

The initial calculation based on the sequence published in the DrugBank database[2] resulted in a mass that did not match the masses obtained in our experiments. Comparison of the DrugBank sequence with a previously published sequence[3] revealed a difference in one amino acid at position 219, located in the conserved region of the heavy chain, Ala versus Val. The sequence containing the Ala at position 219 did match well with the results obtained from intact mass measurements as well as with previously reported results.[4] To further verify this, a series of additional experiments was performed.

After reducing the antibody (without alkylation), the analysis of separated light and heavy chain was performed applying different resolution settings to account for whether or not isotopic resolution can be achieved based on molecular weight. Due to the smaller molecular weight of the light chain, it is possible to obtain an isotopically resolved spectrum, whereas for the heavy chain this is not possible since it is about twice as large as the light chain.

To apply different resolution settings, the method was set up in two segments (140k resolution for the scans acquired from 0 to 15.8 min, and 17.5k resolution from 15.8 to 30 min) and the gradient was optimized to achieve well-separated peaks of the light and heavy chain.

On both monolithic columns evaluated in this study (PepSwift 250 mm x 0.2 mm i.d. and ProSwift RP-10R 50 mm x 0.1 mm i.d.), the separation of the two peaks by more than 2 min was equally possible (Figure 6). The mass spectra obtained from the light chain and from the heavy chain (Figures 6b and 6c) were submitted for deconvolution. The isotopically resolved light chain spectrum was deconvoluted using the Xtract algorithm, resulting in a monoisotopic molecular weight of 23,025.3758 Da, which matches the calculated mass by 2.5 ppm. The heavy chain was deconvoluted using the ReSpect algorithm, resulting in three peaks, each of which represents one of the major glycoforms, G0F, G1F, and G2F (Figure 7).

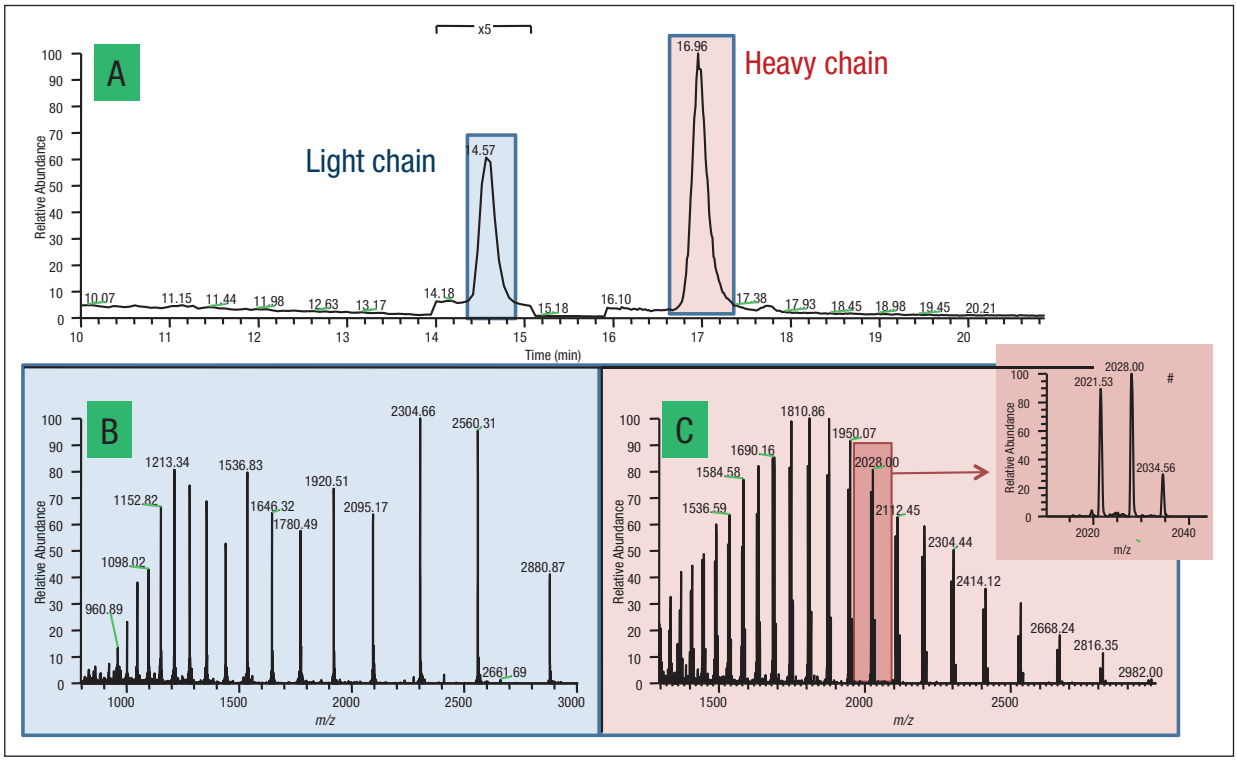


Figure 6. Chromatogram (**A**) and full MS spectra of light (**B**) and heavy chain (**C**) from reduced rituximab. The insert in panel C shows a zoomed in view of charge state z=+25, with the three peaks representing three different glycoforms.

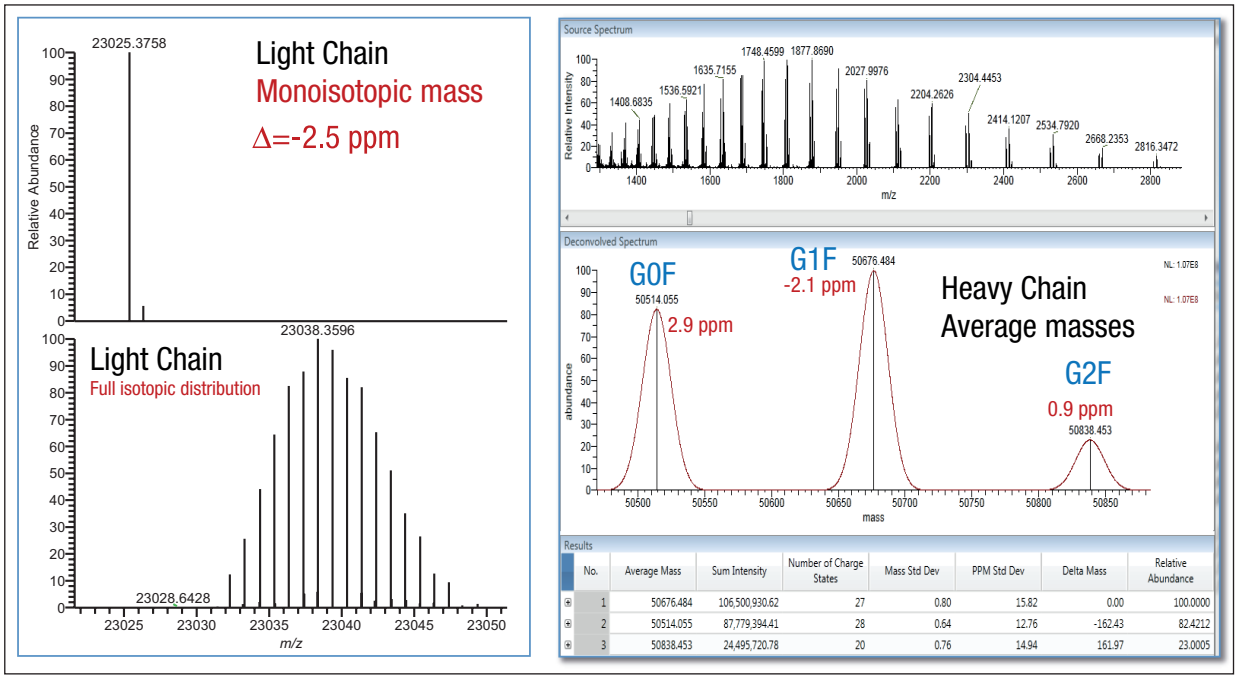


Figure 7. Deconvolution results of the light and heavy chain. The light chain, acquired at a resolution setting of 140,000 in full scan mode, was deconvoluted using the Xtract algorithm, obtaining an accurate monoisotopic mass as well as the full isotopic envelope (left). The heavy chain, detected at 17,500 resolution, was deconvoluted with the ReSpect algorithm providing average masses (right).

To assess the limit of detection of the instrument setup using the 250 x 0.2 mm monolithic PepSwift column, a series of LC/MS runs were acquired. Between 50 pg and 20 ng of the intact antibody was applied on column (Figure 8), starting with the lowest concentration. Two blanks were run before the sequence and between each sample to exclude carryover effects. With this setup, 500 pg was found to be the lowest amount that still achieved a good spectrum for deriving the most abundant glycoforms of the intact antibody. Here it is worth pointing out that for the lowest concentrations it was crucial to prepare the samples fresh without storing them for several hours in the autosampler prior to analysis.

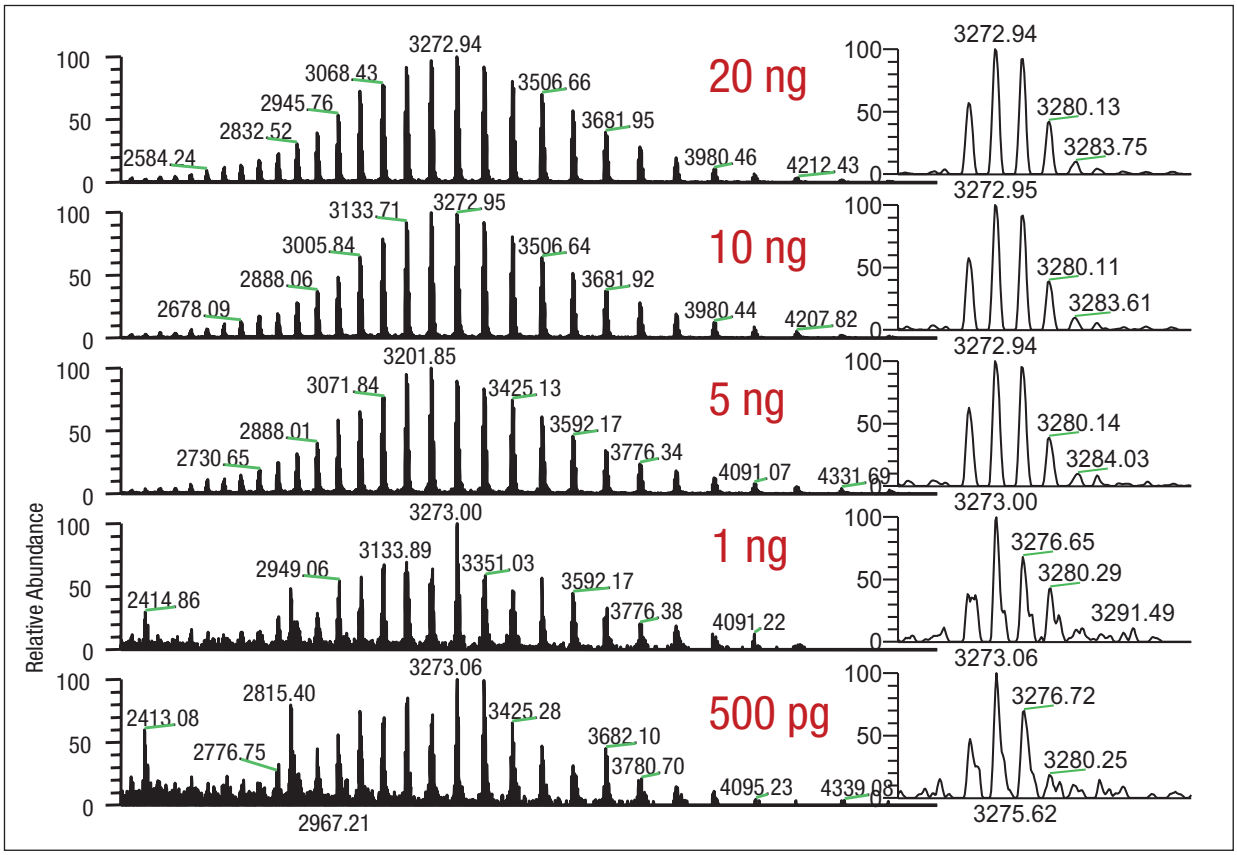


Figure 8. Full MS spectra from a dilution series of 20 ng to 500 pg of intact antibody, applied on a 250 mm x 0.2 mm i.d. monolithic PepSwift column

On the 50 mm x 1 mm i.d. monolithic ProSwift column, 30 ng and 150 ng of intact antibody were applied, both of which produced high quality spectra (Figure 9). Based on the 30 ng load it can be estimated that the lowest amount still yielding a sufficient spectrum quality to be between 5 and 10 ng.

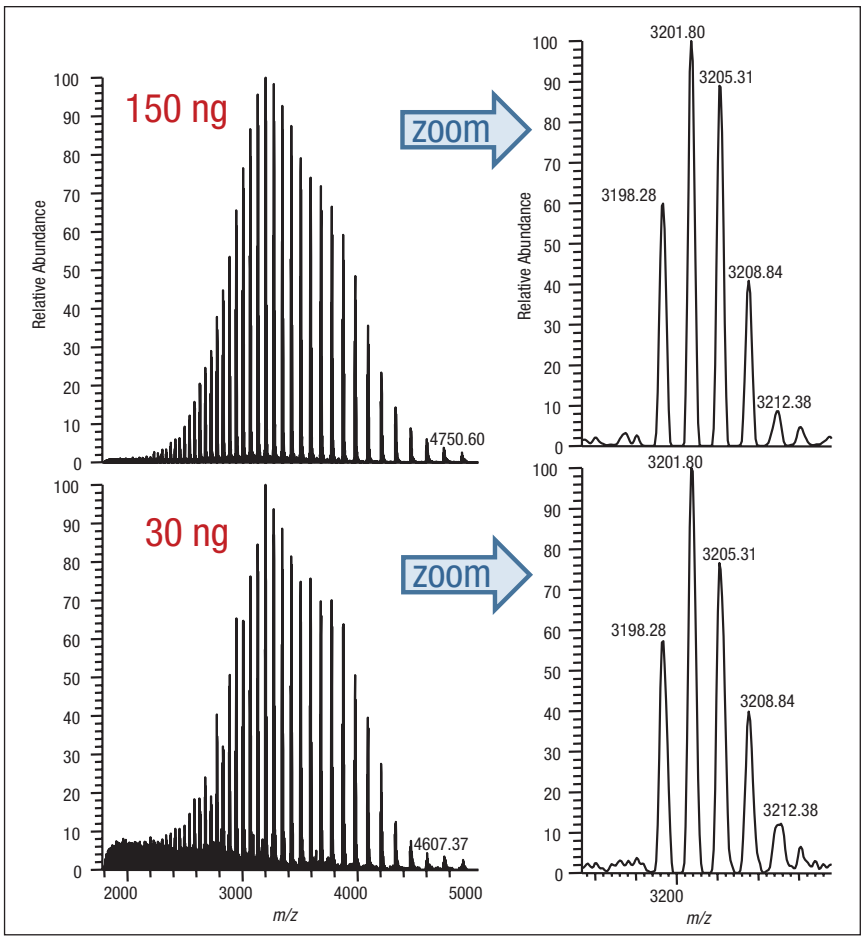


Figure 9. Full MS spectra (left) and zoomed in view of the highest abundant charge state (right) of 150 ng and 30 ng loads of intact rituximab applied on a 50 mm x 1 mm i.d. monolithic ProSwift column

In an attempt to further confirm the sequences of the light and heavy chains, two types of top-down experiments were performed: all-ion fragmentation (AIF) with fragmentation upon collision in the HCD cell and a multiplexed (5-plex), targeted MS^2 experiment on five selected charge states each of the light and heavy chains. All spectra were acquired at 70,000 resolution. For the targeted MS spectrum, a retention-time-dependent mass list was used, targeting first the earlier eluting light chain (RT 13.16 min: *m/z* 1536.96, 1646.6, 1773.3, 1920.7, 2095.4) and later the heavy chain (RT 16–20 min: *m/z* 1584.6, 1635.7, 1684.7, 1748.5, 1810.9). In this type of experiment, the first charge state listed on the inclusion list is selected and sent to the HCD cell for fragmentation. The product ions are stored in the HCD cell while the second charge state is isolated, sent to the HCD cell, fragmented, and stored in the cell until the fifth charge state has also been fragmented. All ions from the five individual isolation and fragmentation steps are sent together to the Orbitrap analyzer, resulting in one single fragment ion spectrum.

The fragment ion assignment for the light chain is displayed in Figure 10. There is good coverage of both the N- and C-terminal ends as well as some fragments in the center of the sequence, resulting in 28% coverage, respectively 15% of the theoretical fragments. For the heavy chain, fragmentation was less efficient with both methods and resulted in about 20 fragments, most of which represent the sequence termini.

To further confirm the sequences, a bottom-up approach was performed using a digest with trypsin following reduction and alkylation of the antibody. The chromatogram obtained from the digest is displayed in Figure 11. A database search against a four-entry database containing the light chain, both variants of the heavy chain, and trypsin revealed a sequence coverage of the light chain of 96% and for the heavy chain of 78.8% (Figure 12). The two short missing peptides from the light chain (LEIK and EAK) could be detected as intact masses only in the full MS spectra, whereas the peptide EAK was identified based on the accurate mass corresponding to the peptide containing a missed cleavage **EAK**VQWK. Taking into account the peptides identified based on MS/MS spectra and based on accurate masses of the small intact peptides, sequence coverage for the light chain is 100%.

Figure 10. Matched sequence coverage of the rituximab light chain based on fragment ions obtained from AIF experiments. Seventeen b- and 50 y-ions were assigned, corresponding to 15.7% of the theoretical number of fragments (67 of 426).

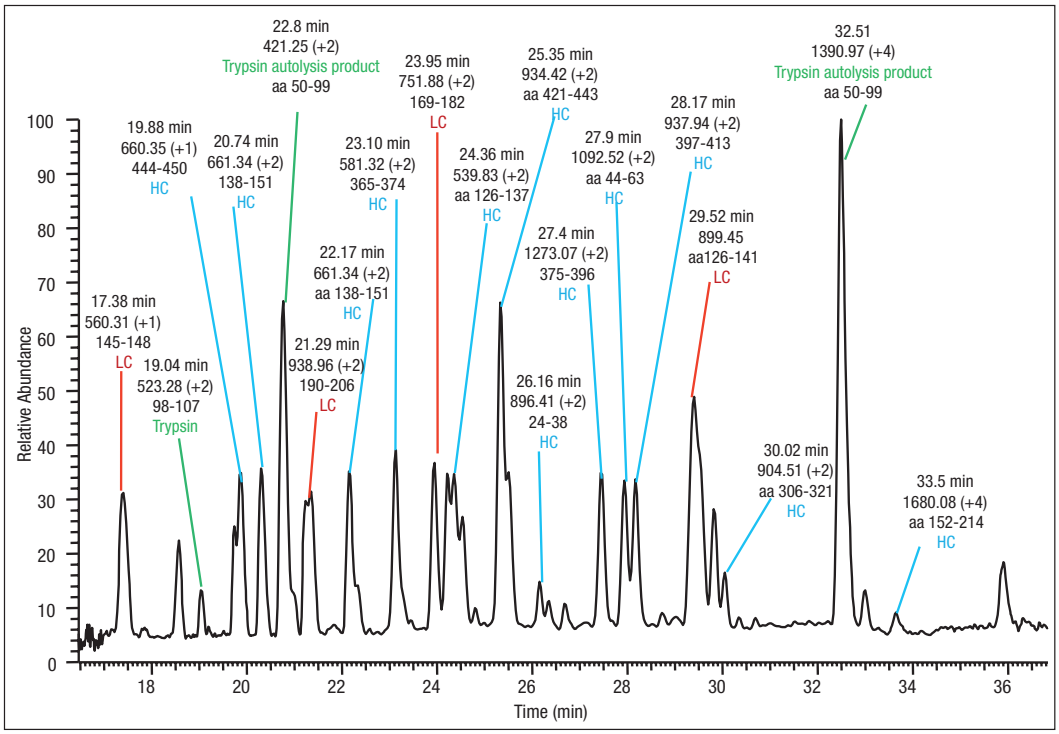


Figure 11. Base peak chromatogram of a digest using trypsin on the reduced and alkylated antibody rituximab

Light chain, length 213 aa

```
1   QIVLSQSPAI LSASPGEKVT MTCRASSSVS YIHWFQQKPG SSPKPWIYAT
51  SNLASGVPVR FSGSGSGTSY SLTISRVEAE DAATYYCQQW TSNPPTFGGG
101 TKLEIKRTVA APSVFIFPPS DEQLKSGTAS VVCLLNNFYP REAKVQWKVD
151 NALQSGNSQE SVTEQDSKDS TYSLSSTLTL SKADYEKHKV YACEVTHQGL
201 SSPVTKSFNR GEC
```

Sequence coverage 100% (96.7% with MS/MS)

Heavy chain, length 451 aa

```
1   QVQLQQPGAE LVKPGASVKM SCKASGYTFT SYNMHWVKQT PGRGLEWIGA
51  IYPGNGDTSY NQKFKGKATL TADKSSSTAY MQLSSLTSED SAVYYCARST
101 YYGGDWYFNV WGAGTTVTVS AASTKGPSVF PLAPSSKSTS GGTAALGCLV
151 KDYFPEPVTV SWNSGALTSG VHTFPAVLQS SGLYSLSSVV TVPSSSLGTQ
201 TYICNVNHKP SNTKVDKKAE PKSCDKTHTC PPCPAPELLG GPSVFLFPPK
251 PKDTLMISRT PEVTCVVVDV SHEDPEVKFN WYVDGVEVHN AKTKPREEQY
301 NSTYRVVSVL TVLHQDWLNG KEYKCKVSNK ALPAPIEKTI SKAKGQPREP
351 QVYTLPPSRD ELTKNQVSLT CLVKGFYPSD IAVEWESNGQ PENNYKTTPP
401 VLDSDGSFFL YSKLTVDKSR WQQGNVFSCS VMHEALHNHY TQKSLSLSPG(K)
```

Sequence coverage 99.5% (95.8% with MS/MS)

Figure 12. Amino sequence of light and heavy chains from rituximab. Amino acids shown in black letters represent the parts identified based on MS/MS spectra. Sequences confirmed based on MS full scan data as intact peptides only are shown in green. The two amino acids shown in red (AK) as part of the heavy chain could neither be covered on the MS nor on the MS/MS level. Resulting sequence coverage for the light chain is 100% (96% with MS/MS) and 99.5% (98.8% with MS/MS) for the heavy chain. Asparagin^{251} in the heavy chain represents the glycosylation site.

For the heavy chain, the peptide GQPR was also identified based on the accurate mass of the intact peptide. Lastly, the peptide containing the glycosylation site at position Asn^{301} was not identified in its unglycosylated form based on an MS/MS spectrum. A database search including the expected glycans as modifications was successful. In addition, the glycopeptides can easily be detected in the full scan spectra in different glycosylated forms and in different charge states, and the MS/MS spectra can easily be spotted due to the presence of a characteristic peak pattern. The G0F-containing peptide is shown as an example in Figure 13, representing the intact precursor and the typical fragmentation pattern obtained from glycopeptides using HCD-type fragmentation: the two hexonium ions at mass 204 (HexNAc) and 366 (Hex-HexNAc) as well as the fragment ions nicely showing the sequence ladder of released hexose (*m/z* 162), N-acetylhexosamine (203), and Fucose (146). Considering all peptides on the MS full scan level and based on MS/MS spectra via database searches, the sequence coverage of the heavy chain is 99.5%, leaving only two amino acids not covered (aa 343-344).

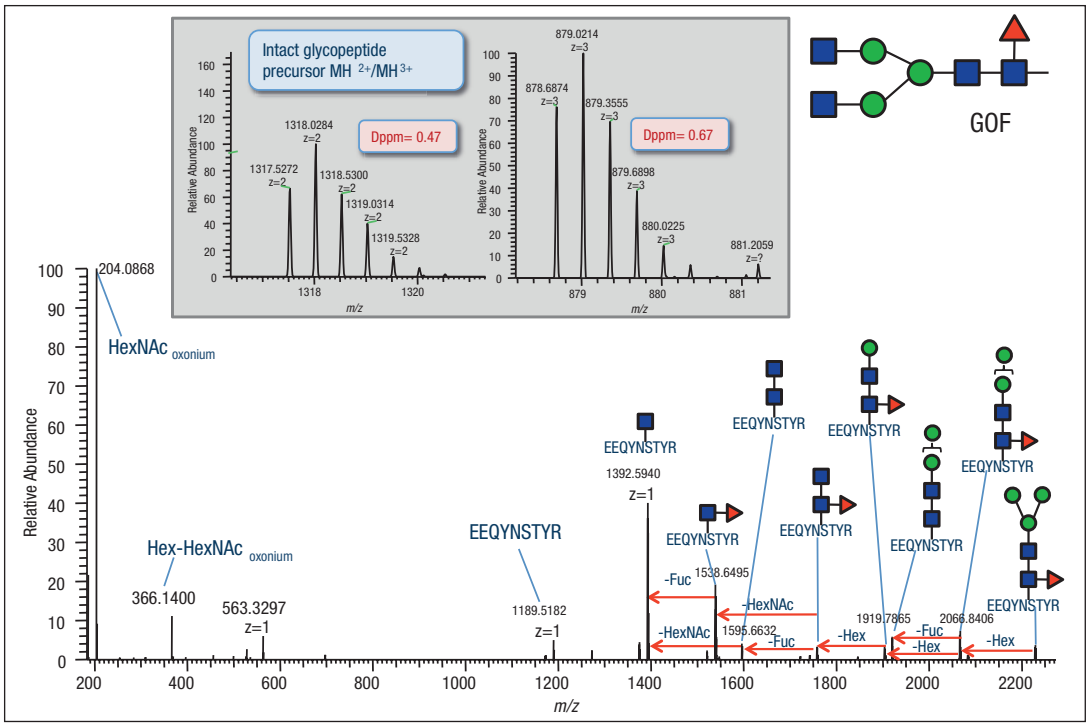


Figure 13. MS/MS spectrum of the glycopeptide aa 297-305 (EEQYN*STYR, *=G0F) obtained from the triply charged glycopeptide precursor. Inserts show the isotope patterns of doubly and triply charged intact precursors detected in the full scan spectrum.

Conclusion

In this study, a workflow is presented that combines fast chromatography, using two sizes of monolithic columns, and high resolution Orbitrap mass spectrometry of intact, as well as reduced, rituximab, sequence verification by AIF, and multiplexed HCD top-down fragmentation, supplemented by a bottom-up approach.

The data presented here also demonstrate the sensitivity of the applied LC-MS instrument setup, still obtaining a good quality MS spectrum from as low as 500 pg of the intact antibody loaded on column. Furthermore, for the analysis of the reduced mAb, a chromatographic separation of the light and heavy chains was achieved allowing for their detection at different resolution settings.

The data obtained from this workflow allow the determination of the molecular weight of the intact antibody, the confirmation/verification of the amino acid sequence of light and heavy chain, and the identification and evaluation of the relative abundance of various glycoforms of rituximab.

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Monoclonal Antibody and Related Product Characterization Under Native Conditions Using a Benchtop Mass Spectrometer

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Application Note 597

Key Words
Orbitrap native MS, extended mass range MS, monoclonal antibody (mAb), monoclonal antibody-drug-conjugate (ADC), monoclonal antibody antigen complexes (mAb/Ag)

Goal
Demonstrate the characterization of mAbs, antibody-drug conjugates (ADC), mAb/antigen (mAb/Ag) complexes, and a mixture of mAbs under their native conditions by using a high-resolution, accurate-mass (HRAM) benchtop mass spectrometer with extended mass range (EMR) in combination with a chip-based electrospray ionization interface.

Introduction
Native mass spectrometry (MS) has emerged as a valuable technique for characterization of intact noncovalent protein complexes, reaching a high level of reliability within the last ten years.[1] For the analysis of intact monoclonal antibodies (mAbs), native MS yields accurate mass measurements of the molecules, glycoform identification, and assessment of higher-order structures (dimer, trimer, tetramer), thus providing a robust, fast, and reliable first-line analytical characterization tool.[2,3] This approach can now be applied to the routine characterization of heterogeneous therapeutic monoclonal antibodies. Native MS has gained interest not only for analysis of intact mAb, but also for analysis of antibody-drug conjugates (ADCs), bispecific mAbs, antibody-antigen complexes, and characterization of antibody mixtures. It benefits from simplified data interpretation due to the presence of fewer charge states compared to classical denaturing MS.

This application note describes the use of a new Orbitrap mass spectrometer with an extended mass range of up to *m/z* 20,000 and improved detection of high-mass ions for the characterization of mAbs, ADCs, mAb/Ag, and mAb mixtures under native conditions.

Figure 1. Exactive Plus EMR mass spectrometer equipped with a TriVersa NanoMate chip-based electrospray ionization interface

Experimental
Sample Preparation
The intact trastuzumab (Herceptin®, Roche), the monoclonal antibody-drug conjugate brentuximab vedotin (ADC, Adcetris®, Seattle Genetics), the mAb/antigen complexes of J10.4 mAb/JAM-A, and one mixture of eleven distinct IgG antibodies were introduced using the TriVersa NanoMate® (Advion, USA) onto the Thermo Scientific™ Exactive™ Plus EMR Orbitrap™ mass spectrometer.

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Brentuximab vedotin was deglycosylated using EndoS endoglycosidase (IgGZERO™, Genovis). Titration experiments involving J10.4 mAb and JAM-A were monitored by native MS in order to determine the binding stoichiometry. The fixed amount of J10.4 (5 µM) was incubated with increasing amounts (1:1, 1:2, 1:4, 1:8) of JAM-A up to 40 µM. The mixture of eleven distinct deglycosylated humanized IgG antibodies included two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb [4, 5]. They were mixed together prior to PNGase-F deglycosylation.

Finally, all the samples were buffer exchanged against 150 mM ammonium acetate (AcONH4) pH 7.5. Trastuzumab, deglycosylated Brentuximab vedotin, and the mAb/antigen complexes of J10.4 mAb/JAM-A were injected at 5 µM, and the deglycosylated IgG mixture was injected at 1 µM on the Exactive Plus EMR Orbitrap mass spectrometer.

Direct-Infusion Native MS Conditions

Chip-based infusion conditions	
Instrumentation	TriVersa NanoMate® (Advion, USA) system
Ionization voltage (kV)	1.6–1.8
Gas pressure (psi)	0.3–0.6

The ESI Chip® consists of an array of 400 nanoelectrospray emitters with 5 µm inner diameters.

MS conditions	
Instrumentation	Exactive Plus EMR Orbitrap MS system (Figure 1)
EMR mode	ON
Mass range *(m/z)*	350–20,000
Resolution	17,500 to 140,000, depending on spectral complexity
Target value	3×10^6
Microscans	10
Max injection time (ms)	300
Insource CID energy (eV)	60 to 150, manually tuned for optimized desolvation
S-lens level (%)	100 to 200, manually tuned for optimized transmission and avoiding in-source fragmentation
Injection flatapole DC (V)	8
Inter flatapole lens (V)	7
Bent flatapole DC (V)	6
C-Trap entrance lens tune offset (V) EMR	0
Trapping gas pressure setting factor	4
Spectra average	Enabled (10 to 50 scans are averaged to achieve S/N ratio of >100)

Data Processing

Software	Thermo Scientific™ Protein Deconvolution software version 2.0 SP2 and version 3.0
Deconvolution parameters	
Number of iterations	4
Noise compensation	On
Minimum adjacent charges	1 to 3

Results and Discussion

High-Resolution Native MS Analysis of Intact Monoclonal Antibody Trastuzumab

Trastuzumab (Herceptin®) is a humanized IgG1 mAb, approved for HER2-overexpressing breast cancer treatment since 1998. Several mechanisms of action are thought to contribute to trigger the tumor-inhibitory effect of this protein therapeutic. Among them, trastuzumab can mediate the effector functions of immune cells through its constant region (Fc) by binding to the Fc gamma receptor III (FcγRIII) and triggering antibody-dependent, cell-mediated cytotoxicity (ADCC).

Based on the published amino acid sequence of both the light and heavy chain of trastuzumab, the calculated mass of this protein is $C_{6560}H_{10132}O_{2090}N_{1728}S_{44}$ = 148,057 Da. This calculation includes 16 disulfide bridges (–32 Da), two main glycoforms (G0F; +1445 Da), and near 99% cleavage of two heavy chain C-terminal lysines (–128 × 2 Da). Partial cyclization of one or two N-terminal glutamic acids (–18 Da) may also occur as well as methionine oxidations (+16 Da). Three Asn deamidation/Asp isomerization hot spots have also been described in the CDRs and shown to negatively impact HER2 antigen binding when degraded (Figure 2A).

Trastuzumab was analyzed on the Exactive Plus EMR MS with resolution set at both 17,500 and 35,000. The deconvoluted mass spectrum calculated using Protein Deconvolution software version 2.0 SP2 represents the classical glycosylation pattern of a mAb with baseline-resolved glycan peaks. Figure 2B shows the complete mass spectrum at resolution of 35,000 and a zoom of the corresponding 23^+ charge state of trastuzumab acquired with the resolution set at both 17,500 and 35,000 in native conditions. Compared to the raw spectrum acquired at 17,500 resolution, an interference peak can be resolved by using a resolution of 35,000 or higher. The high resolution can resolve the analyte from the interferences, therefore, ensuring the low ppm mass accuracy. Molecular weights of each trastuzumab glycoform were measured with good mass accuracy in the low ppm range, as shown in Figure 2C. The mass differences between species are +146 Da and +162 Da, corresponding to a fucose or to the addition of multiple hexose units, respectively.

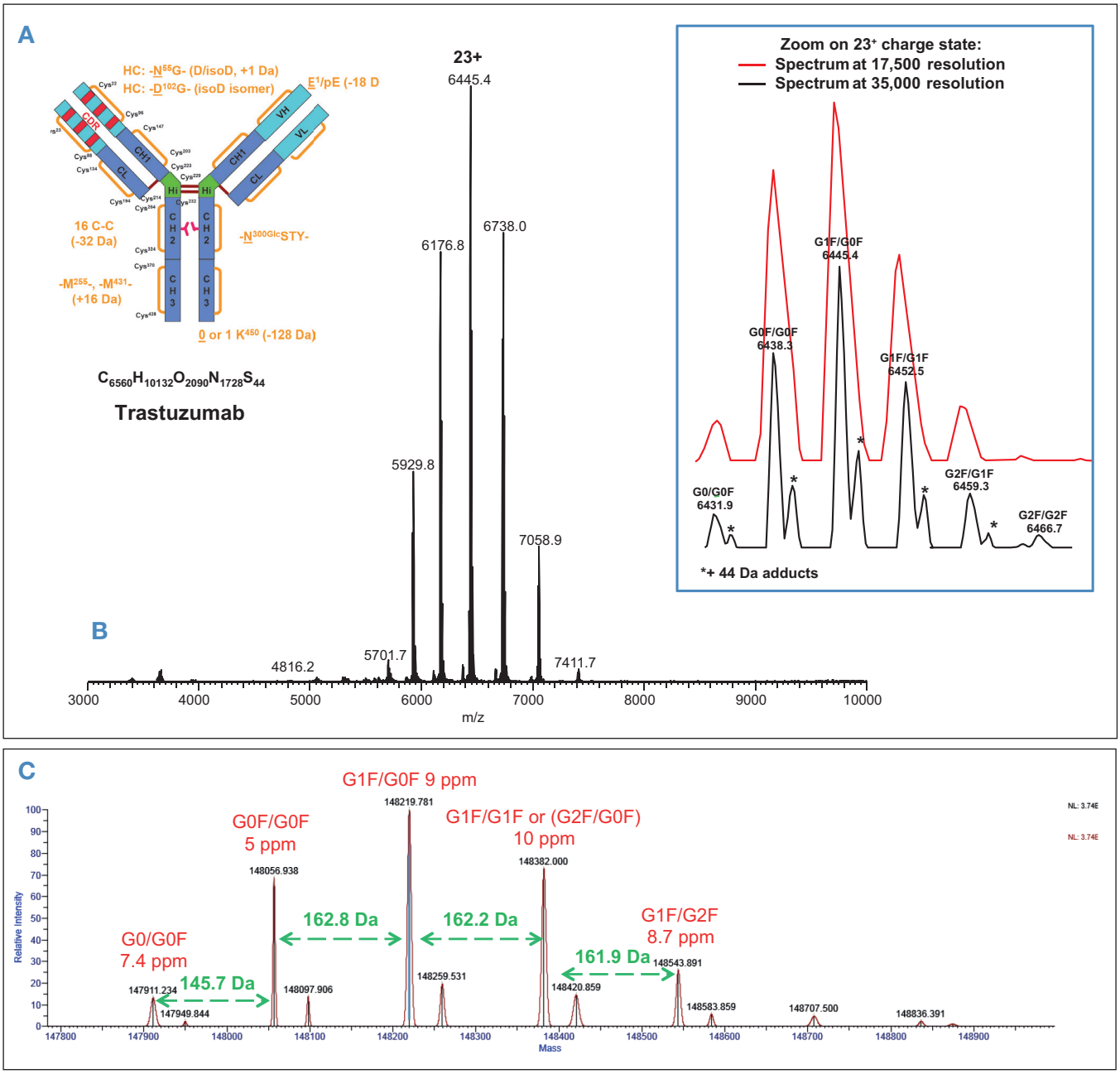

Figure 2. Orbitrap native MS detection of intact monoclonal antibody trastuzumab. A. Intact mAb trastuzumab. B. High-resolution, native MS showing complete mass spectrum and zoom of corresponding 23^{+} charge state. C. Deconvoluted spectrum showing molecular weights of each trastuzumab glycoform with low ppm mass accuracy.

Orbitrap Native MS Analysis of a Monoclonal Antibody-Drug-Conjugate (ADC) Brentuximab Vedotin

Antibody-drug conjugates (ADCs) are an increasingly important modality for treating several types of cancer. The impact of ADCs in this field is due to the exquisite specificity of antibodies that deliver the conjugated cytotoxic agent to targeted tumor cells preferentially, thus reducing the systemic toxicity associated with traditional chemotherapeutic treatments. ADCs are differentiable on the basis of the drug, linker, and also the amino acid residue of attachment on the antibody. Recently, two ADCs were approved by the FDA (Adcetris®, brentuximab vedotin, and Kadcyla®, trastuzumab emtansine) and 35 more are being investigated in clinical trials.

The brentuximab vedotin mass spectrum was recorded at a resolution of 35,000 and in-source CID voltage was set to 75 eV. Figure 3A shows the native deconvoluted mass spectrum of the deglycosylated ADC. Populations with zero (grey), two (black), four (blue), six (red), and eight (green) molecules loaded onto the antibody (payloads) were detected with a mass difference between peaks corresponding to the addition of two payloads (+2,634 Da). The drug loading clearly increases in steps of two, which corresponds to binding of one payload to the two accessible cysteine amino acids after disulfide bridge reduction. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined. Relative ratios of each detected compound were determined using MS peak intensities and served to estimate the mean DAR (4.2), which is in agreement with hydrophobic chromatography data (data not shown). Figure 3B shows the corresponding raw mass spectrum with the entire charge state distribution of brentuximab vedotin under native conditions.

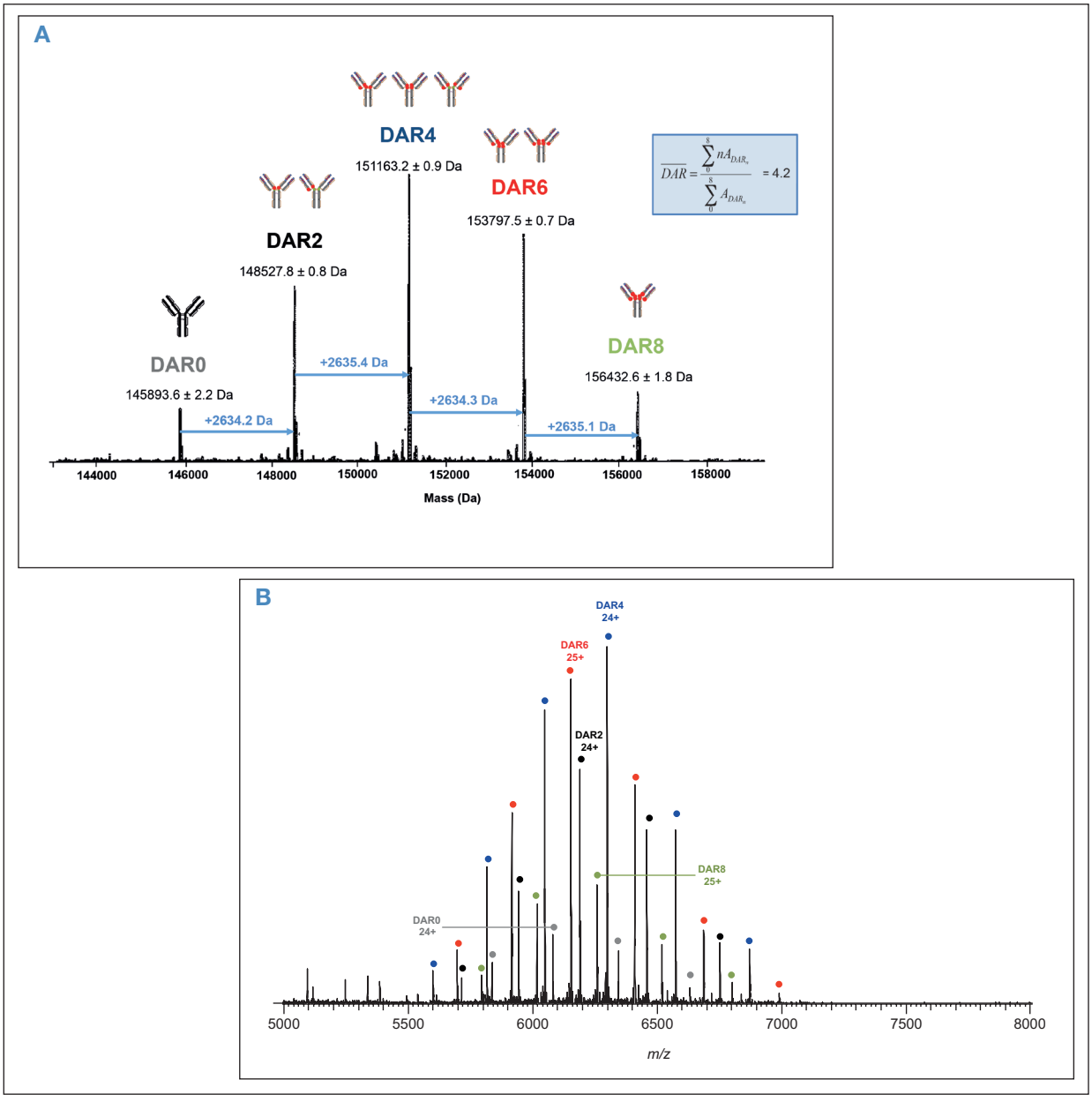

Figure 3. Orbitrap Native MS analysis of a monoclonal Antibody-Drug-Conjugate (ADC). A. Native deconvoluted mass spectrum showing the determination of drug-to-antibody ratio (DAR). B. Raw mass spectrum with the entire charge state distribution of ADC under native conditions.

Orbitrap Native MS Analysis of Immune mAb/Antigen Complexes

Native MS can also be used to analyze mAb/antigen (mAb/Ag) complexes, providing additional information including mAb/antigen binding stoichiometries, specificities and affinities.[4] These properties are essential for originator and biosimilar candidates comparison studies. ESI-MS presents the advantage to allow the direct observation of noncovalent immune complexes without any chemical modification. J10.4 is a commercial mouse monoclonal IgG1 raised against recombinant JAM fusion protein of human origin that is recommended for detection of JAM-A by western blotting and immunopurification techniques. JAM-A, used here as antigen, is a single transmembrane protein belonging to the immunoglobulin superfamily. JAM-A localizes in tight junctions in normal epithelial and endothelial cells where homophilic JAM-A interactions have been shown to be important for regulation of epithelial barrier function.[4,5] This newly identified target is over-expressed in many tumor tissues and therefore is of prime interest as a target in oncology. Two JAM-A molecules are expected to bind to one J10.4 mAb.

The native mass spectrum of mAb/antigen complexes was recorded at a resolution of 35,000 with the in-source CID voltage set to 150 eV. As shown in Figure 4A, when an 4-fold excess of JAM-A (20 µM) is added to J10.4 mAb (5 µM), three species are detected: the intact free mAb (MW 150237.1 ± 1.1 Da, black), 1:1 (MW 174304.4 ± 2.0 Da, blue) and 1:2 (MW 198369.6 ± 2.3 Da, red) mAb:JAM-A complexes. Native MS thus confirmed that two JAM-A molecules can bind to J10.4 mAb. MWs correspond to the main G0F/G0F glycoforms. Relative abundances were estimated from MS peak intensities and proportions of mAb:Ag complexes at 1:1 and 1:2 stoichiometries were observed to be 37% and 30%, respectively, while free mAb represents 33%. Figure 4B shows the corresponding mass spectrum with the entire charge state distribution in native conditions.

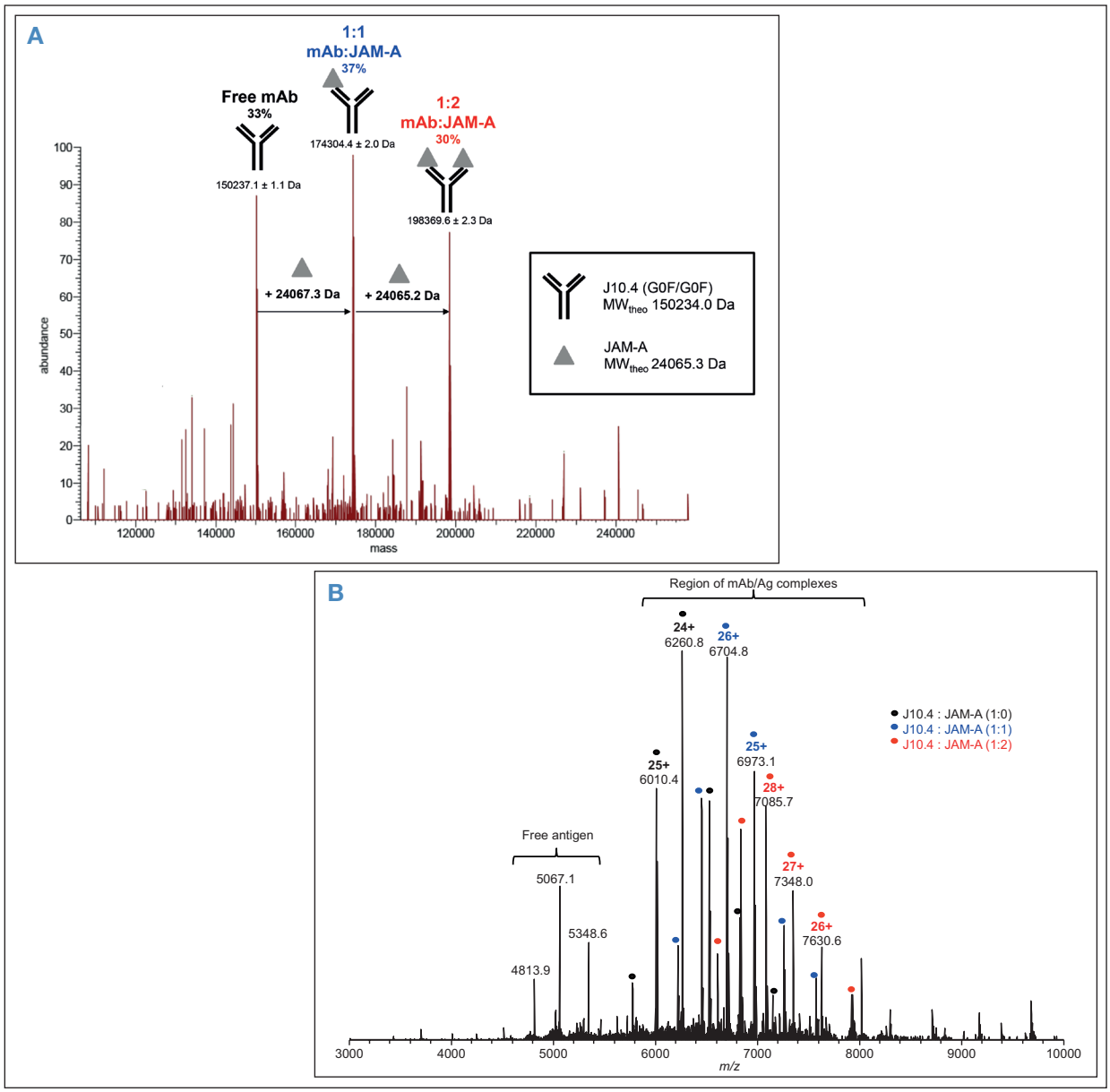

Figure 4. Orbitrap native MS detection of immune mAb/antigen complexes. A. Deconvoluted mass spectrum showing mAb/antigen binding stoichiometries. B. Charge state distribution in native conditions.

Native MS Analysis of a Mixture of Eleven N-deglycosylated Humanized Antibodies

Analysis of mAb mixtures is of utmost interest for high-throughput screening purposes and for therapeutic use to block simultaneously multiple epitopes. Indeed cocktails of mAbs with additive or synergic effects are increasingly foreseen as potential new therapeutic entities.

Figure 5A presents a convoluted mass spectrum of a mixture of eleven distinct deglycosylated humanized IgG antibodies. This mix includes two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb.[4,5] Figure 5B shows a full native mass spectrum of the mAb mix with an in-source CID energy set to 100 eV.

The well-resolved ion signals at a detection resolution of 140,000 and accurately measured masses enable the unambiguous assignment of ten out of the eleven compounds. Trastuzumab and Hz6F4-2v6 could not be differentiated due to very close molecular weights (2 Da). Peaks corresponding to Hz6F4-2 and Hz6F4-2v3, which differ by only 21 Da in mass, are clearly distinguished on the mass spectrum. However, they are not baseline resolved, and when combined with the low signal-to-noise (S/N) ratio (S/N < 20), that causes a relatively low mass accuracy for Hz6F4-2. However, with a good signal-to-noise ratio (S/N > 50), even without baseline-resolved peaks, for example, peaks of Hz6F4-2v9 and 6F4-2v10, the mass accuracies are achieved in the low ppm range for both species. The measured and theoretical masses for the mixture of eleven N-deglycoslated humanized antibodies are listed in Table 1.

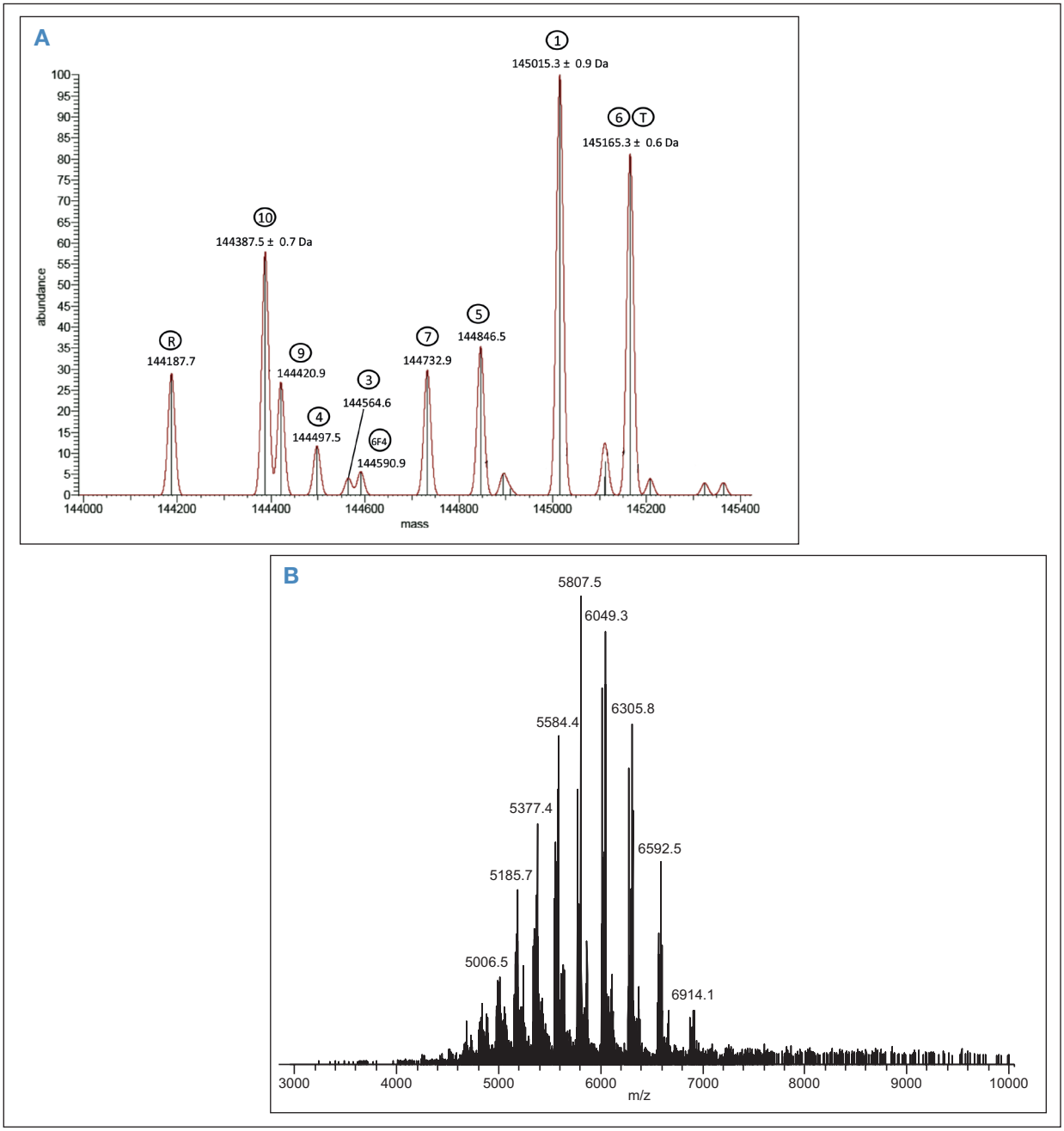

Figure 5. Native MS analysis of a mixture of eleven N-deglycosylated humanized antibodies. A. Deconvoluted mass spectrum. B. Charge state distribution in native conditions.

Table 1. Measured and theoretical masses for the mixture of eleven N-deglycosylated humanized antibodies at an Orbitrap detection resolution of 140,000

	Species	Theoretical Masses (Da)	Measured Masses (Da)	Mass Accuracy (ppm)
R	Rituximab	144186.3	144187.7	9.7
10	6F4-2 v10	144388.3	144387.5	5.5
9	6F4-2 v9	144420.5	144420.9	2.8
4	6F4-2 v 4	144498.4	144497.5	6.2
3	6F4-2 v3	144564.4	144564.6	1.4
6F4	6F4-2	144585.5	144590.9	37.3
7	6F4-2 v7	144732.5	144732.9	2.8
5	6F4-2 v5	144846.9	144846.5	2.8
1	6F4-2 v1	145015.3	145015.3	0
6	6F4-2 v6	145163.3	N.D	N.D
T	Trastuzumab	145165.5	145165.3	1.4

Conclusion

In the analysis (0.3–5 min) using the Exactive Plus EMR MS, molecular weight measurements of mAb and related products in the low ppm mass deviation range allowed the identification of all species simultaneously present in solution. The number of DAR and relative abundance of mAb/Ag complexes was also assessed with the peaks intensities serving for relative quantification of the detected species.

- The high resolving power of the Orbitrap mass analyzer can baseline resolve a native mAb's glycan peaks, as well as the interference peaks, ensuring an excellent mass accuracy in the low ppm range.
- The Exactive Plus EMR MS is able to sensitively characterize ADC complexes with mass differences between peaks corresponding to different additional number of payloads/drugs. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined as well as the relative ratio of each detected compound in order to assess the mean DAR value.
- Native Orbitrap MS can reveal the number of antigens bound to mAbs. Relative abundances of mAb/Ag complexes at different stoichiometries can be achieved from MS peak intesities.
- The Exactive Plus EMR MS enables the high throughput screening of mAb mixtures, ensuring a excellent mass accuracy for each individual mAb.

Acknowledgements

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Analysis of Monoclonal Antibodies, Aggregates, and Their Fragments by Size Exclusion Chromatography Coupled with an Orbitrap Mass Spectrometer

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Overview

Purpose: Demonstrate SEC-MS as a characterization platform for a monoclonal antibody (mAbs) and its fragments.

Methods: Coupling size-exclusion chromatography with high resolution Orbitrap mass spectrometer (SEC-MS) enables accurate mass measurement of mAb, its aggregates, and its fragments.

Results:

1. mAb monomer and dimer aggregate intact masses can be measured by SEC-MS under non-denaturing condition using near neutral pH eluent
2. Thermo Scientific™ Exactive Plus™ EMR Orbitrap mass spectrometer enables the accurate detection of mAb at *m/z* 350- 20,000
3. Thermo Scientific™ MAbPac™ SEC-1 column successfully separates the HC and LC, and partially separates Fab and Fc fragments using denaturing eluent.

Introduction

The biopharmaceutical industry has continued its focus on the development of biotherapeutic monoclonal antibody (mAbs) drugs[1]. mAbs produced from mammalian cell culture may contain significant amounts of dimers and higher-order aggregates. Size exclusion chromatography (SEC) is a well-accepted technique for the detection and accurate quantification of protein aggregates in biological drug products. It is routinely used for the characterization and quality control of mAb products.

There is a growing trend to obtain intact mass information as well as the glycan profile in the QC of monoclonal antibodies using high resolution mass spectrometry. The most commonly employed LC/MS method is to desalt the mAb via reversed phase chromatography followed by the MS analysis. However, the extreme low pH and organic solvent used in the reverse phase chromatography often denatures the mAb. In the case of antibody-drug conjugate (ADC) with interchain cysteine linked drugs, the harsh solvent condition will dissociate the heavy and light chains of the ADC and prevent the measurement of intact mass. A non-denaturing SEC-based desalting mass spectrometry method enables mass measurement of the mAb in its native state. The volatile ammonium formate buffer is compatible with MS and preserves intact protein structure.[2]

Full characterization of mAb includes determination of mass of the mAb fragments, such as heavy chain (HC) and light chain (LC) generated by reduction of interchain disulfide bonds, as well as Fab and Fc generated by papain digestion. Using denaturing eluent containing 20% acetonitrile, 0.1% TFA, and 0.05% formic acid, SEC can baseline separate HC and LC, as well as partially separate Fab and Fc. It serves as a platform method for mAb fragment analysis.

The Exactive Plus EMR mass spectrometer combines high-resolution, accurate mass data with an extended mass range (EMR). It has an *m/z* range up to 20,000 and improved transmission of higher mass ions for stronger signals. All these features make the Exactive Plus EMR mass spectrometer a superb tool for accurate intact mass measurement of mAb and high performance screening of mAb glycosylation profile.

The mAbPac SEC-1 is a size exclusion chromatography (SEC) column designed for monoclonal antibody (mAb) analysis, including monomers, aggregates, and fragments. Its stable surface bonding leads to low column bleed and compatibility with MS detection. In this study, we demonstrate the compatibility of mAbPac SEC-1 with Exactive Plus EMR Mass Spectrometer. SEC-MS enables intact mass detection of mAb monomer, dimer aggregate under non-denaturing condition and fragments (including heavy chain, light chain, Fab, and Fc) under denaturing conditions.

Methods

Chemicals and reagents
High purity ammonium formate (≥99.995%) was purchased from Sigma®. Other reagents were purchased from reputable suppliers. Monoclonal antibodies mAb1 and mAb2 were gifts from a biotech company.

Columns
MAbPac SEC-1, 5 µm, 4 × 300 mm (P/N 074696)
MAbPac SEC-1, 5 µm, 2.1 × 150 mm (P/N 088462)

Liquid Chromatography
HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 RSLCnano System equipped with: SRD-3400 Membrane Degasser, NCS-3500RS dual-gradient pump and column compartment , and WPS-3000TPL Rapid Separation Thermostatted Autosampler. SEC analysis was carried out in isocratic mode. For the 4.0 mm ID column, flow rate was set at 200 µL/min. For the 2.1 mm ID column, flow rate was set at 50 µL/min.

Reduction of mAb to heavy chain (HC) and light chain (LC) subunits
Reduction of inter-chain disulfides in a mAb (1 mg/mL) was achieved by incubation of mAb with 20 mM DTT at 50 °C for 30 min. The reduced sample was acidified with formic acid to final concentration at 0.1%.

Papain digestion of mAb to generate Fab and Fc subunits
The digestion was carried out by incubating mAb (1 mg/mL) with papain (0.04 mg/ml) in 100 mM Tris-HCl, pH 7.6, 4 mM EDTA and 5 mM Cysteine buffer at 37 °C. After 4 hours, the digestion was stopped by addition of formic acid to final concentration at 0.1%.

Non-denaturing SEC mobile phase
20 mM ammonium formate (pH 6.3).

Denaturing SEC mobile phase
20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid acid (TFA).

MS Conditions
The Exactive Plus EMR mass spectrometer was used for this study. Intact mAb or mAb fragments were analyzed by ESI-MS. HESI probe was used. See Table I for details.

Data processing
Full MS spectra of intact mAbs, HC, LC, Fab, and Fc fragments were analyzed using Thermo Scientific™ Protein Deconvolution software (v 3.0) that utilizes the ReSpect algorithm for molecular mass determination.

TABLE 1. MS conditions. The Exactive Plus EMR Orbitrap mass spectrometer was used for this study. The following parameters were employed for all measurements: Source DC Offset: -25V; Injection Flatapole DC: -8V; Inter Flatapole Lens: -7V; Bent Flatapole DC: -6V. Other ion transfer parameters in the tune file were set as default.

Instrument Conditions	Non-denaturing, MAb Monomer	Non-denaturing, MAb Dimer	Denaturing
EMR mode	On	On	On
Mass range	m/z 400–20,000	m/z 2,000–15,000	m/z 400–6,000
Spray voltage	4.3 kV	4.3 kV	4.3 kV
Sheath gas	30 arb. units	15 arb. units	30 arb. units
Auxiliary gas	10 arb. units	3 arb. units	10 arb. units
Capillary temperature	275 °C	275 °C	275 °C
S-lens level	200	200	200
In-source CID	100 eV	100 eV	100 eV
HCD CE	10	100	n/a
Microscans	5	5	1
AGC target	1×10^6	3×10^6	1×10^6
Maximum IT	300 ms	200 ms	200 ms
Resolving power	35,000	17,500	17,500
Probe temperature	400 °C	100 °C	200 °C

Results

Analysis of mAb by non-denaturing SEC-MS

The analysis of mAbs by SEC is typically performed under non-denaturing conditions at near-physiological pH range (6.8). The commonly used buffer is phosphate buffer with 300 mM NaCl. However, the non-volatile nature of phosphate buffer and high salt content makes this buffer non-compatible with online mass spectrometry detection. Therefore, we explored using a volatile buffer such as 20 mM ammonium formate for SEC separation and directly coupling the SEC column to the Exactive Plus EMR instrument. Figure 1 shows the SEC-MS analysis of mAb1, with Figure 1a showing the extracted ion chromatogram of *m/z* at 5483.08-5483.31 and Figure 1b showing the charge envelope of +24 to +29 in the *m/z* range of 5100-6200. Normally under acidic condition, the charge envelope of mAb is in the *m/z* range of 2000-4000. Since the 20 mM ammonium formate eluent has near neutral pH (at 6.3), the charge envelope of mAb shifts to higher mass range. The detection of such high *m/z* charge envelope (*m/z* above 6000) is made possible with the extended mass range of the Orbitrap instrument. Figure 1c shows the deconvoluted mass spectra of the mAb, with a main peak at mass 148,033 u and adjacent peaks at mass 148,198 u, and mass148,359 u, corresponding to different glycoforms with 1 and 2 additional hexoses. The adjacent peak at mass 148,163 u, is 130 u above the main peak, corresponding to a lysine variant.

Separation of mAb2 dimer aggregate and monomer was achieved on a short SEC column (2.1 × 150 mm) within 8 min (Figure 2a). Both dimer aggregate and monomer were successfully detected (Figure 2b and 2d). The deconvoluted spectra of aggregates show dimer peaks at mass 296,785 u and 297,105 u (Figure 2c), corresponding to the homo-dimers of monomers at mass148,393 u and 148,554 u (Figure 2e). The mass differences between measured mass and calculated mass derived from the monomer mass are 3 and 7 ppm respectively. In addition, the dimer aggregate peak at mass 296,949 u corresponds the hetero-dimer of monomers at mass 148,393 u and 148,554 u.

FIGURE 1: SEC-MS analysis of mAb1 under non-denaturing condition using 20 mM NH_4HCO_2. mAb was injected onto a MAbPac SEC-1 4 x 300 mm column and the flow rate was set at 200 µL/min. (a) extracted ion chromatogram of mAb, (b) mass spectrum of mAb, (c) deconvoluted spectrum of mAb.

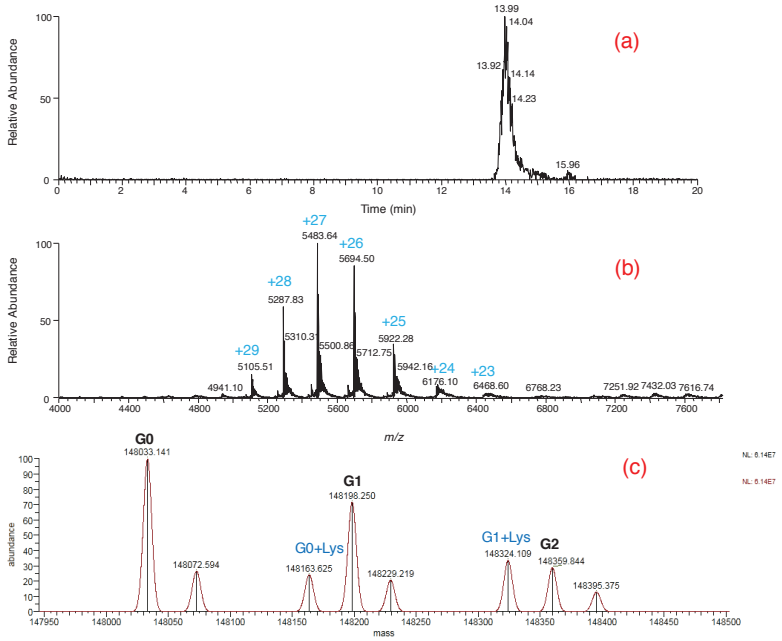
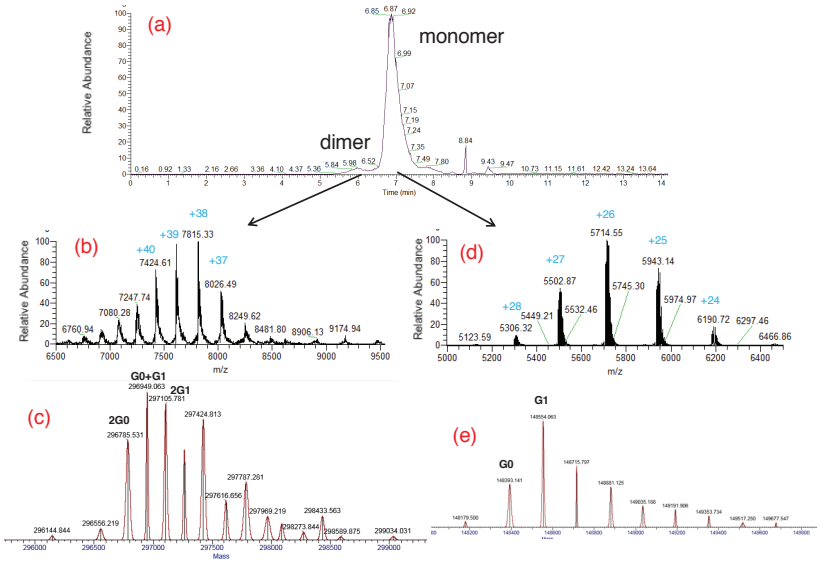


FIGURE 2: SEC-MS analysis of mAb2 dimer aggregate and monomer under non-denaturing condition using 20 mMNH_4HCO_2. mAb was injected onto a MAbPac SEC-1 2.1 x 150 mm column and the flow rate was set at 50 µL/min. (a) extracted ion chromatogram of mAb monomer and dimer. (b) mass spectrum of mAb dimer, (c) deconvoluted spectrum of mAb dimer. (d) mass spectrum of mAb monomer, (e) deconvoluted spectrum of mAb monomer.

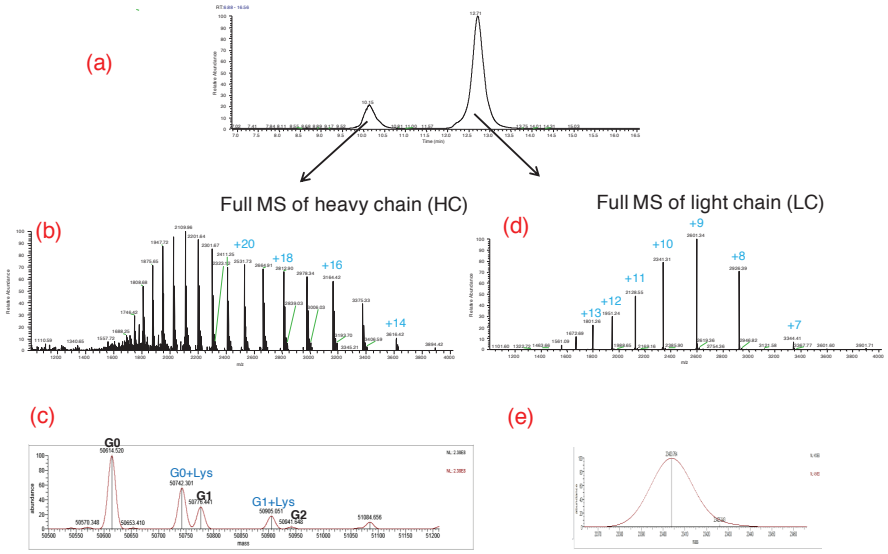


Analysis of mAb fragments by denaturing SEC-MS

Comprehensive analysis of the mAb post translational modifications, such as deamidation, C-terminal lysine truncation, N-terminal pyroglutamation, methionine oxidation, and glycosylation, requires complete digestion of the mAbs and sequencing of all the peptides. However, "peptide mapping" is time consuming. A simpler and faster way to analyze the mAb variants and locate the modifications is to measure the mass of heavy chain and light chain, or Fab and Fc fragments. Heavy chain and light chain are generated by the reduction of mAb. Fab and Fac fragments are generated by papain digestion. For example, the glycan modification is located in the Fc region of the heavy chain, glycan variants can be detected in the heavy chain and Fc fragment mass profiles, while light chain and Fab fragment mass profiles should only show a single polypeptide chain.

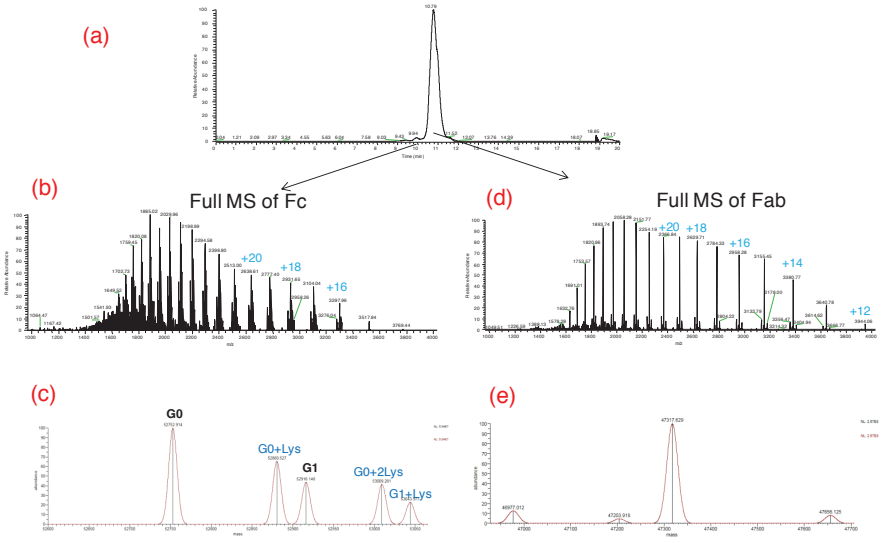
Figure 3 shows the SEC-MS analysis of HC and LC of mAb1 using 20% acetonitrile, 0.1% formic acid, and 0.05% TFA. Figure 3a shows the extracted ion chromatogram of HC with *m/z* at 3163.70-3164.89 and LC with *m/z* at 2600.78-2601.88. Using this denaturing eluent system, mAb HC elutes at about 10.15 min and mAb LC elutes at about 12.71 min. Different mAbs have been tested and their HC and LC have similar retention time. Therefore, denaturing SEC can be used as a platform method for the separation of HC and LC of mAbs. Figure 3b shows the charge envelope of mAb HC in the *m/z* range of 1900-3600 and Figure 3c shows the deconvoluted mass spectra of the mAb HC, with a main peak at mass 50614.5 u and adjacent peaks at mass 50,742.3 u, and 50,776.4 u, corresponding to a lysine variant and a different glycoform with 1 additional hexose. The lysine variant is located at the C-terminal of the HC. Figure 3d shows the charge envelope of mAb LC in the *m/z* range of 1500-3500 and Figure 3e shows the deconvoluted mass spectra of the mAb LC, with a single peak at mass 23,403.7 u. The mAb light chain is not glycosylated and does not have C-terminal lysine variants. The intact mass of mAb is determined at mass148,029 u using the equation 2x(HC+LC)-8. The calculated mass is in good agreement with the measured mass at mass 148,035 u.

FIGURE 3: SEC-MS analysis of mAb1 heavy chain and light chain under denaturing condition using 20% acetonitrile, 0.1% formic acid and 0.05% trifluoroacetic acid. mAb was injected onto a MAbPac SEC-1 4 x 300 mm column and the flow rate was set at 200 µL/min. (a) extracted ion chromatogram of heavy chain (HC) and light chain (LC), (b) mass spectrum of heavy chain (HC), (c) deconvoluted spectrum of heavy chain (HC), (d) mass spectrum of light chain (LC), (e) deconvoluted spectrum of light chain (LC).



Using the same chromatographic method, Fc and Fab fragments from mAb1 are eluted off the SEC column at 9.94 and 10.79 min (Figure 4a), although the separation is not as good as the HC and LC due to the fact that Fab and Fc fragments are very similar in size. Figure 4b shows the charge envelope of Fc in the *m/z* range of 1500-3500 and Figure 4c shows the deconvoluted mass spectra of the Fc, with a main peak at mass 52,752.9 u and adjacent peaks at mass 52,880.5 u, and 52,916.1 u, corresponding to a lysine variant and a different glycoform with 1 additional hexose. Figure 4d shows the charge envelope of Fab in the *m/z* range of 1600-3700 and Figure 4e shows the deconvoluted mass spectra of the Fab, with a single peak at mass 47317.6 u. The intact mass of mAb is determined at mass 147,387 u using the equation 2xFab+Fc. The calculated mass is more than 700 u away from the measured mass at mass148,035 u, indicating an additional fragment generated from the papain digestion.

FIGURE 4: SEC-MS analysis of mAb1 Fc and Fab under denaturing condition using 20% acetonitrile, 0.1% formic acid and 0.05% trifluoroacetic acid. mAb was injected onto a MAbPac SEC-1 4 x 300 mm column and the flow rate was set at 200 µL/min. (a) extracted ion chromatogram of Fc and Fab, (b) mass spectrum of Fc, (c) deconvoluted spectrum of Fc, (d) mass spectrum of Fab, (e) deconvoluted spectrum of Fab.



Conclusions

- mAb monomer and dimer aggregate intact mass can be measured by SEC-MS under non-denaturing condition using near neutral pH eluent, such as 20 mM ammonium formate.
- The Exactive Plus EMR mass spectrometer enables the accurate detection of mAb at *m/z* 350- 20,000.
- mAbPac SEC-1 column successfully separates the HC and LC, and partially separates Fab, and Fc fragments using denaturing eluent such as 20% acetonitrile, 0.1% formic acid, and 0.05% trifluoroacetic acid.

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Highly Sensitive, Robust MS-Based Workflow for Therapeutic Monoclonal Antibody Analysis from Complex Matrices

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Overview

Purpose: To demonstrate Mass Spectrometric Immunoassay (MSIA) workflows for the analysis of low level therapeutic monoclonal antibodies from human plasma. The model system demonstrated targeted adalimumab.

Methods: Adalimumab was purified from human plasma by using MSIA™ Streptavidin D.A.R.T.'S.™ that were pre-treated with biotinylated TNF-α. Adalimumab was selectively purified from human plasma by binding to an immobilized TNF-α antigen. The purified adalimumab was then either (1) reduced for downstream intact analysis or (2) reduced/alkylated/ trypsinized for bottom-up analysis by High Resolution Accurate Mass Spectrometry (HRAM MS) detection with the Thermo Scientific™ Q Exactive™ mass spectrometer.

Results: The ability to reproducibly detect adalimumab at concentrations as low as 5 ng/mL directly from human plasma.

Introduction

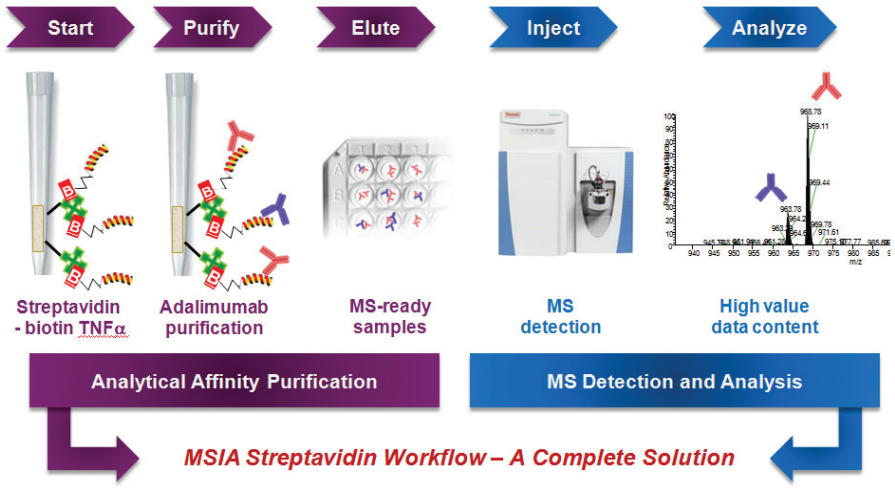
Monoclonal antibodies, and their derivative forms (Antibody Drug Conjugates, Single Domain Antibodies, Fragment Antibodies, etc), are rapidly becoming the preferred drug choice in the treatment of numerous diseases. This preference stems from their improved efficacy, selectivity, and decreased side effects, relative to conventional small molecule therapeutics. As this therapeutic class is rapidly evolving and expanding utility into other indications, it is quickly being recognized that new analytical tools are required for for the accurate and consistent analytical measurement of these drugs. Improved methods that determine quantity, character (Drug Antibody Ratio, Biotransformation, etc) and functionality, are all paramount to any drug sponsor. Described here is application of the MSIA Streptavidin Workflow for the affinity purification and MS detection of adalimumab.

Methods

Sample Preparation

Human plasma was spiked with adalimumab in varying concentrations. The sample size used was 500 µL of plasma and it was diluted with 250 µL PBS.

Figure 1. MSIA Streptavidin Workflow for Therapeutic Antibodies



Analytical Affinity Purification

MSIA Streptavidin D.A.R.T.'S were treated with a solution of biotinylated TNF-α to immobilize the affinity ligand on the microcolumn surface. Plasma samples spiked with adalimumab were incubated with the TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S. Analytical affinity purification of adalimumab was achieved by repetitive pipetting (aspirating and dispensing) of the sample solution though the functionalized MSIA Streptavidin D.A.R.T.'S. Following purification, the MSIA Streptavidin D.A.R.T.'S containing bound adalimumab were washed and then treated with elution buffer to release purified adalimumab. Affinity purification steps were automated by using the Thermo Scientific™ Versette™ automated liquid handler.

In these experiments the purified adalimumab underwent two types of treatment just prior to MS analysis. Following elution, the adalimumab either subjected to (1) reduction or (2) reduction, alkylation and digestion (120 ng of trypsin) for bottom up analyses. Digested samples were internally calibrated through the addition of 5.95 fmol/µL of the Pierce Retention Time Calibration (PRTC) peptide mixture to each sample.

MS Detection and Analysis

Liquid Chromatography

For bottom-up peptide analysis, purified adalimumab (ranging from 5-500 ng/mL in plasma) was prepared by Analytical Affinity Purification (described above) and then reduced/alkylated/digested. The resulting peptide fragments were injected onto a Thermo Scientific Hypersil™ Gold aQ 2.1 x 100 mm column heated to 70 °C. Peptides were eluted at 150 µL/min using a gradient of 2-35% formic acid (0.1%) in acetonitrile in 45 minutes on an Ultimate 3000 RSLC.

For intact analysis of the reduced adalimumab, 1.8 µg/mL was recovered from plasma and injected into a Thermo Scientific™ ProSwift™ column (RP-4H 0.5 mm x 100 mm) heated to 60 °C. The heavy chain and light chain were eluted at 200 µL/min using a gradient of 15-35% formic acid (0.1%) in acetonitrile in 8.2 minutes on an Ultimate 3000 RSLC.

Mass Spectrometry

All samples were analyzed on a Q Exactive mass spectrometer.

Adalimumab digests were analyzed using a top 10 data-dependent method. Full scans were acquired at a resolving power of 70,000 (FWHM) at *m/z* 200 and an AGC target value of 1E6. HCD spectra were acquired at a resolving power of 17,500 (FWHM) at *m/z* 200 and an AGC target value of 1E5 with a normalized collision energy of 27 and a 20 second dynamic exclusion duration.

Intact heavy chain and light chain were analyzed with a full scan taken from m/z 900-4500 at a resolving power of 17,500 (FWHM) at *m/z* 200 and an AGC target value of 3E6.

Data Analysis

Bottom-up data was searched using SEQUEST® HT in Thermo Scientific™ Proteome Discoverer™ Software (1.4 SP1) against a database of adalimumab heavy chain and light chain appended with 115 common contaminant proteins. Spectra were searched with a 15 ppm precursor tolerance and a 0.02 Da fragment ion tolerance with dynamic pyroglutamylation of N-termini, oxidation of methionines, carbamidomethylation of cysteines and deamidation of asparagines and glutamines. Results were filtered to 1% FDR using Percolator.

Reduced, intact data was deconvolved using Thermo Scientific™ Protein Deconvolution™ Software (3.0) with the ReSpect™ algorithm.

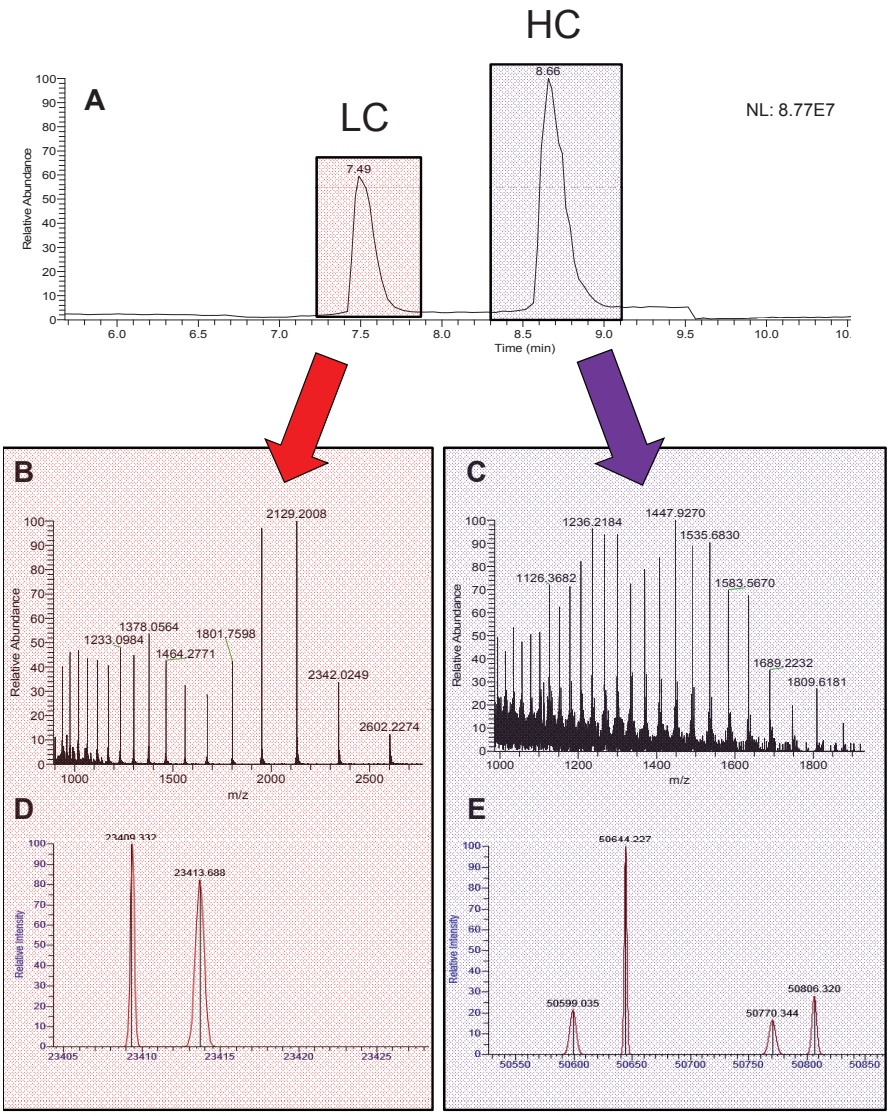
Results

The MSIA Streptavidin Workflow combines traditional ligand binding with MS detection to provide a highly sensitive, robust and reproducible method to therapeutic mAb analytics. By using the innate affinity of the therapeutic mAb for its target antigen, a metric of function is also provided as a part the general analysis, while HRAM MS detection provides additional analytical flexibility over other developing QQQ methods.

Intact Analysis of Reduced Adalimumab

Figure 1 shows the elution profile of adalimumab (500 ng spiked into plasma at 1 µg/mL) purified from human plasma. The Ab was reduced to heavy chain (HC) and light chain (LC). Deconvolution of the LC mass spectrum generated provided two masses, 23409.33 and 23413.68. This 4 Da separation is indicative of incomplete reduction of the two disulfide linkages of the LC. The mass at 23413.72 is within 1 Da of the theoretical LC average mass of adalimumab. Deconvolution of the HC mass spectrum gave a mass at 50644.22, which is suggestive of a modification by loss of the C-terminal lysine and the addition of one N-linked glycan. The mass at 50806.32 represents the addition of a hexose group.

FIGURE 1: Intact analysis of reduced adalimumab A) Base peak chromatogram of reduced adalimumab showing the elution profiles of light chain (LC) and heavy chain (HC). B) and C) Raw MS spectra for LC and HC, respectively. D) The deconvolved average mass (M+H) of LC. E) The deconvolved average mass (M+H) of HC.



Bottom-Up Analysis of Adalimumab

Plasma samples were spiked with adalimumab at 5 ng/mL, 10 ng/mL, 50 ng/mL, 500 ng/mL and 5000 ng/mL. Adalimumab was retrieved from matrix by using TNF-α-derivatized MSIA Streptavidin D.A.R.T.'S. Then, the purified analyte was subject to reduction, alkylation, and trypsin digestion prior to the LC-MS/MS sequence coverage determination and PTM analyses.

FIGURE 2. LC-MS chromatograms for 5 ng/mL adalimumab from plasma. A) Base peak chromatogram. B-D) Extracted ion chromatrograms for three adalimumab peptides. E) Extracted ion chromatogram for one PRTC standard peptide at 200 fmol.

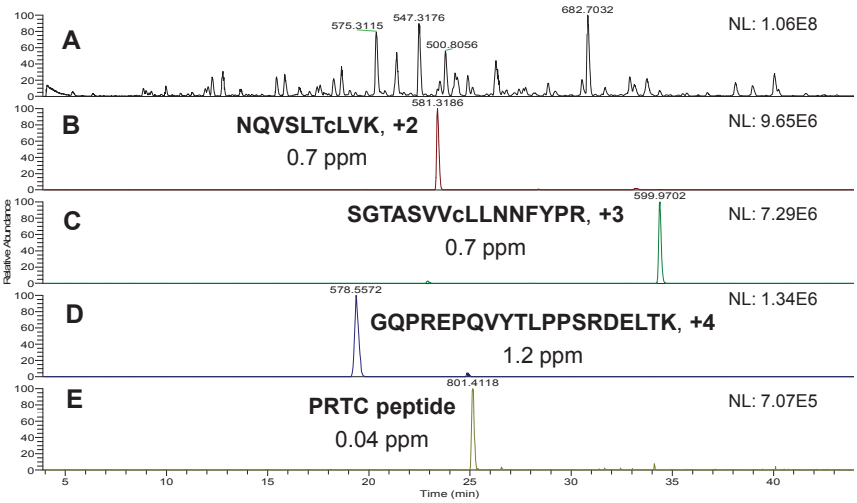


FIGURE 3. Bottom-up sequence coverage for adalimumab HC at 5 ng/mL. Note the sequence underlined in red was determined to be heavily glycosylated based on MS full scan data. C = carbamidomethylation, O = oxidation, D = deamidation.

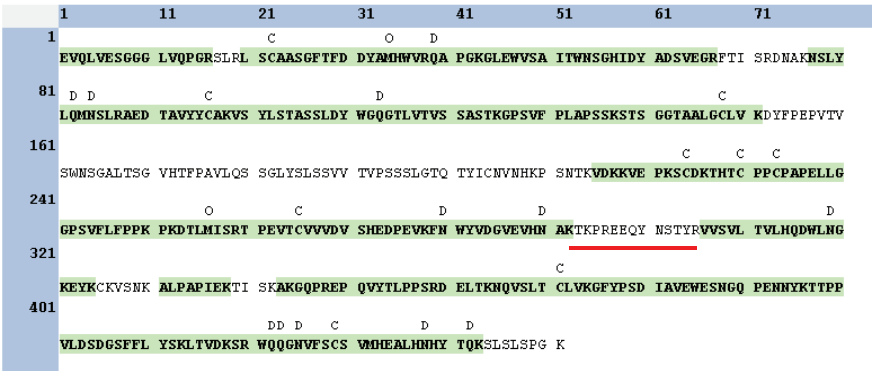
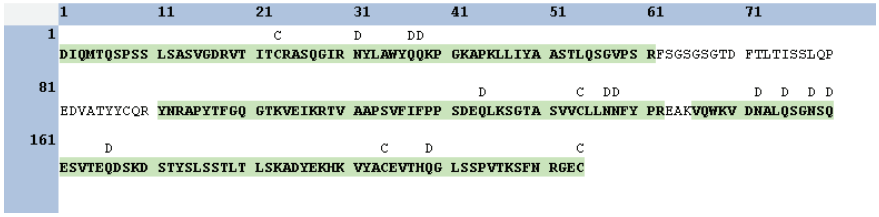


FIGURE 4. Bottom-up sequence coverage for adalimumab LC at 5 ng/mL. C = carbamidomethylation, O = oxidation, D = deamidation.



Note that we did not expect to detect the heavy chain tryptic peptide D_{152}-K_{214} nor the light chain tryptic peptide F_{62}-R_{90} due to their extreme hydrophobicities and thus incompatibilities with C18 chromatography. Therefore, with trypsinization only 81% of the heavy chain and 85% of the light chain sequences are LC-MS/MS exceptional.

TABLE 1. Bottom-up analysis of adalimumab peptides: Coverage for adalimumab from varying concentrations. Note that replicates were not performed.

	5 ng/mL	10 ng/mL	50 ng/mL	500 ng/mL	5000 ng/mL
HC % Sequence Coverage	79%	73%	63%	67%	78%
LC % Sequence Coverage	85%	75%	73%	77%	75%

*The percentage sequence coverages are based on the full heavy chain and light chain sequences.

FIGURE 5. Full MS trace showing the the adalimumab glycoform variation on heavy chain peptide TKPREEQYNSTYR at 5 ng/mL. Note the ion clusters in brackets are salt adducts.

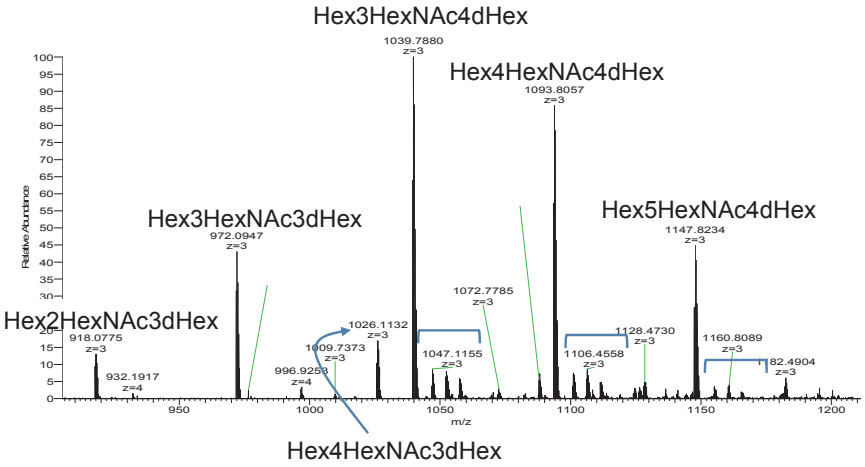
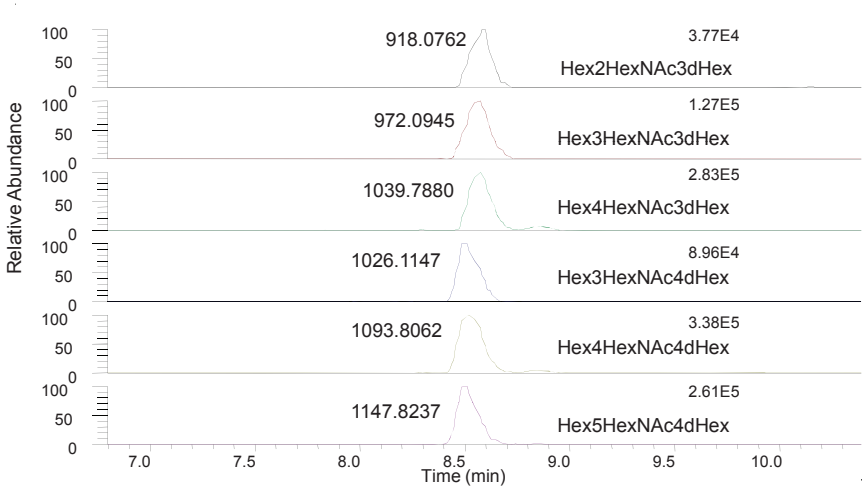


FIGURE 6. Relative retention times and abundances of the observed glycoforms of the HC peptide TKPREEQYNSTYR.



Conclusion

- The data demonstrates the capabilities of MSIA Streptavidin Workflow as a reproducible and robust method for analysis of therapeutic antibodies.
- The analytical detection limit of 5 ng/mL for adalimumab, using high flow LC, from human plasma samples was observed.
- MSIA Streptavidin Workflows overcome cross reactivity issues that plague traditional generic Fc targeting capture Ab based methods by using the target antigen as an affinity ligand.
- The described method provides highly specific characterization data. In the adalimumab model, multiple glycoforms were detected. However, this sets the foundation for other high value characterization methods, such as Drug Antibody Ratio determination of Antibody Drug Conjugates.
- The MSIA Streptavidin Workflow provides a remedy to chronic issues observed with therapeutic neutralization events and protein complexation.
- The described method sets the foundation for quantitative analysis for the simultaneous determination of quantity, character and functionality of the targeted therapeutic molecule.

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Monoclonal Antibody and Related Product Characterization under Native Conditions using a Benchtop Exactive Plus EMR MS

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Overview

Purpose: Demonstrate the characterization of mAbs, antibody-drug conjugates (ADCs), mAb/antigen (mAb/Ag) complexes, and a mixture of mAbs under their native conditions by using a high-resolution, accurate-mass (HRAM) benchtop mass spectrometer with extended mass range (EMR).

Methods: All antibodies and their related products samples were introduced using an Advion TriVersa NanoMate with chip based nanospray ionization in the positive mode to the Thermo Scientific™ Exactive Plus™ Extended Mass Range mass spectrometer. Thermo Scientific™ Orbitrap™ detection parameters were set according to the type of analyte measured. Deconvolution of signals measured for determination of molecular masses and relative abundances of the analytes were carried out using Thermo Scientific™ Protein Deconvolution software.

Results: In the analysis (0.3–5 min) using the Exactive Plus EMR MS, molecular weight measurements of mAb and related products in the low ppm mass deviation range allowed the identification of all species simultaneously present in solution. The number of drug antibody ratio (DAR) and relative abundance of mAb/Ag complexes was also assessed with the peak intensities serving for relative quantification of the detected species.

Introduction

Native mass spectrometry (MS) has emerged as a valuable technique for characterization of intact non-covalent protein complexes, reaching a high level of reliability within the last ten years. [1] For the analysis of intact monoclonal antibodies (mAbs), native MS yields accurate mass measurements of the molecules, glycoform identification, and assessment of higher-order structures (dimer, trimer, tetramer), thus providing a robust, fast, and reliable first-line analytical characterization tool. [2,3]

This poster describes the use of a new Orbitrap mass spectrometer with an extended mass range of up to *m/z* 20,000 and improved detection of high-mass ions for the characterization of mAbs, ADCs, mAb/Ag, and mAb mixtures under native conditions.

FIGURE 1. Exactive Plus EMR mass spectrometer equipped with a TriVersa NanoMate chip-based electrospray ionization interface

Methods

Sample Preparation

The intact trastuzumab (Herceptin®, Roche), the monoclonal antibody-drug conjugate (ADC) brentuximab vedotin (Adcetris®, Seattle Genetics), the mAb/antigen complexes of J10.4 mAb/JAM-A, and one mixture of eleven distinct IgG antibodies were introduced using the TriVersa NanoMate® into the Exactive Plus EMR Orbitrap mass spectrometer. Titration experiments involving J10.4 mAb and JAM-A were monitored by native MS. The fixed amount of J10.4 (5 µM) was incubated with increasing amounts (1:1, 1:2, 1:4, 1:8) of JAM-A up to 40 µM. The mixture of eleven distinct deglycosylated humanized IgG antibodies included two marketed therapeutic mAbs (rituximab and trastuzumab) and nine point mutation variants of the Hz6F4-2 mAb [4, 5]. They were mixed together prior to PNGase-F deglycosylation. Finally, all the samples were buffer exchanged against 150 mM ammonium acetate (AcONH4) pH 7.5. trastuzumab, deglycosylated brentuximab vedotin, and the mAb/antigen complexes of J10.4 mAb/JAM-A were injected at 5 µM, and the deglycosylated IgG mixture was injected at 1 µM on the Exactive Plus EMR Orbitrap mass spectrometer.

Direct-Infusion Native MS Conditions

Chip-based infusion conditions	
Instrumentation	TriVersa NanoMate® (Advion, USA) system
Ionization voltage 9kV)	1.6-1.8
Gas pressure (psi)	0.3 – 0.6
The ESI ChipR consists of an array of 400 nanoelectrospray emitters with 5 µm inner diameters.	

MS conditions	
Instrumentation	Exactive Plus EMR Orbitrap MS system (Figure 1)
Mass Range	350 – 20,000
Resolution	17,500 to 140,000, depending on spectral complexity
Target value	3×10^6
Microscans	10
Maximal injection time (ms)	300
Insource CID energy (eV)	60 to150 eV, manually tuned for optimized desolvation
S-lens level (%)	100 to 200, manually tuned for optimized transmission and avoiding in-source fragmentation
Trapping gas pressure setting factor	4
Spectra average	Enabled (10 to 50 scans are averaged to achieve S/N ratio > 100)

Data Analysis	
Software	Protein Deconvolution software version 2.0 Sp2 and version 3.0
Deconvolution parameters	
Number of iterations	4
Noise compensation	On
Minimum adjacent charges	1 to 3

Results

High-Resolution Native MS Analysis of Intact Monoclonal Antibody Trastuzumab

Trastuzumab was analyzed on the Exactive Plus EMR MS with resolution set at both 17,500 and 35,000 (Figure 2A). The major glycoforms of the antibody are baseline resolved at 17,5000 resolution. An interference peak can be resolved by using a higher resolution, 35,000. Molecular weights of each trastuzumab glycoform were therefore measured with good mass accuracy in the low ppm range, as shown in Figure 2B. The mass differences between species are +146 Da and +162 Da, corresponding to a fucose or to the addition of multiple hexose units, respectively.

Orbitrap Native MS Analysis of a Monoclonal Antibody-Drug-Conjugate (ADC) Brentuximab Vedotin

The brentuximab vedotin mass spectrum was recorded at a resolution of 35,000 and in-source CID voltage was set to 75 eV. Figure 3A shows the native deconvoluted mass spectrum of the deglycosylated ADC. Populations with zero (grey), two (black), four (blue), six (red), and eight (green) molecules loaded onto the antibody (payloads) were detected with a mass difference between peaks corresponding to the addition of two payloads (+2,634 Da). For each set of peaks, the drug-to-antibody ratio (DAR) can be determined. Relative ratios of each detected compound were determined using MS peak intensities and served to estimate the mean DAR (4.2), which is in agreement with hydrophobic chromatography data (data not shown).

FIGURE 2. Orbitrap native MS detection of intact monoclonal antibody trastuzumab. A. High-resolution, native MS showing complete mass spectrum and zoom of corresponding 23+ charge state. B. Deconvoluted spectrum showing molecular weights of each trastuzumab glycoform with low ppm mass accuracy.

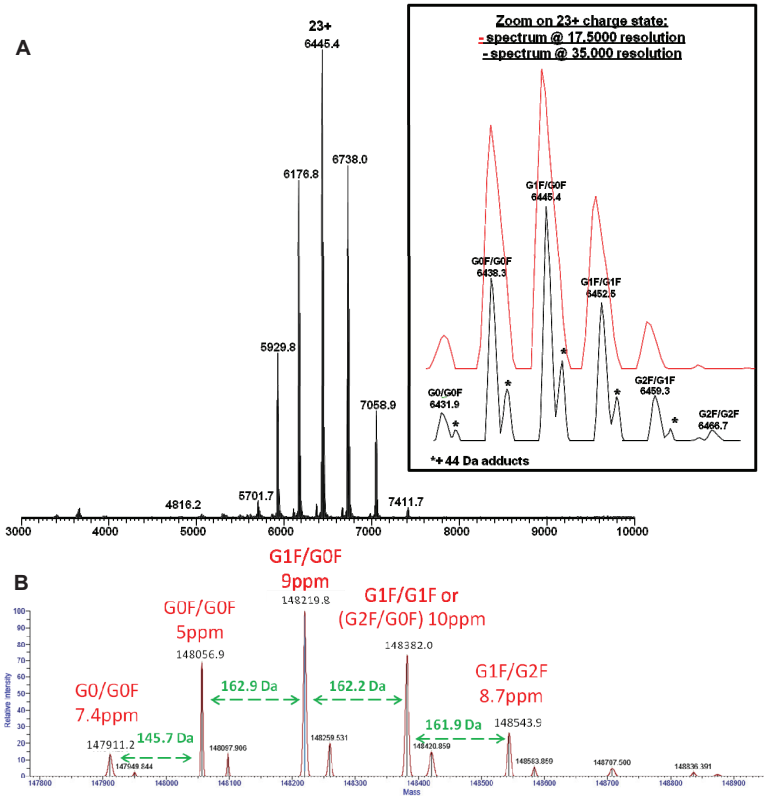
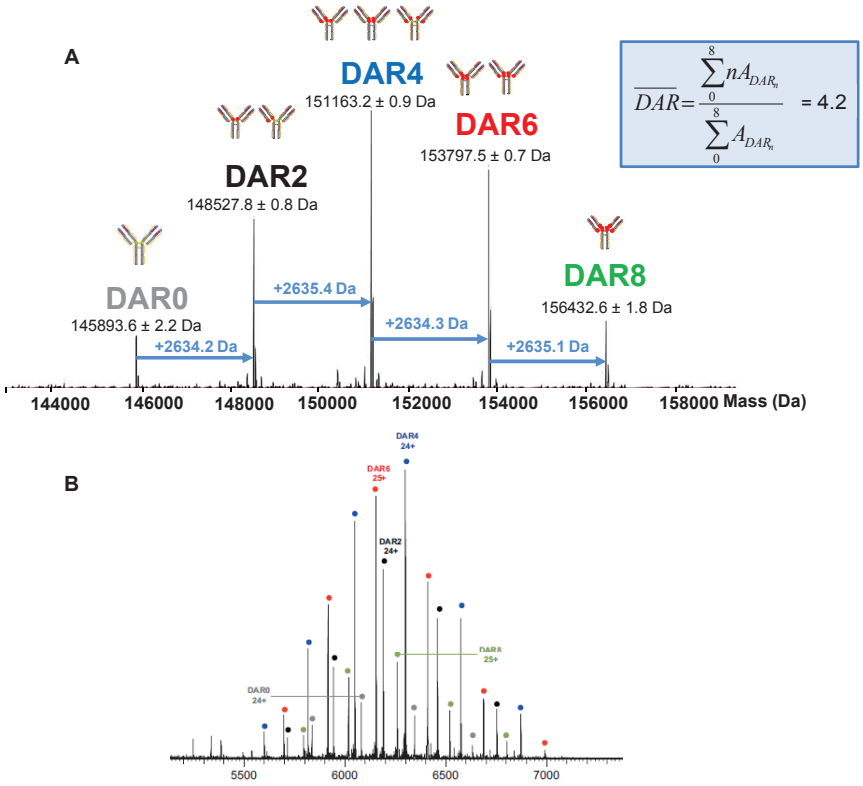


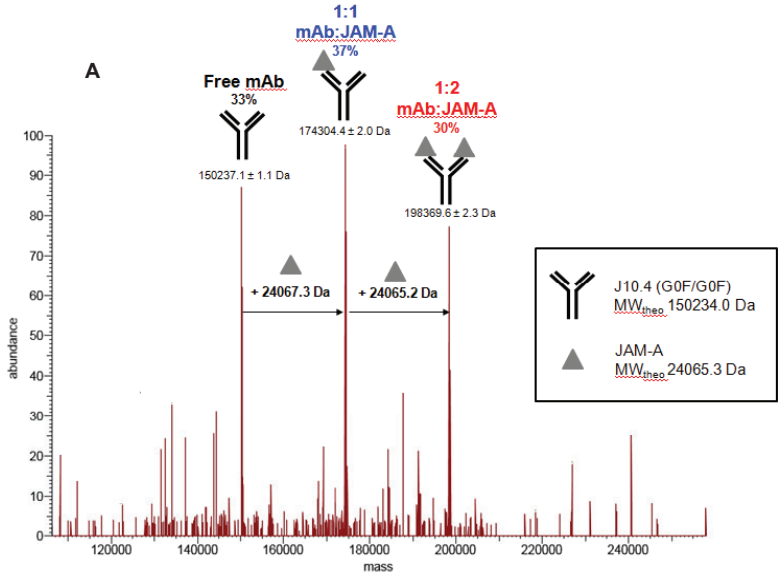
FIGURE 3. Orbitrap Native MS analysis of a monoclonal Antibody-Drug-Conjugate (ADC). A. Native deconvoluted mass spectrum showing the determination of drug-to-antibody ratio (DAR). B. Raw mass spectrum with the entire charge state distribution of ADC under native conditions.

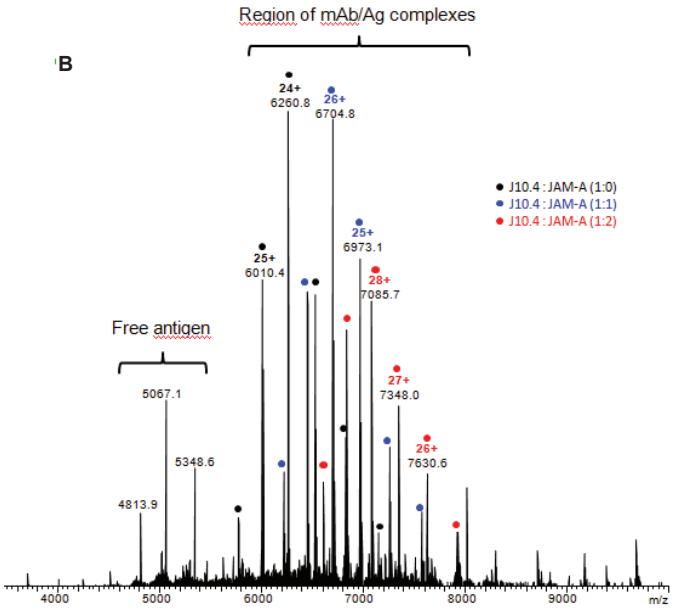


Orbitrap Native MS Analysis of Immune mAb/Antigen Complexes

The native mass spectrum of mAb/antigen complexes was recorded at a resolution of 35,000 with the in-source CID voltage set to 150 eV. As shown in Figure 4A, when an 4-fold excess of JAM-A (20 µM) is added to J10.4 mAb (5 µM), three species are detected: the intact free mAb (MW 150237.1 ± 1.1 Da, black), 1:1 (MW 174304.4 ± 2.0 Da, blue) and 1:2 (MW 198369.6 ± 2.3Da, red) mAb:JAM-A complexes. Native MS thus confirmed that two JAM-A molecules can bind to J10.4 mAb. MWs correspond to the main G0F/G0F glycoforms. Relative abundances were estimated from MS peak intensities and proportions of mAb:Ag complexes at 1:1 and 1:2 stoichiometries were observed to be 37% and 30%, respectively, while free mAb represents 33%. Figure 4B shows the corresponding mass spectrum with the entire charge state distribution in native conditions.

Figure 4. Orbitrap native MS detection of immune mAb/antigen complexes. A. Deconvoluted mass spectrum showing mAb/antigen binding stoichiometries. B. Charge state distribution in native conditions.





Native MS Analysis of a Mixture of Eleven N-deglycosylated Humanized Antibodies

Figure 5 presents a deconvoluted mass spectrum of a mixture of eleven distinct deglycosylated humanized IgG antibodies. The well-resolved ion signals at a detection resolution of 140,000 and accurately measured masses enable the unambiguous assignment of ten out of the eleven compounds. Trastuzumab and Hz6F4-2v6 could not be differentiated due to very close molecular weights (2 Da). Peaks corresponding to Hz6F4-2 and Hz6F4-2v3, which differ by only 21 Da in mass, are clearly distinguished on the mass spectrum. However, they are not baseline resolved, and when combined with the low signal-to-noise (S/N) ratio (S/N < 20), that causes a relatively low mass accuracy for Hz6F4-2. However, with a good signal-to-noise ratio (S/N > 50), even without baseline-resolved peaks, for example, peaks of Hz6F4-2v9 and 6F4-2v10, the mass accuracies are achieved in the low ppm range for both species (see Table 1).

Figure 5. Deconvoluted mass spectrum of the Native MS analysis of a mixture of eleven N-deglycosylated humanized antibodies.

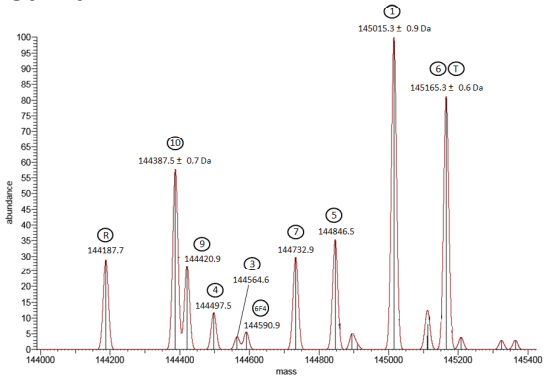


TABLE 1. Measured and theoretical masses for the mixture of eleven N-deglycosylated humanized antibodies at an Orbitrap detection resolution of 140k.

	Species	Theoretical masses (Da)	Measured masses (Da)	Mass accuracy (ppm)
R	Rituximab	144186.3	144187.7	9.7
10	6F4-2 v10	144388.3	144387.5	5.5
9	6F4-2 v9	144420.5	144420.9	2.8
4	6F4-2 v 4	144498.4	144497.5	6.2
3	6F4-2 v3	144564.4	144564.6	1.4
6F4	6F4-2	144585.5	144590.9	37.3
7	6F4-2 v7	144732.5	144732.9	2.8
5	6F4-2 v5	144846.9	144846.5	2.8
1	6F4-2 v1	145015.3	145015.3	0
6	6F4-2 v6	145163.3	N.D	N.D
T	Trastuzumab	145165.5	145165.3	1.4

Conclusion

- The Orbitrap mass analyzer can baseline resolve a native mAb's glycan peaks, as well as the interference peaks, ensuring excellent mass accuracy in the low ppm range.
- The Exactive Plus EMR MS is able to sensitively characterize ADC complexes with mass differences between peaks corresponding to different additional number of payloads/drugs. For each set of peaks, the drug-to-antibody ratio (DAR) can be determined as well as the relative ratio of each detected compound in order to assess the mean DAR value.
- Native Orbitrap MS can reveal the number of antigens bound to mAbs. Relative abundances of mAb/Ag complexes at different stoichiometries can be achieved from MS peak intensities.
- The Exactive Plus EMR MS enables the high throughput screening of mAb mixtures, ensuring excellent mass accuracy for each individual mAb.

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Structure Characterization of Intact Monoclonal Antibody Using an Orbitrap Tribrid Mass Spectrometer

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Overview

Purpose: Structural characterization of intact mAb method coupling capillary LC with new generation Orbitrap mass spectrometer.

Methods: An intact monoclonal antibody (mAb) molecule mass was measured by Thermo Scientific™ Orbitrap™ Fusion™ MS. Mass spectra of the intact protein were analyzed by Thermo Scientific™ Protein Deconvolution™ software. Both Thermo Scientific™ Orbitrap Elite™ MS and Orbitrap Fusion MS systems were used for mAb top-down analysis. ETD and HCD MS/MS data were analyzed by Thermo Scientific™ ProSightPC™ 3.0 software.

Results: mAb was accurately measured with low ppm mass error and multiple glycoforms were accurately identified. About 70% backbone sequence coverage for light chain and 20% for heavy chain was achieved using top-down analysis.

Introduction

Monoclonal antibodies (mAbs) are increasingly being developed for the detection and treatment of diseases. The quality of the antibodies is crucial for both safety and efficacy. Due to the heterogeneity of mAb products, thorough characterization is very challenging. Among the analytical tools used for the analysis of therapeutic mAb, high resolution accurate mass (HRAM) mass spectrometry (MS) has become increasingly important for the determination and quantification of the various glycoforms, confirmation of amino acid sequence and post-translational modifications, and detection of minor impurities and high order structure. In this study, new methods coupling capillary LC with a new generation Orbitrap mass spectrometer were developed and optimized for structural characterization of an intact mAb.

Methods

Sample Preparation

A monocolonal antibody (mAb) (NIST and purchased from Waters) were diluted with 0.1% FA in water for intact protein analysis. The reduced monoclonal antibody was prepared in 6 M Guanidine , 100 mM DTT, and heated at 60 °C for 30 min prior to top-down analysis.

Liquid Chromatography

Thermo Scientific™ Dionex™ ProSwift™ RP-10R monolithic columns (200 μm x 25 cm or 1 mm x 5 cm) were used for desalting prior to intact mass measurement. 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B) were used as the LC solvents. The column was heated to 60 °C and the flow rate was 800 nL/min. After injection of 100 ng mAb, an 8 min gradient was used to elute mAbs from the 200 μm x 25 cm column (0.0min, 10%B; 1.0min, 90%). For the 1 mm x 5 cm column, the flow rate was 80 μL/min using the same gradient.

A Thermo Scientific™ Dionex™ PepSwift™ 500 µm x 5 cm column was used for heavy and light chain separation. Flow rate was 10 μL/min. 2 μg of reduced antibody was injected, a 3 to 5 min 5%B to 25%B and 5 min to 11 min 25%B to 40% gradient was used to separate the heavy and light chain.

Mass Spectrometry

The samples were directly injected from the capillary LC to either an Orbitrap Fusion or Orbitrap Elite mass spectrometer. The Orbitrap Fusion MS was used for both antibody intact mass measurement and top-down analysis. An Orbitrap Elite MS was also used for top-down analysis. For the intact antibody, various parameters including ion transfer tube temperature, in-source activation energy, s-lens values, AGC target and others were optimized. For top-down analysis, the ETD and HCD were performed in separate runs to achieve maximum combined sequence coverage. For ETD MS/MS, the activation time was 5 to 50 msec while for HCD MS/MS, normalized collision energy level was 10%-25 %. For the source conditions, the spray voltage was set to 4kV, the sheath gas flow rate was set at 8, the capillary temperature was 225 °C, the S-lens level was set at 55 and in-source CID was set at 45 eV. Resolution was set at 120,000 and AGC target was set to 1E6 for top-down MS/MS while maximum IT was set at 250 ms.

Data Analysis

The MS full spectra for intact mAb were analyzed using Protein Deconvolution software using ReSpect™ or Xtract deconvolution. The top-down ETD and HCD spectra were analyzed using ProSightPC 3.0 software.

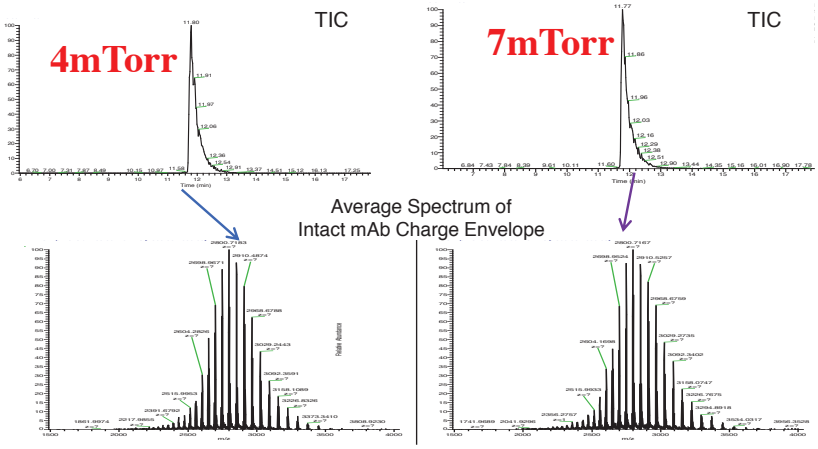
Results

Intact mAb Mass Measurement

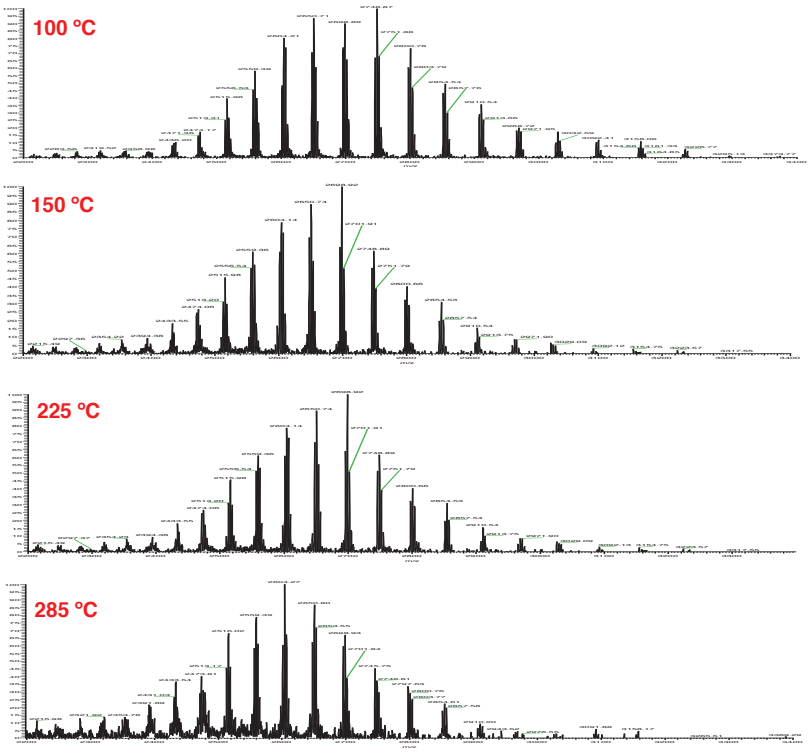
Several mass spectrometer experiment parameters including HCD cell pressure, source fragmentation energy, AGC target and source heating temperature of the ESI probe were optimized for intact mAb mass measurement. 4 mTorr and 7 mTorr HCD cell pressure (Figure 1a), source fragmentation energy of 60 and 100, MS full AGC target 5e4 and 1e5 were used and the results showed that these parameters had no significant impact on the intact mAb mass measurement. However, it was found that the capillary temperature plays a critical role for detection of the intact mAb. Figure 1b shows the mass spectra for the intact mAb at four capillary temperatures ranging from 100 ºC to 285 ºC As the temperature increased from 100 ºC to 285 ºC, the charge envelope for the intact mAb moved from higher to lower *m/z* with more than one order of magnitude sensitivity drop. The mAb also started to show signs of degradation at the highest temperature. 225 ºC was the temperature that still maintained the intact mAb and the sensitivity as a operation temperature.

FIGURE 1. Experiment parameter optimization

a) Example mAb analysis under different HCD cell pressure



b) Capillary temperature optimization



Using the optimized experiment parameters, 100 ng of mAb triplicate runs were used to evaluate the reproducibility of the experiment. Figure 2 shows the results of this reproducibility analysis, with the results looking highly similar between the three runs. 100 ng of mAb was then analyzed with the optimized experiment condition. The molecular mass was measured with less than 15 ppm mass error for the analyzed mAb. All five different glycoforms were also accurately identified. The results showed in Figure 3.

FIGURE 2. Reproducibility of mAb analysis with Optimized experiment condition

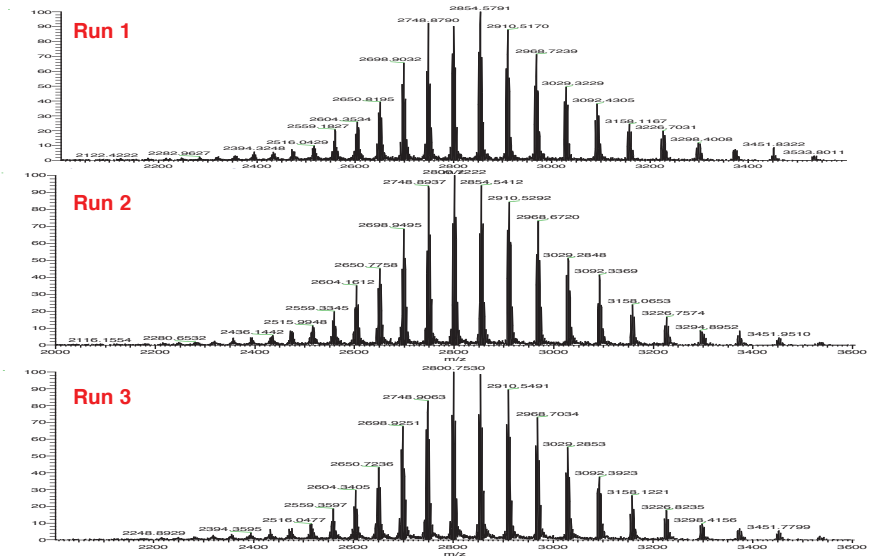
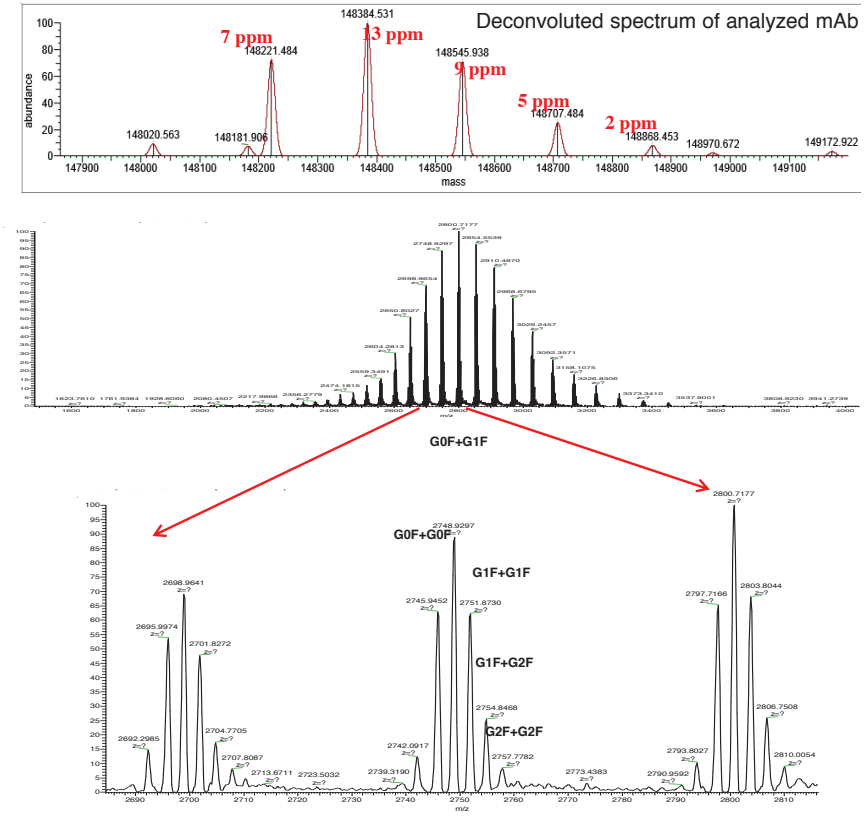


FIGURE 3. mAb mass measurement with optimized experiment conditions, 100ng on PepSwift column.



Top-down LC-MS/MS of mAb light and heavy chain

To characterize the mAb sequence, top-down LC-MS/MS analysis was performed for reduced mAb light and heavy chain with both ETD and HCD. Figure 4 a) is the workflow diagram for the analysis. ETD and HCD MS/MS spectra were acquired in two separate LC-MS runs. The molecular mass of both the light and heavy chains were measured with low ppm mass accuracy (data not shown). ETD with a reaction time of 15 ms generated the most fragmentation coverage with 52% of light chain backbone sites fragmented. An HCD collision energy of 18% yielded the most fragmentation with a fragmentation coverage of 39%. The combination of the ETD and HCD data produced around 67% fragmentation coverage (Figure 5).

Figure 4 Top-down LC-MS/MS of mAb light and heavy chain

a) The online top-down MS/MS of NIST mAb light and heavy chain analysis with both ETD and HCD

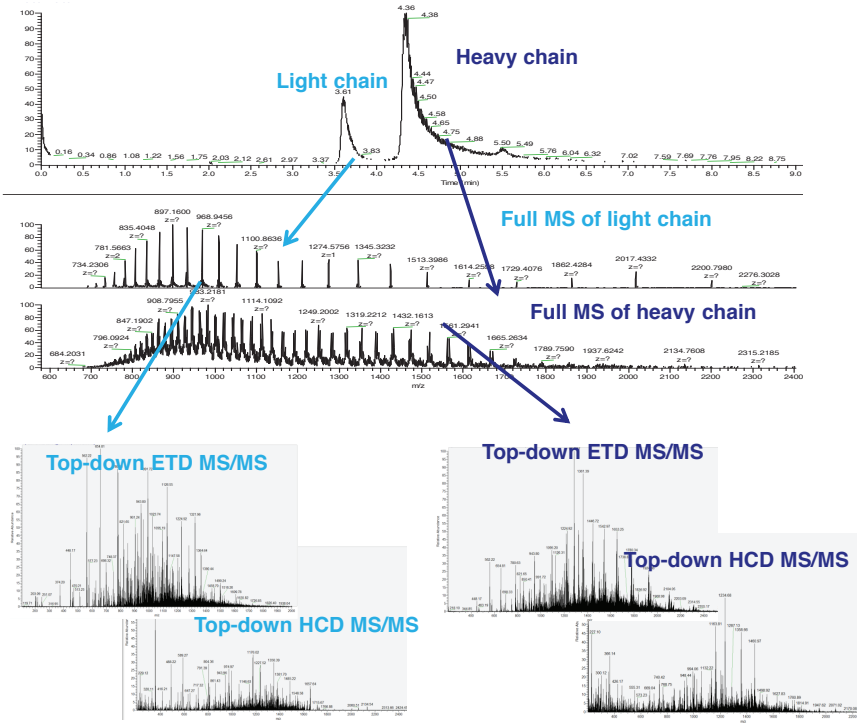
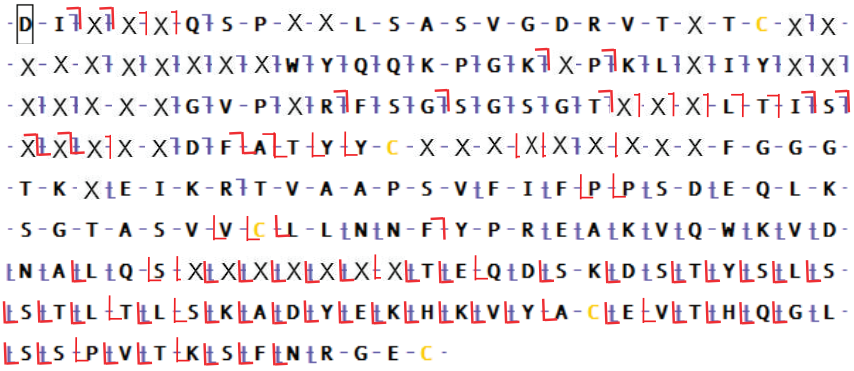


FIGURE 5. Top-down LC-MS/MS of NIST mAb light chainusing Orbitrap Elite. Sequence coverage from ETD (blue) and HCD (red) using Orbitrap Elite.



Deglycosylated and reduced mAb was infused and analyzed by Orbitrap Fusion. Six ms ETD reaction time and 14% HCD collision energy were used for the separate ETD and HCD fragmentation spectra. Around 22% backbone fragmentation coverage was achieved combining both ETD and HCD (Figure 6).

FIGURE 6. Example mAb heavy chain sequence coverage of ETD (blue, 6ms) and HCD (red, 15%) using Orbitrap Fusion MS.

Conclusion

- The monoclonal antibody could be successfully analyzed by both Orbitrap Elite and Orbitrap Fusion mass spectrometers.
- With optimized experiment conditions, a molecular mass for several glycoforms of the example monoclonal antibody was accurately measured with low ppm mass error using Orbitrap Fusion MS.
- 67% fragment coverage was obtained for NIST mAb light chain using Orbitrap Elite with LC-MS analysis.
- 22% fragment coverage was obtained for example mAb heavy chain using Orbitrap Fusion MS with infusion.

Acknowledgements

We would like to thank NIST for providing us with the monoclonal antibody.

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Analysis of Intact Macromolecular Assemblies On A Bench Top Orbitrap MS System

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Thermo SCIENTIFIC

Overview

Purpose: Measure proteins in native like state to study the biologically active conformations.

Methods: Protein samples were measured using a Thermo Scientific™ Exactive Plus EMR™ mass spectrometer with chip based static nanospray using an Advion™ TriVersa™ Nanomate™.

Results: It could be shown that for proteins of various molecular weight clear separation of isotope resp. isoform signals could be achieved due to full desolvation even under native conditions.

Introduction

Intact proteins are routinely measured using ESI-MS instrumentation under acidic, denaturing conditions, destroying large, non covalent protein assemblies and substrate bound complexes. Under native conditions, fully active protein assemblies can be studied, but these experiments are challenging due to the limited surface area of protein complexes for protonation at physiological pH. Ion signals are shifted to higher *m/z* values, which until recently, only TOF instruments were capable of detecting. TOF measurements have limited achievable resolution, making it difficult to resolve specific isoforms or substrate complexes. With an Exactive Plus EMR bench top Orbitrap instrument we were able to detect ion signals up to *m/z* 20,000 with high mass resolving power thus achieving remarkable signal distribution and precision for various large protein assemblies.

Methods

Sample Preparation

Carbonic Anhydrase, Herceptin and Pyruvate Kinase are commercially available and were purchased from Sigma-Aldrich, Germany. GroEL samples were provides by the laboratory of Prof. Dr. Albert Heck, Utrecht, The Nehterlands. All samples were desalted prior measurement using Bio-Rad™ Micro BioSpin™ columns, following the instructions of the BioSpin column manual. Desalting changed any storage buffer system to 5 µM ammonium acetate buffer, pH 6.8 ± 0.2.

Sample introduction

Samples were introduced using an Advion TriVersa NanoMate with chip based nanospray ionization in positive mode, using an Advion nozzle chip with an internal spray nozzle diameter of 5 µm. According to the manufacturer's specifications this should result in a sample flow of approx. 100 nL/min.

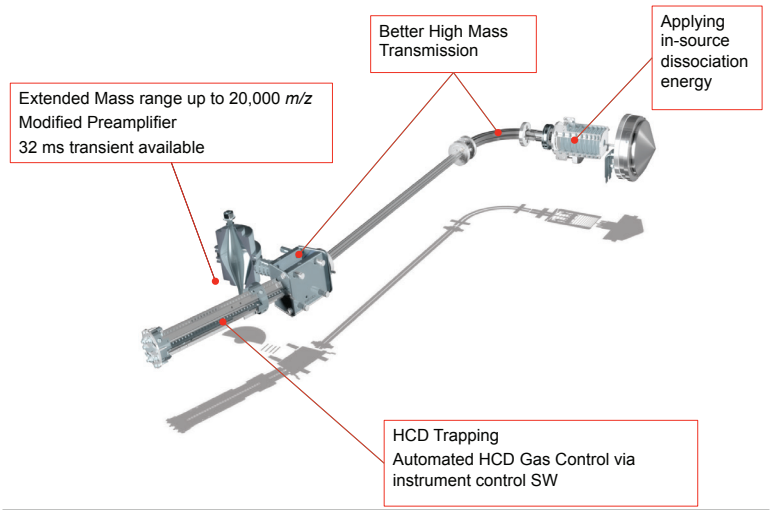
Mass Spectrometry

All analyses were carried out on a Thermo Scientific Exactive Plus EMR mass spectrometer. Detection parameters were set according to the type of analyte measured.

Data Analysis

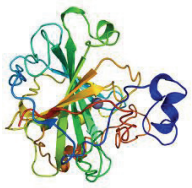
Deconvolution of signals measured for determination of molecular masses of the analytes were carried out using ProteinDeconvolution 2.0 SP2 software.

FIGURE 1. Improved ion path in the Exactive Plus EMR mass spectrometer



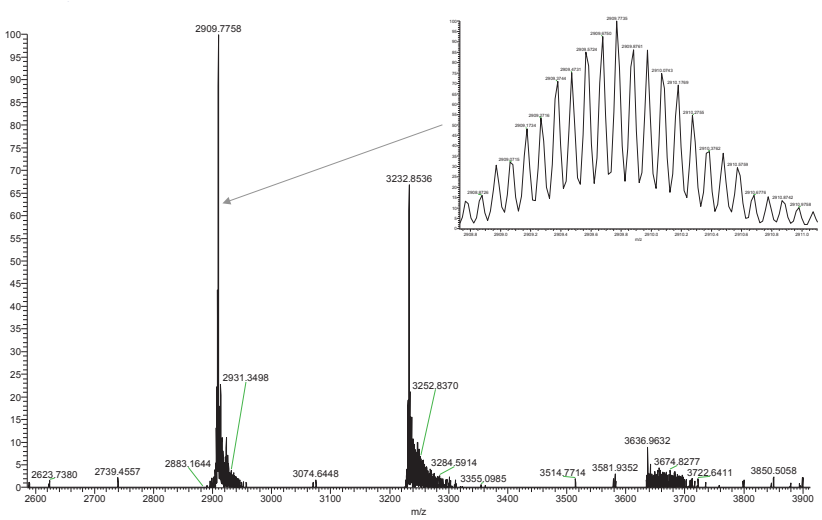
Results

Carbonic Anhydrase



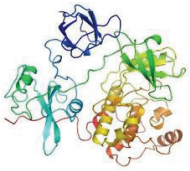
Smaller proteins can easily measured by mass spectrometry, but under native conditions even these show quite different behavior. The signal of carbonic anhydrase for example, as member of this group of proteins, is reduced to two major charge states, making a classical deconvolution based on charge envelop pattern difficult. Due to the high resolution of the Orbitrap detection system, this 29 kDa protein still can be isotopically resolved (see fig. 2).

FIGURE 2. Spectrum of carbonic anhydrase in native state. Isotopic resolution is easily achieved.



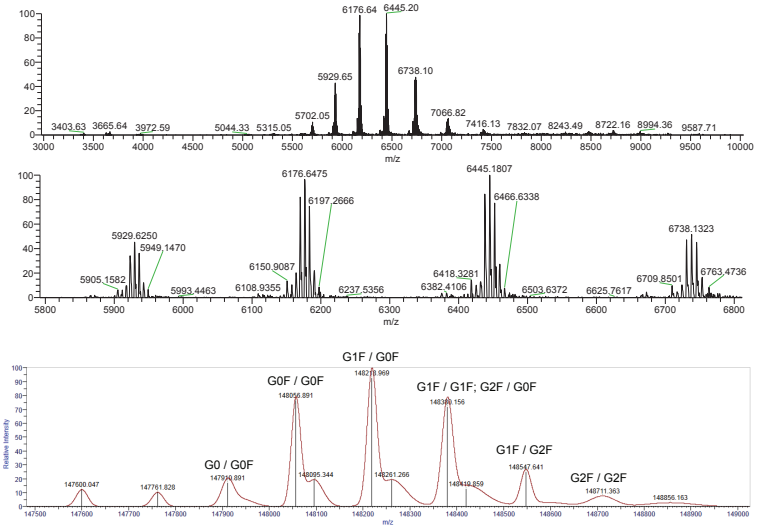
Here the +9 charge state deconvolutes to a mass of 29069.6107 Da. The active protein zinc containing molecule has a theoretical mass of 29069.6034, relating to a mass accuracy of 0.25 ppm.

Herceptin

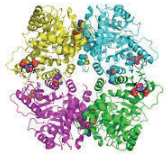


Herceptin is a therapeutic antibody in cancer treatment. The determination of the glycolysation status is important for characterization and quality control. For Herceptin, we could achieve a clear baseline separation and assignment of the major glycoforms known (see fig. 3). In addition, present interfering adducts could be resolved clearly enough to separate them from the antibody signal, so a correct mass assignment could be achieved.

FIGURE 3. Experimental and deconvoluted spectrum of Herceptin, showing clear resolution of glycoforms and even resolving smaller adducts which would affect mass accuracy if not resolved

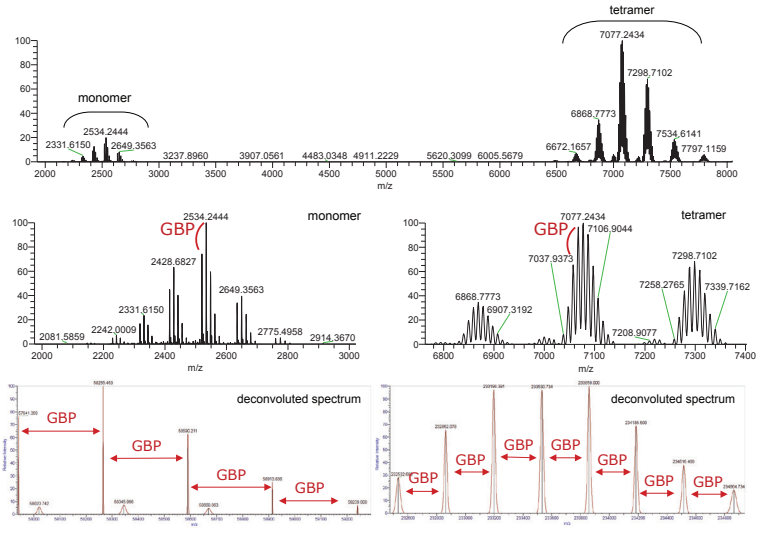


Pyruvate Kinase

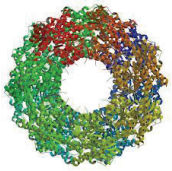


In native state Pyruvate kinase is a tetrameric protein assembly of intermediate size. The full protein assembly appeared as a strong signal in the spectrum and due to full desolvation of the molecules in the mass spectrometer the full pattern of isoform combinations is visible (see fig. 4). Upon slight application for fragmentation energy the monomeric subunits with their isoform pattern are visible together with the full assembly. The mass difference of 324 amu is clearly visible in the deconvoluted spectra of the subunit as well as the multitude of isoform combinations for the tetrameric complex.

FIGURE 4. Experimental and deconvoluted spectra of Pyruvate Kinase showing the resolution of the isoforms with bound 2,5-anhydro-D-glucitol 1,6-bisphosphate (GBP)

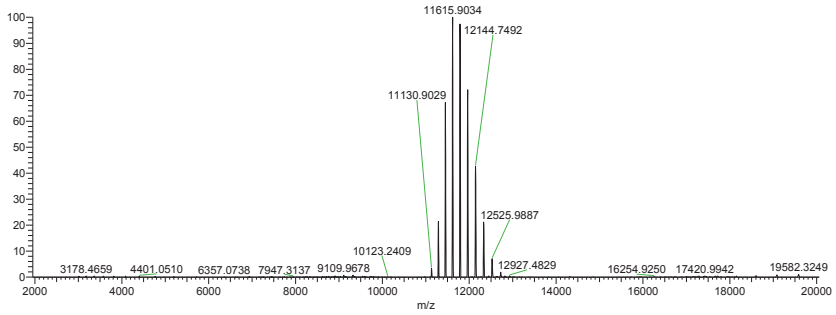


GroEL



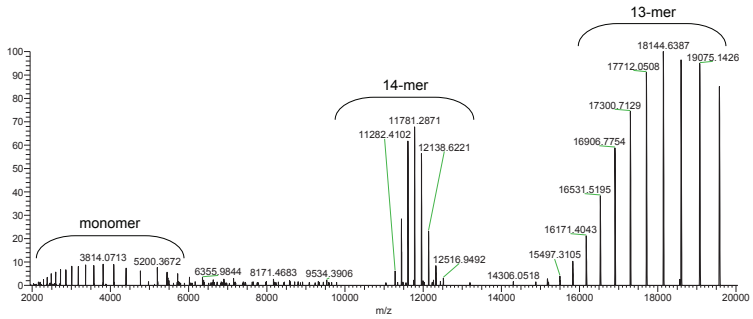
The chaperone protein GroEL from E. coli belongs to the group large proteins with a mass of roughly 800 kDa of the fulllyactive 14-mer complex. With proteins of this size, the resolving power of the MS instrumentation becomes a minor part for successful resolution of isoforms and conjugates, but desolvation capabilities are the limiting factor. With GroEL we could achieve full desolvation resulting in sharp baseline separation of the different charge states (see fig. 5).

FIGURE 5. Spectrum of GroEL, showing the full assembly of the 14-mer



Application of elevated fragmentation energy lead to fragmentation of the assembly. The charge envelop of the first fragment, the 13-mer became the dominant signal reaching up to the upper mass range limint of *m/z* 20000, while the monomer signals were visible at the lower end of the spectrum at the same time (see fig 6).

FIGURE 6. HCD spectrum of GroEL, showing the full assembly of the 14-mer together with the first fragmentation step (13-mer) and the according monomer signals.



Conclusion

With a Thermo Scientific Exactive Plus EMR mass spectrometer proteins easily can be studied in their native state, revealing the accurate mass of the fully active protein complex as well as clear separation of isoforms and conjugates. Large protein assemblies can be fragmented down to significant sub-assemblies and monomers for in-detail evaluation of quarternary structures.

For small proteins up to 35 kDa isotopic resolution can be achieved for determination of the monoisotopic mass. Larger proteins show clear separated signals for isoforms and conjugates. Acquisition time per compound lies in the rang of seconds.

Sample introduction with the Advion TriVersa NanoMate proved to be easy to handle, providing reproducible and stable spray conditions for best quality spectra acquired with minimum time consumption. It allows for automated data acquisition for maximum sample throughput.

Acknowledgements

We would like to thank Professor Albert Heck and his group from the University of Uthrecht, The Netherlands, for supplying samples of E. coli GroEL.

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Intact Mass Analysis of Monoclonal Antibody (MAb) Charge Variants Separated Using Linear pH Gradient

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Overview

Purpose: Intact mass analysis of monoclonal antibody (MAb) charge variants separated using linear pH gradient.

Methods: The separation of MAb charge variants is achieved using linear pH gradient method on a cation-exchange column. The intact mass information is acquired on the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer.

Results: This linear pH gradient enables the high resolution separation of MAb charge variants. The intact mass analysis characterizes the structural difference of the MAb variants.

Introduction

Monoclonal antibodies can be highly heterogeneous due to modifications such as sialylation, deamidation, and C-terminal lysine truncation. Salt gradient cation-exchange chromatography has been used with some success in characterizing MAb charge variants. However, additional effort is often required to tailor the salt gradient method for an individual MAb. In the fast-paced drug development environment, a platform method is desired to accommodate the majority of the MAb analyses.

In 2009, Dell and Moreno reported a method to separate MAb charge variants using pH gradient ion-exchange chromatography. The buffer employed to generate the pH gradient consisted of piperazine, imidazole, and tris, covering a pH range of 6 to 9.5. While good separation was observed, the slope of the pH increase was shallow at the beginning and steep towards the end.[1] In this study, we present a novel pH gradient method for cation-exchange chromatography that is more linear. This method features a multi-component buffer system in which the linear gradient was run from 100% eluent A (low pH buffer) to 100% eluent B (high pH buffer). Using an online pH meter, it was confirmed that a linear pH gradient was achieved. Furthermore, a plot of measured pH values at the retention time of model proteins versus their pI values exhibited a high correlation. Once the approximate pH elution range of the target MAb has been established in the initial run, further optimization of separation can simply be achieved by running a shallower pH gradient in a narrower pH range.

Methods

Sample Preparation:

All standard proteins were purchased from Sigma. Harvest cell culture and monoclonal antibodies were a gift from a local biotech company. Proteins and MAb were dissolved in deionized water.

Column and Buffer

Thermo Scientific™ MAbPac™ SCX-10, 10 µm, 4 × 250 mm (P/N 074625)

CX-1 pH Gradient Buffer Kit (P/N 083274)

Liquid Chromatography

HPLC experiments were carried out using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS System equipped with:

- SRD-3600 Membrane Degasser
- DGP-3600RS Biocompatible Dual Gradient Rapid Separation Pump
- TCC-3000SD Thermostatted Column Compartment with two biocompatible 10-port valves
- WPS-3000TBRS Biocompatible Rapid Separation Thermostatted Autosampler
- VWD-3400RS UV Detector equipped with a Micro Flow Cell
- PCM-3000 pH and Conductivity Monitor

Column and Buffer

The CX-1 pH buffer kit consists of one bottle of 10X buffer A (pH 5.6) and one bottle of 10X buffer B (pH 10.2). Eluent A and B each was prepared by simply diluting the corresponding 10 X buffer 10 fold using deionized water.

Linear pH Gradient Chromatography
The linear pH gradient was generated by running linear gradient from 100% eluent A (pH 5.6) to 100% eluent B (pH 10.2). For pH gradient analysis carried out on the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation-exchange columns, the gradient method in Table 1 was used unless further stated.

Table 1. 30 min linear gradient method for the MAbPac SCX-10, 10 µm, 4 × 250 mm, cation exchange columns. Total run time is 40 min. The linear pH range covers from pH 5.6 to pH 10.2. UV wavelength was set at 280 nm.

Time (minutes)	Flow rate (mL/min)	%A	%B
0-1	1	100	0
1-31	1	100-0	0-100
31-34	1	0	100
34-40	1	100	0

LC-MS
First dimension HPLC: in a scale up purification, 1 mL of IgG was purified from 3.8 mL of HCC using Thermo Scientific™ Pierce Protein A beads. The protein concentration was determined at ~ 0.5 mg/mL. About 33 µL of the purified IgG was injected onto a MAbPac SCX-10, 10 µm, 4 × 250 mm column and separated via linear pH gradient from pH 6.52 to pH 9.28. The column was equilibrated at 40% B. Three minutes after sample injection, a linear gradient was run from 40% to 100% B in 30 minutes. Fractions were collected onto a 96-well plate at a rate of 0.2 min per fraction from 10 to 26 min.

Second dimension LC-MS: Thermo Scientific™ ProSwift™ RP-10R monolithic column (1 × 50 mm) was used for desalting. LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 50 ºC during analysis. Flow rate was 100 µL/min. After injection of MAbs, a 5 min gradient from 10% B to 95% B was used to elute the mAbs from the column.

MS: The Q Exactive Orbitrap mass spectrometer was used for this study. Intact MAb was analyzed by ESI-MS for intact molecular mass. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 ºC. S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500. The AGC target was set at 3E6 for full scan. Maximum IT was set at 200 ms.

Data Processing: Full MS spectra of intact MAbs were analyzed using Thermo Scientific™ Protein Deconvolution 1.0 software that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the MAb. The averaged spectra were subsequently deconvoluted using an input m/z range of 2000 to 4000 m/z, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and a minimum of at least eight consecutive charge states from the input m/z spectrum to produce a deconvoluted peak.

Results

Linear pH gradient
The linear pH gradient was achieved by employing a multi-component buffer system containing multiple zwitterionic buffer species with pI values ranging from 6 to 10. Eluent A was titrated to pH 5.6 and eluent B was titrated to pH 10.2. In this pH range, each buffer species was either neutral or negatively charged. Therefore they were not retained by cation-exchange column stationary phase and served as good buffers for the mobile phase and the stationary phase.

Using the gradient method shown in Table 1, six proteins with a range of pI values from 6 to 10 were effectively separated on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. These proteins were lectin (including three isoforms, lectin-1, lectin-2, and lectin-3), trypsinogen, ribonuclease A, and cytomchrome C. The chromatogram was shown in Figure 1. The pH value measured in this experiment as a function of time was plotted in Figure 2. The pH gradient was essentially linear from pH 5.6 to pH 10.2 over a 30 minute period. The correlation coefficient value R^2 was 0.9996.

An analysis was performed to show that there is a correlation between the elution pH for the peaks and the corresponding pI values of the protein components. Figure 3 is a graph comparing the measured pH values for six protein component peaks in Figure 1 as a function of the corresponding pI values. The measured pH values for the six protein component peaks exhibited a strong linear correlation to the literature based pI values. Thus, after a calibration procedure, this example supports the fact that linear regression coupled with the gradient method described here can be used to estimate the pI of a protein component based on the peak retention time and measured pH.

FIGURE 1. Chromatogram of six proteins separated on a 30-min linear pH gradient on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. Protein name, retention time, and corresponding pH values are labeled for each protein peak.

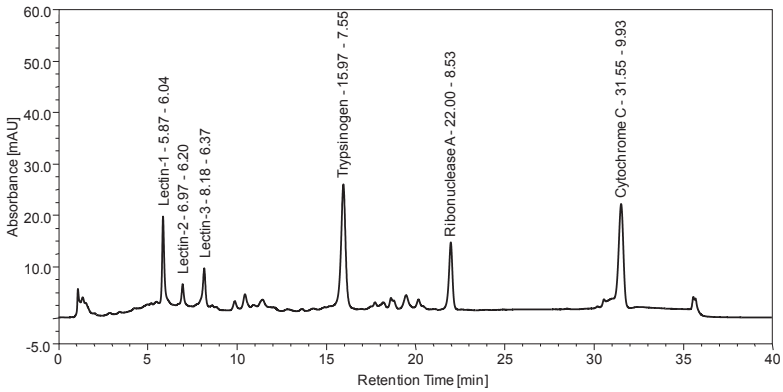


FIGURE 2. A graph showing measured pH values as a function of time. The measured pH values were exported from the same experiment shown in Figure 1. The measured pH values are labeled using blue diamond shape.

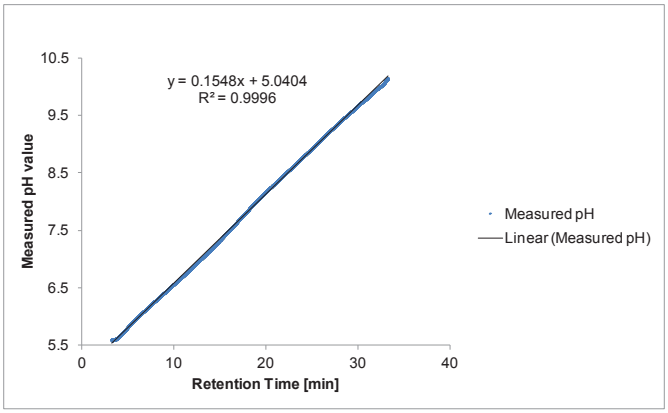
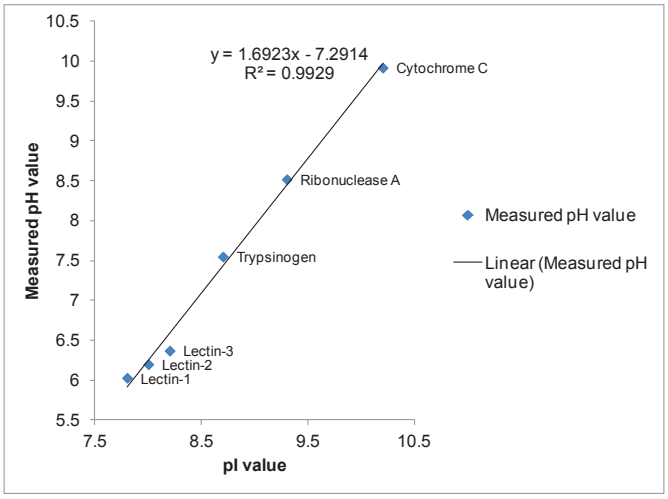


FIGURE 3. A graph plotting the measured pH values for six protein component peaks as a function of the corresponding pI value. The measured pH values of all six components were exported from the same experiment shown in Figure 1.

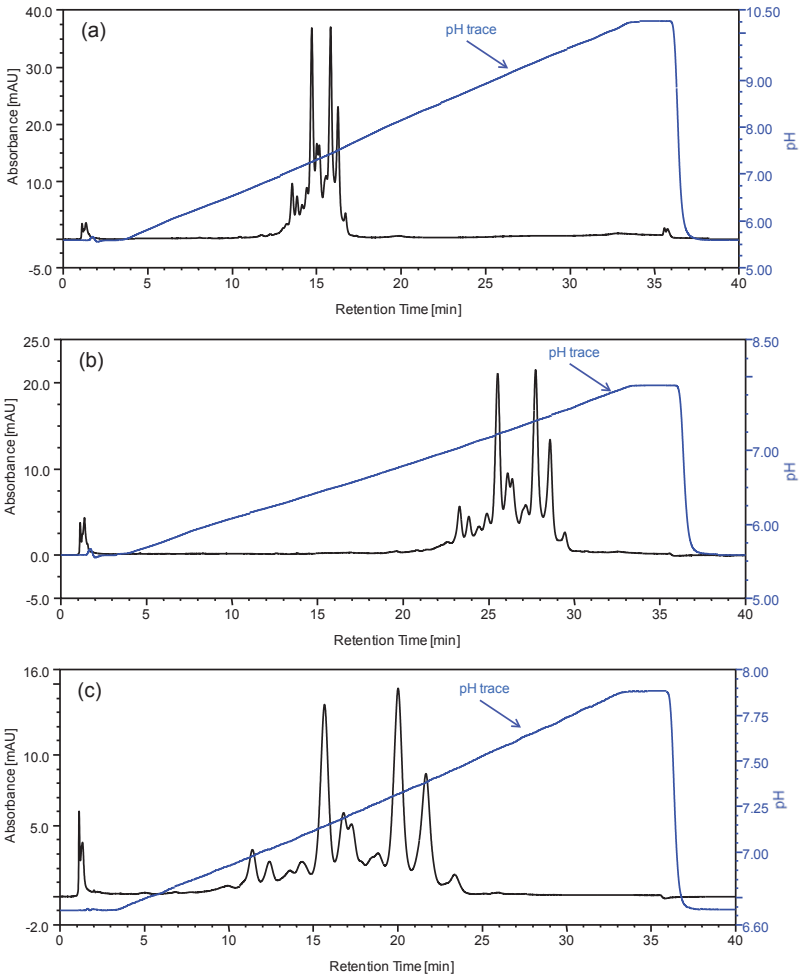


pH Gradient Separation Platform for MAb Variants

Most MAbs have pI values in the range of 6 to 10. Our pH gradient separation method can serve as a platform for charge variant separation. Using a full range of pH gradient from pH 5.6 to pH 10.2, we established the pH elution range in the initial run (Figure 4a) with a pH gradient slope of 0.153 pH unit/min. Further optimization of separation can simply be achieved by running a shallower pH gradient in a narrower pH range. Figure 4b showed the separation profile from pH 5.6 to pH 7.9 with pH gradient slope at 0.076 pH unit/min. Figure 4c showed the separation profile from pH 6.75 to pH 7.9 with pH gradient slope at 0.038 pH unit/min. The pH traces in Figure 4a, 4b, and 4c demonstrated that the pH gradient maintain linear when the slope was reduced to ½ or ¼ of the initial run.

Because the chromatographic profile of the variants were predictable when running a shallower pH gradient. Pump methods for chromatogram shown in Figure 4b and 4c can be automatically generated by writing a post-acquisition script using the MAb variant pH elution range information collected in the initial run (Figure 4a). This example illustrates the advantages of using pH gradient separation platform, which is to simplify and automate the method development for MAb charge variant separation.

FIGURE 4. An example of MAb charge variant separation by linear pH gradient. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column. (a) Separation by pH gradient, 0% B (pH 5.6) to 100% B (pH 10.2), gradient method was shown in Table 1; (b) Separation by pH gradient, 0% B (pH 5.6) to 50% B (pH 7.9); (c) Separation by pH gradient, 25% B (pH 6.75) to 50% B (pH 7.9).



Intact Mass of MAb variants

An IgG sample was purified from harvest cell culture using the Protein A bead. This sample was analyzed via linear pH gradient and the fractions were collected via a time-based method (Figure 5). Major fractions collected off the pH gradient were analyzed on a Q Exactive mass spectrometer. On-line desalting using a reversed-phase monolithic column was carried out prior to MS detection. Figure 6 showed the deconvoluted mass spectra of peak 1, 2, 3, 4, and 5. The deconvoluted spectra showed that the major component in Peak 1 has a *m/z* at 147993. Adjacent peaks at *m/z* 148155 and 148317 correspond to different glycoforms with 1 and 2 additional hexoses. The major component in peak 2 has a *m/z* at 148121. The delta mass between Peak 1 and Peak 2 is 128 amu, corresponding to one lysine. The deconvoluted spectra of Peak 3 and peak 4 have the same MS profile as Peak 1 and Peak 2, suggesting they are structural isomers. The major component in Peak5 has a *m/z* at 148250. The delta mass between Peak 4 and Peak 5 is 129 amu. These data suggest that Peak 3 and Peak 4 correspond to lysine truncation variants of Peak 5.

FIGURE 5. pH gradient separation of purified IgG on a ion-exchange column. The separation was carried out on a MAbPac SCX-10, 10 µm, 4 × 250 mm column via a 30 min linear pH gradient from 40% B (pH 6.52) to 80% B (pH 9.28

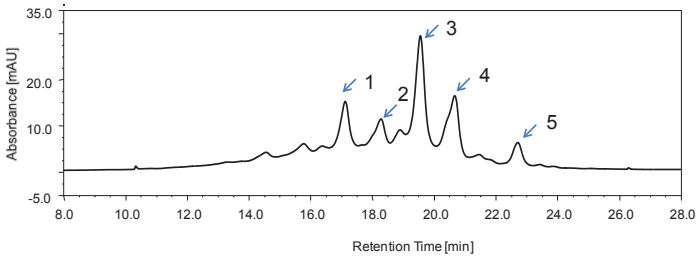
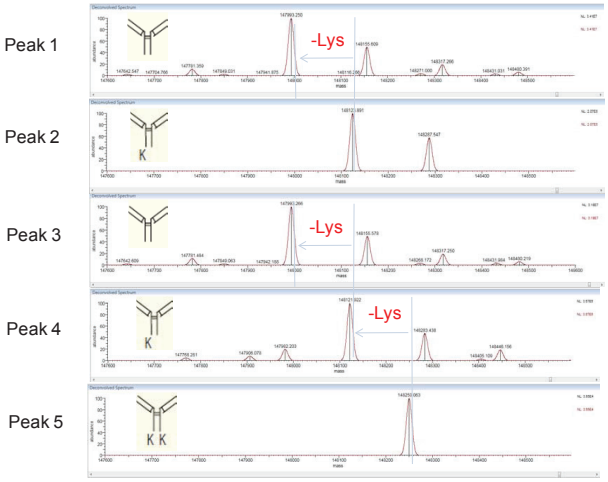


FIGURE 6. Deconvoluted MS Spectra.



Conclusions

- A linear pH gradient from pH 5.6 to pH 10.2 was generated using a multi-component zwitterionic buffer system on a cation-exchange column.
- A linear pH gradient separation platform enables high resolution, fast and rugged MAb charge variant analysis and automation of method optimization.
- The combination of off-line IEC separation and on-line LC mass spectrometry detection provides an efficient way to obtain structural information of MAb variants.

References

1. Farnan, D and Moreno, T. Multiproduct high-resolution monoclonal antibody charge variant separations by pH gradient ion-exchange chromatography. *Anal. Chem.,* 2009, 81, 8846–57.

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Automated MAb Workflow: from Harvest Cell Culture to Intact Mass Analysis of Variants

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Overview

Purpose: Demonstrate an automated monoclonal antibody (MAb) analysis two-dimensional (2D) workflow and intact mass detection.

Methods: Automated analysis is achieved with the Thermo Scientific Dionex UltiMate 3000 x2 Dual Titanium Biocompatible Analytical LC System using Thermo Scientific Dionex Chromeleon Chromatography Data System (CDS) software. The intact mass information is acquired on the Thermo Scientific Q Exactive mass spectrometer.

Results: This workflow enables the completion of affinity purification, size-exclusion analysis, and charge variant analysis in less than one hour. The intact mass analysis characterizes the structural difference of the MAb variants.

Introduction

During development of recombinant MAbs, a large of number of harvest cell culture (HCC) samples must be screened for IgG titer, aggregations, and charge variants. Affinity chromatography is often used first to purify MAbs, with typical yields of more than 95%. Size-exclusion chromatography (SEC) is used to identify and quantify MAb aggregations. Finally, ion-exchange chromatography (IEC) characterizes charge variants. For the final biopharmaceutical product approval and subsequent manufacturing processes, a comprehensive characterization of MAb purity, aggregate forms, and charge variants is required by the regulatory agencies.

In the present study, we automate a 2D high-performance liquid chromatography (HPLC) workflow using an integrated HPLC system. This system consists of a dual-gradient pump, a UV/VIS detector, a column oven, and an autosampler capable of both sample injection and fraction collection. First, the HCC is injected onto the POROS® A Protein A Affinity column and IgG fractions are collected by the autosampler. Subsequently, the IgG fractions are injected separately onto Thermo Scientific MAbPac SEC-1 and MAbPac™ SCX-10 columns for further analysis. The MAbPac SCX-10, 3 µm column was recently introduced in 4 × 50 mm format for high-throughput MAb variant analysis. This column delivers high resolution separation with a shorter run time using either salt or pH gradients. Incorporating this column into the workflow, we completed affinity purification, SEC and charge variant analyses in less than one hour. Furthermore, the fractions collected off the MAbPac SCX-10 column were analyzed by mass spectrometry (MS), and intact mass information of the MAbs demonstrated the presence of lysine variants.

Methods

Harvest Cell Culture

MAb HCC was a gift from a local biotech company. The HCC was filtered through a 0.22 µm membrane prior to sample injection.

Columns

- MAbPac SCX-10, 3 µm, 4 × 50 mm (P/N 077907)
- MAbPac SCX-10, 10 µm, 4 × 250 mm (P/N 074625)
- MAbPac SEC-1, 4 × 300 mm (P/N 074696)
- POROS A Protein A Affinity 20 µm Column, PEEK™, 4.6 mm x 50 mm, 0.8 ml (P/N 1-5022-24)

Liquid Chromatography System

HPLC experiments were carried out using an UltiMate™ 3000 x2 Dual Titanium System equipped with SRD-3600 Integrated Solvent and Degasser Rack, DGP-3600BM x 2 Dual-Gradient Micro Pump, TCC-3000SD Thermostated Column Compartment with two biocompatible 10-port valves, WPS-3000T(B)FC Analytical Dual-Valve Wellplate Sampler, VWD-3400RS Four Channel Variable Wavelength Detector equipped with a Micro Flow Cell, and PCM-3000 pH and Conductivity Monitor.

pH-Based Ion-Exchange Chromatography

In a scale-up purification, 1 mL of IgG was purified from the 3.8 mL HCC using Thermo Scientific Pierce Protein A Plus Agarose beads (P/N 22810). The protein concentration was determined at ~ 0.5 mg/mL. Approximately 100 µL of the purified IgG was injected onto a MAbPac SCX-10, 10 µm, 4 × 250 mm column and separated via pH gradient from pH 7.8 to pH 10.8. Mobile phase buffers contained 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine with pH values of either 6.8 (Buffer A) or 10.8 (Buffer B). The column was equilibrated at 40% B. Three min after sample injection, a linear gradient was run from 40% to 100% B in 30 min. Fractions were collected onto a 96-wellplate at a rate of 0.2 min per fraction from 17 to 27 min.

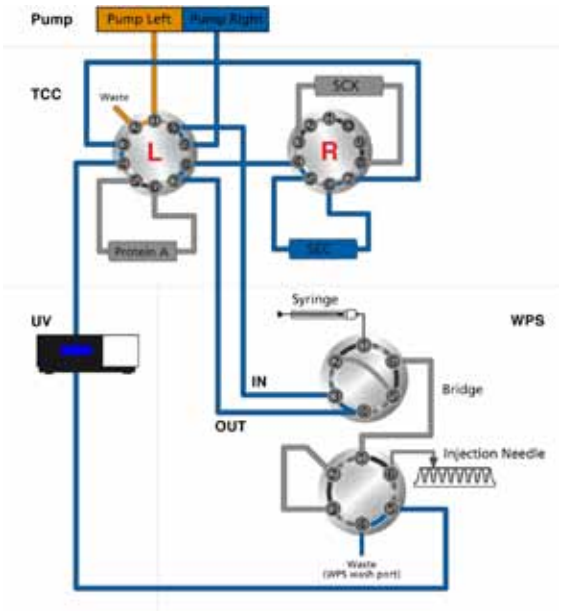
2D-LC Workflow

The workflow and LC conditions for automated off-line 2D-LC include the following:

- Injection of 50 µL of an unpurified HCC sample
- A first-dimension (^{1}D) affinity chromatography separation at a flow rate of 2.0 mL/min using the following steps:
  - A column wash/equilibration step of 0.75 min
  - An elution step of 1 min
  - Automated time-based fraction collection into a wellplate in the autosampler
  - Protein A column is regenerated by a 20% acetonitrile wash and reconditioned for the next analysis

  Total analysis time is approximately 3 min.
- A second-dimension (^{2}D) separation of the collected fraction includes one of the following:
  - SEC separation at a flow rate of 0.3 mL/min using an isocratic mobile phase
  - Strong cation-exchange separation at a flow rate of 0.6 mL/min using a salt gradient

FIGURE 1. Fluidic configuration of the automated off-line 2D-LC system using the wellplate bio-inert autosampler



LC-MS

HPLC: Thermo Scientific ProSwift RP-10R Monolithic Capillary Column (1.0 mm i.d. × 5 cm) was used for desalting. LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 50 ºC during analysis. Flow rate was 100 µL/min. After injection of MAb, a 5 min gradient from 10% B to 95% B was used to elute MAbs from the column.

MS: Using Q Exactive™ instruments, intact MAb was analyzed by ESI-MS for intact molecular mass. The spray voltage was 4 kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 ºC . S-lens level was set at 55. In-source CID was set at 45 eV. Resolution was 17,500. The AGC target was set at 3E6 for full scan. Maximum IT was set at 200 ms.

Data Processing: Full MS spectra of intact MAbs were analyzed using Thermo Scientific Protein Deconvolution software 1.0 that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the MAb. The averaged spectra were subsequently deconvoluted using an input *m/z* range of 2000 to 4000 *m/z*, an output mass range of 140000 to 160000 Da, a target mass of 150000 Da, and minimum of at least 8 consecutive charge states from the input *m/z* spectrum to produce a deconvoluted peak.

Results

In the first step of the chromatographic separation, HCC was injected onto the Protein A Affinity column. In order to collect sufficient amounts of IgG material for the ^{2}D analysis, 50 µL of HCC was injected. The IgG fraction was collected into a 96-wellplate using time-based triggers (Figure 2). The total collection time was 0.1 min. At 2 mL/min flow rate, the total volume collected was 200 µL. Chromeleon CDS software is capable of fraction collection using UV-based peak triggers, or both time and peak triggers together. In the configuration presented here, there was a 0.1 min delay time in fraction collection.

A transition sequence was used to switch the valves and direct the flow path to each ^{2}D analysis column. The ^{2}D analyses can be either SEC (Figure 3) or IEC (Figure 4). Collected fractions can be directly injected onto the ^{2}D column without further modifications. The injection volume for each ^{2}D was 25 µL.

The IEC analysis of the Protein-A purified fractions which used a linear salt gradient revealed many variants in the purified IgG fractions. A one-hour carboxypeptidase digestion (data not shown) eliminated several peaks and enhanced others, suggesting the presence of lysine variants. Use of the MAbPac SCX-10 3 µm column reduced the analysis time from ~60 to 20 min. The total analysis time for all three chromatographic steps was <60 min, which included the transition programs between different analyses. All these steps are automated, and therefore multiple HCC samples can be cycled through without user intervention.

Over the last few years, researchers have demonstrated that pH-gradient-based IEC is an effective method to separate acidic and basic proteins. In this study, we applied pH gradient to the separate MAb variants on a MAbPac SCX-10 column. As shown in Figure 5, separation of at least three variants was achieved. Major peaks 1, 2, and 3 eluted at 19.8, 20.8, and 22.1 min, respectively. Use of the PCM-3000 allowed real-time monitoring of the pH and conductivity of the eluent during all the analyses. The pH values for fractions containing Peaks 1, 2, and 3 were 8.5, 8.6, and 8.7, respectively. These fractions were analyzed on a Q Exactive mass spectrometer (Figure 6). On-line desalting using a reversed phase monolithic column was carried out prior to MS detection. The deconvoluted spectra (Figure 7) showed that the major component in Peak 1 has a 147992.703 *m/z*. Adjacent peaks at 148155.503 and 148315.903 *m/z* correspond to different glycoforms with 1 and 2 additional hexoses. The major component in Peak 2 has a 148210.650 *m/z*. The delta mass between Peak 1 and Peak 2 is 128 amu, corresponding to one lysine. Similarly, the delta mass between Peak 2 and Peak 3 (at *m/z* 148248.641) is also 128 amu. These data suggest that Peak 1 and Peak 2 correspond to lysine truncation variants of Peak 3.

FIGURE 2. Example of a ^{1}D affinity purification of IgG from HCC: the vertical yellow stripe indicates fractionation time.

Protein A Affinity Separation Conditions:

Column: AB Poros® A 20 µm, 4.6 × 50 mm
Mobile Phase A: 50 mM NaH_2PO_4, 150 mM NaCl, pH 7.5
Mobile Phase B: 50 mM NaH_2PO_4, 150 mM NaCl, pH 2.5
Mobile Phase C: Acetonitrile
Gradient: Wash and equilibration step for 0.75 min at 100% A,
followed by 1 min elution step at 100% B,
followed by 0.5 min regeneration step at 80% B and 20% C
Flow Rate: 2.0 mL/min
Temperature: 30 °C

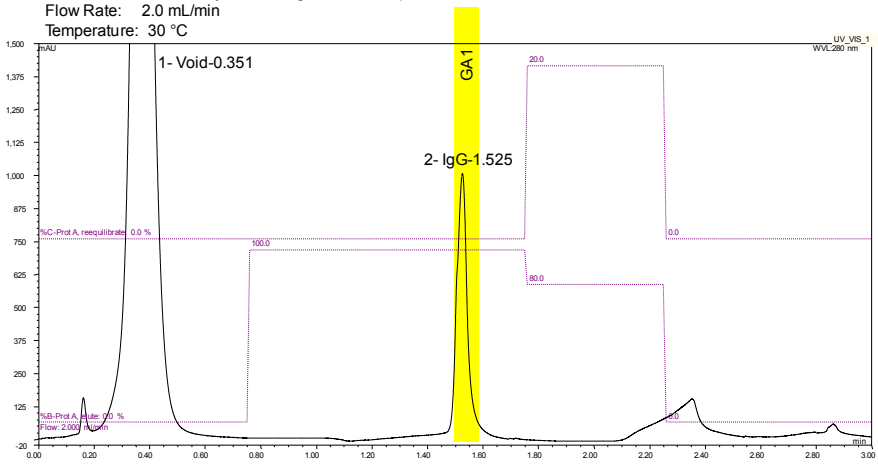


FIGURE 3. Example of an isocratic ^{2}D SEC separation of a purified IgG fraction collected from the MAbPac SEC-1, 4 × 300 mm column

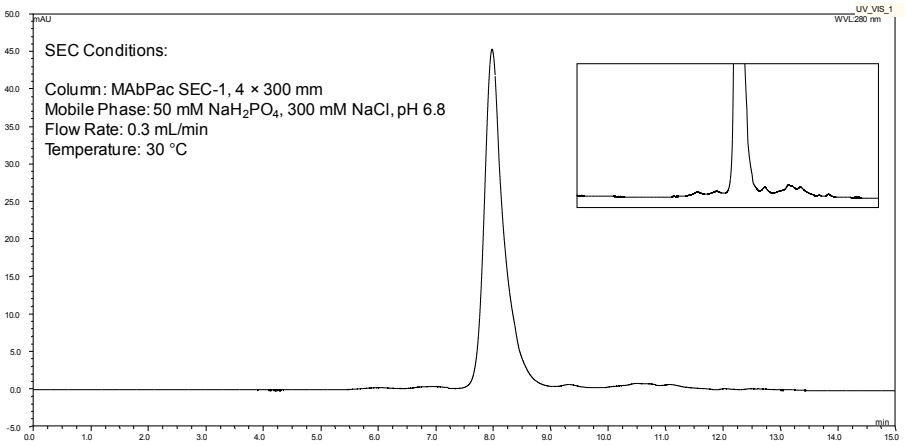


FIGURE 4. Example of a ^{2}D SCX separation of a purified IgG fraction collected from the MAbPac SCX-10, 3 µm, 4 × 50 mm column

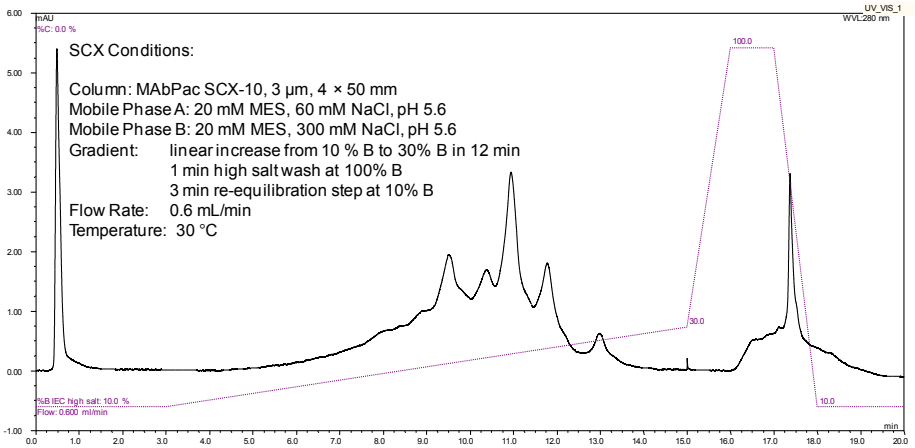


FIGURE 5. pH gradient separation of purifed IgG on a MAbPac SCX-10 column

pH-Gradient Separation Conditions:

Column: MAbPac SCX-10, 10 µm, 4 × 250 mm
Mobile Phase A: 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine, pH value 6.8
Mobile Phase B: 9.6 mM Tris, 11 mM imidazole, and 6 mM piperazine, pH value 10.8
Gradient: 3 min pre-equilibration at 40% B
followed by linear increase from 40 % B to 100% B in 30 min
followed by 7 min high pH wash at 100% B
followed by 15 min re-equilibration step at 40% B
Flow Rate: 1.0 mL/min
Temperature: 30 °C
Fraction Collection Rate: 0.2 min/well

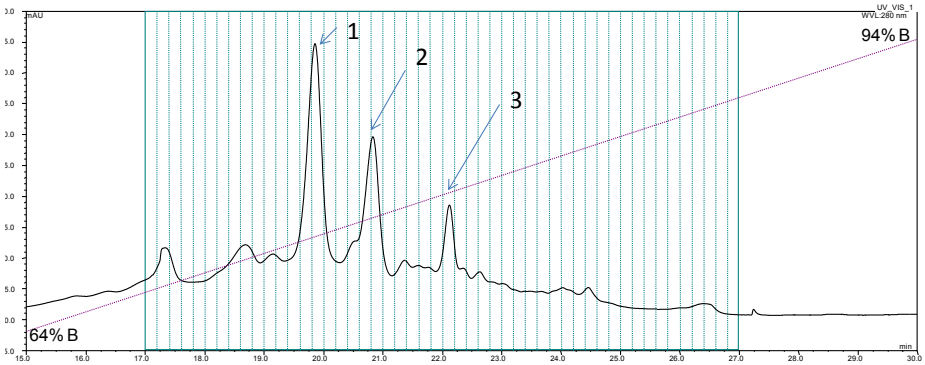


FIGURE 6. Full scan MS spectra

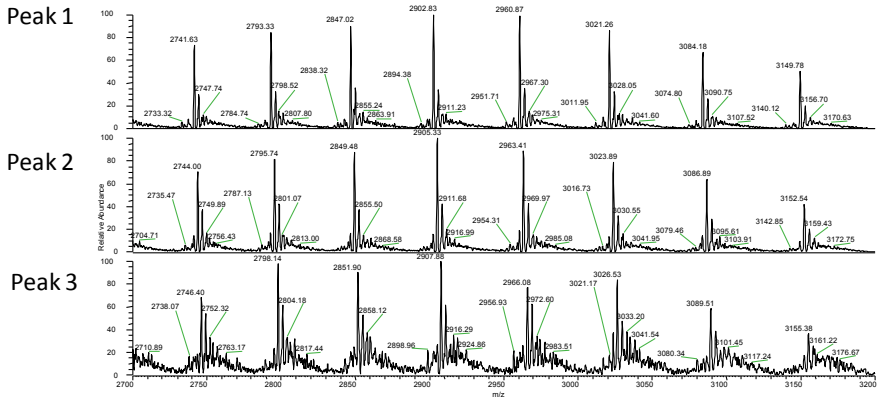
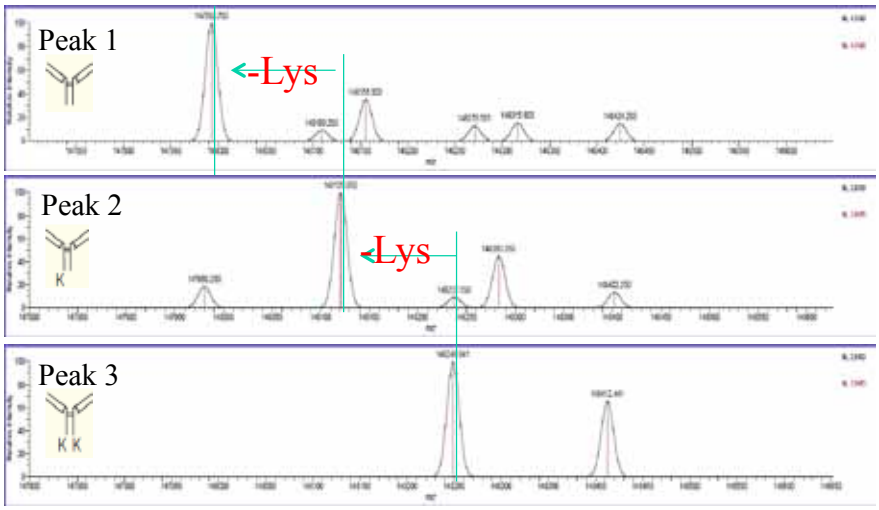


FIGURE 7. Deconvoluted MS spectra



Conclusion

- Using Protein-A Affinity, MAbPac SEC, and MAbPac SCX-10 columns, HCC was characterized by affinity purification, followed by SEC and charge variant analysis in less than one hour.
- The separation of the lysine variants demonstrated that the pH-based gradient method is an effective approach, orthogonal to salt gradient separation.
- The combination of off-line IEC separation and on-line LC MS detection provides an efficient way to obtain structural information of MAb variants.

References

1. Decrop, W.; Swart, R. Development of an Automated Method for Antibody Purification and Analysis. *J. Biomol. Tech*. **2011**, October; 22 (Supplement).
2. Rea, J., Moreno, T., Lou, Y., and Farnan, D. Validation of a pH Gradient-Based Ion-exchange Chromatography Method for High-Resolution Monoclonal Antibody Charge Variant Separations. *J. Pharm. Biomed. Anal*. **2011** *54* (2), 317–23.

Acknowledgements

We would like to thank Terry Zhang at Thermo Fisher Scientific for assistance in acquiring the MS data.

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A High Resolution Bench-Top Orbitrap LC-MS Workflow Solution for Comprehensive Intact Monoclonal Antibody Characterization

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Overview

Purpose: To develop a high resolution LC/MS-based workflow solution for robust, accurate and comprehensive intact monoclonal antibody (mAb) characterization.

Methods: Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometers were used for intact mass measurement and top-down sequencing. Full MS spectra of intact and reduced mAbs were analyzed using Thermo Scientific™ Protein Deconvolution™ software version 2.0 that utilizes the ReSpect™ algorithm for molecular mass determination. The top-down msx HCD spectra were analyzed using Thermo Scientific™ ProSightPC™ software version 2.0.

Results: A mass error of less than 10 ppm was routinely achieved for intact mAb mass measurement. Low mass modifications, such as oxidation, can be confidently identified on substructure level such as intact Fab, or light chain. Using an on-line high resolution top-down MS/MS approach, over 30% of the fragmentation site was covered for intact light chain as well as for Fab heavy chain. Sequence coverage from top-down approach also confirmed disulfide linkage on partially reduced samples.

Introduction

Monoclonal antibodies (mAbs) are increasingly being developed and utilized for diagnosing and therapeutic treatment of diseases including cancer. Due to the heterogeneity of mAb products, thorough characterization is necessary for their reproducible and safe production. Among the analytical tools used for the analysis of therapeutic mAbs, mass spectrometry has become more and more important in providing valuable information on various protein properties. Such information includes intact mass, amino acid sequence, post-translational modifications including glycosylation form distribution, minor impurities due to sample processing and handling, and high order structure. Characterization at the intact protein level is usually the first step. In this study, a high resolution LC-MS based workflow solution was developed for robust, accurate and comprehensive mAb characterization at the intact protein level. The fast chromatography, superior resolution and mass accuracy provided by the Q Exactive LC/MS system, and accurate data analysis of this workflow, provides a high-confident screening tool to accelerate biopharmaceutical product development.

Methods

Samples: Four intact mAbs were used in this study. To reduce intact mAb, the sample was incubated for one hour at 60 °C or 37 °C in 6 M guanidine-HCl containing 5 mM DTT for complete or partial reduction, respectively. Fab was generated using papain in 1mM EDTA, 10 mM Cys, 50 mM sodium phosphate buffer, pH 7.0. Before digestion, the enzyme suspension (10 mg/ml) was activated for 15 min at 37 °C in the same buffer at an enzyme: buffer ratio of 1:9. The digestion was performed at 37 °C overnight using an enzyme: antibody ratio of 1:99 w/w.

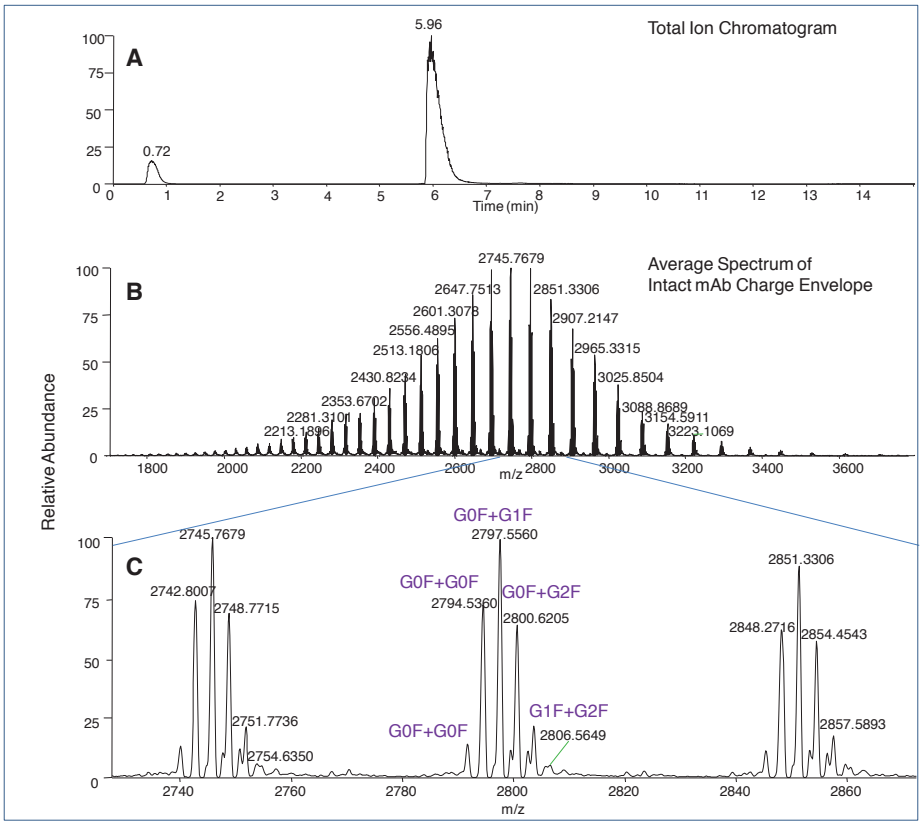
HPLC: A Thermo Scientific™ ProSwift™ RP-10R monolithic column (1 x 50mm) was used for desalting and separation of light and Fab heavy chain. LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). The column was heated to 80 °C during analysis. The flow rate was 60 µL/min. After injection of 1 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0 min, 20% B; 1.0 min, 35% B; 3.0 min, 55% B; 4.0 min, 98% B; 7.0 min, 98% B; 7.1 min, 20% B; 15.0 min, 20% B).

Mass Spectrometry: Q Exactive instruments were used for this study. Intact and reduced mAbs were analyzed by ESI-MS for intact molecular mass. Top-down MS/MS was performed using high energy collision dissociation with a unique spectrum multiplexing feature (msx HCD). In this data acquisition mode, fragment ions produced from several individual HCD events, each on a precursor of a different charge state of the reduced mAb, were detected together in the Thermo Scientific™ Orbitrap™ mass analyzer. The spray voltage was 4 kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 °C. S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb and intact Fab average mass measurement, or 140,000 for light chain and Fab heavy chain monoisotopic mass measurement. Resolution was set at 140,000 for top-down MS/MS. The AGC target was set at 3E6 for full scan and 2E5 for MS/MS. Maximum IT was set at 250 ms.

Data Processing: Full MS spectra were analyzed using Protein Deconvolution software that utilizes the ReSpect algorithm for molecular mass determination. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input m/z spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses with the various combinations of commonly found glycoforms. The top-down msx HCD spectra were analyzed using ProSightPC software in the single protein mode with a fragment ion tolerance of 5 ppm.

Results

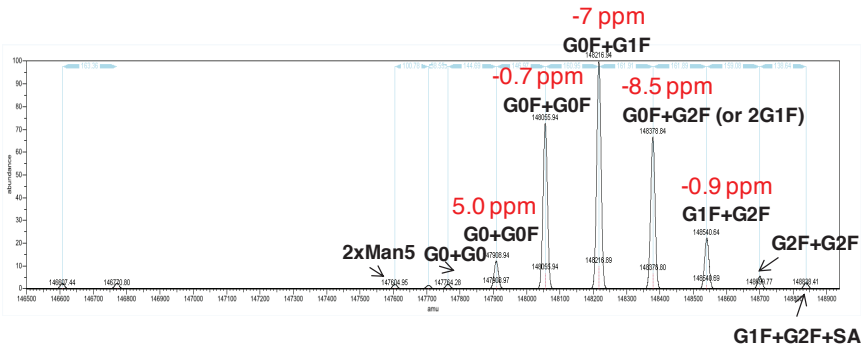
Figure 1: Intact mAb analysis using LC MS



One microgram of mAb was desalted and eluted from a ProSwift RP-10R monolithic column using a 15 min gradient and analyzed using ESI-MS on the Q Exactive MS. As shown in Figure 1, the mAb was eluted over one minute as shown in (A). The average spectrum over the elution time shows a nicely distributed complete charge envelope of the mAb (B). A zoom-in view of each charge state reveals five major glycosylation forms that are baseline separated (C).

After each of the mAb datasets were analyzed using the Protein Deconvolution software, the masses were compared to the masses expected for the known amino acid sequence with the various combinations of glycoforms commonly found on mAbs. One such result is shown below in Figure 2.

Figure 2: Deconvoluted spectrum for a mAb with known composition and mass errors of average molecular mass



To measure the mass accuracy and reproducibility of mAb samples on the Q Exactive MS in conjunction with Protein Deconvolution software, the mAb sample was analyzed several times using two different instruments over three different days. The results for ppm mass accuracy are shown in Table 1 and the results for relative abundance of the various glycoforms are shown in Table 2.

Table 1: ppm mass deviations from expected target masses for the 5 most abundant glycoforms

RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	-10.5	0.7	-10.5	-13.8	-18.0
2	1	-11.6	-1.1	-8.8	-11.2	-12.0
3	1	5.1	-5.0	-2.6	5.1	5.6
4	2	-14.3	3.0	-6.9	-5.4	-5.9
5	2	-8.6	-2.2	-12.2	-12.5	-12.9
6	2	-14.3	-6.6	-12.3	-14.8	-10.1

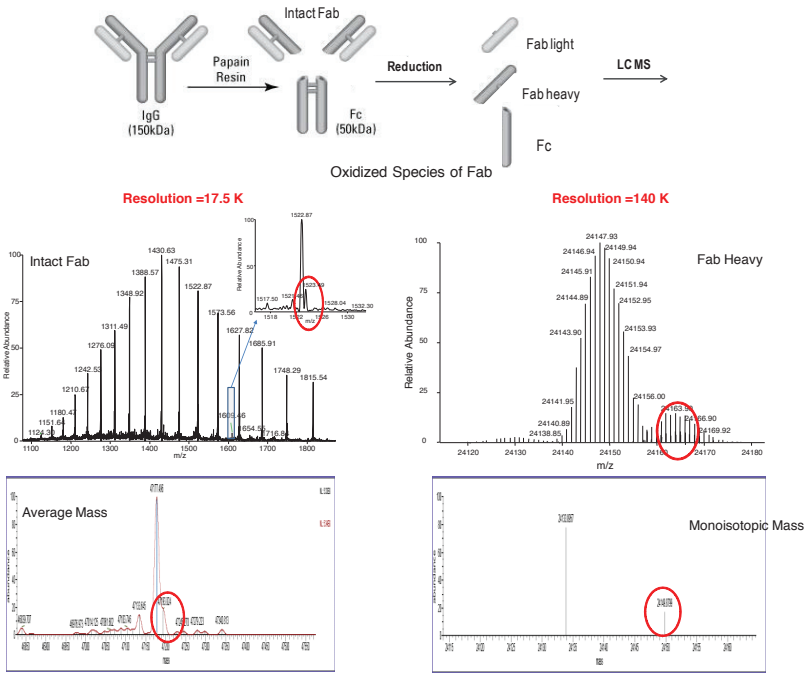
The average ppm error for all 34 measurements of four different mAbs on multiple instruments was **6.9 ppm** with a standard deviation of **6.4 ppm** (not all the data are shown here). This indicates that the Q Exactive MS is a very powerful platform for confirmation of protein primary structure.

Table 2. Relative abundance for the 5 most abundant glycoforms

RAW file	Q Exactive	G0+G0F	G0F+G0F	G0F+G1F	G0F+G2F	G1F+G2F
1	1	12.9	74.1	100.0	67.0	23.4
2	1	12.0	72.8	100.0	66.2	22.0
3	1	12.2	75.0	100.0	67.0	23.6
4	2	12.7	75.7	100.0	63.6	21.6
5	2	13.2	75.4	100.0	64.8	21.0
6	2	12.9	76.6	100.0	64.7	21.6
CV		3.4%	1.6%	N.A.	3.9%	4.4%

For the top 5 glycoforms, the relative intensity reproducibility is within a few percent.

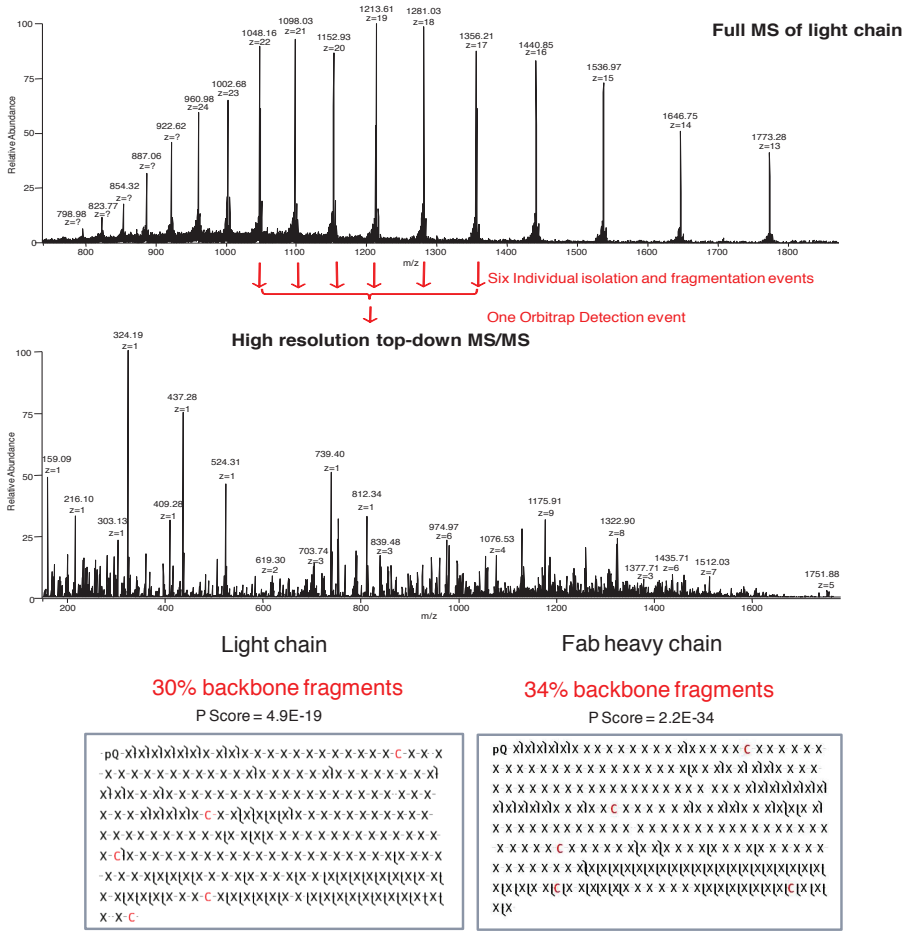
Figure 3: Identification of oxidation on intact Fab, light and Fab heavy chain



Sub-structure	Resolution	Delta Mass from non-oxidized (Da)	Protein level mass error (ppm)
Fab	17.5K	16.3	6.4
Fab heavy	140K	16.0158	0.7
Fab light	140K	16.0152	0.7

Further characterization at substructure level is shown in Figure 3 to Figure 5. Fab was generated using papain which cleaves this molecule at hinge. Fab was then reduced to generate light chain and Fab heavy chain (Figure 3, top). LC/MS of intact Fab, light chain and Fab heavy chain identified oxidation species as shown above (Figure 3, middle). The mass errors of the identification at protein level were 6.4 ppm at resolution 17,500 for Fab and less than 1 ppm at resolution 140,000 for light chain and Fab heavy chain (Figure 3 bottom).

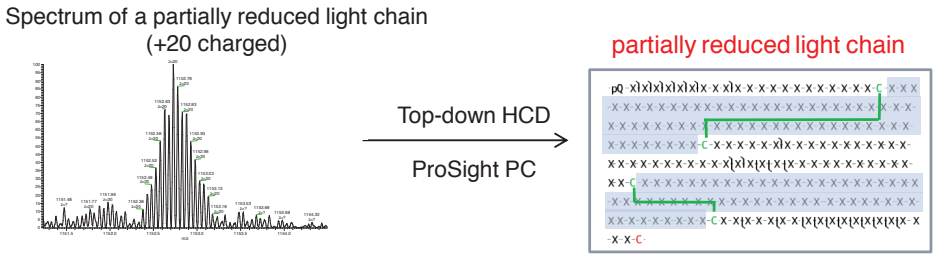
Figure 4: Top-down sequencing of light and Fab heavy chain using LC-MS/MS



Besides molecular mass, amino acid sequence can be obtained at the intact protein level using a top-down LC-MS/MS approach. High resolution top-down HCD was performed using a multiplexing mode where multiple precursors, which were the same protein molecule carrying different number of charges, were isolated, fragmented separately and the resulting fragment ions were then detected all together in a single Orbitrap detection event (Figure 4 top). More than 30% of fragments from backbone cleavage were detected for both light chain and Fab heavy chain (Figure 4 bottom) with excellent P score from ProSight PC software.

Top-down sequencing was also performed on a partially reduced light chain which is 4.02 Da less in molecular mass than the fully reduced species. Analysis of the HCD spectrum in ProSight PC software matched two disulfide linkages, which is typical of this type of IgG molecule (Figure 5).

Figure 5: Top-down sequencing maps disulfide linkage on partially reduced light chain



Conclusion

In this study, a workflow was developed that combines high resolution Orbitrap MS, fast chromatography, high throughput msx HCD and accurate data analysis to characterize intact mAb. The precise mass measurement and extensive, high confident amino acid sequence obtained from this workflow provides the following information for intact mAb and its substructure:

- Accurate measurement of intact molecular mass
- Reproducible quantification of glycoform relative abundance
- Confident amino acid sequence information and structural information

BioPharma_PN63944_E 11/13S

Top-Down Analysis of Intact Antibodies Using Orbitrap Mass Spectrometry

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Thermo Fisher Scientific (Bremen), Germany

Thermo SCIENTIFIC

Overview

Purpose: Perform top-down analysis of intact antibodies in native and denatured forms using Thermo Scientific™ Orbitrap™ mass spectrometry

Methods: Orbitrap MS and MS/MS (HCD, ETD, and EThcD) analysis of intact Herceptin IgG monoclonal antibody

Results: Demonstrated the ability of Orbitrap instrumentation to perform top-down analysis of intact antibodies in native and denatured forms

Introduction

Therapeutic monoclonal antibodies (mAbs) have gained considerable importance over the past years due to their use to treat cancer and autoimmune diseases. Mass spectrometry plays a significant role among the analytical tools used for the analysis of therapeutic mAbs, being able to provide valuable information on antibody properties such as intact mass, amino acid sequence, disulfide bridges, and post-translational modifications (PTMs) including glycosylation. Usually mass spectrometric analysis is performed at the peptide level, which requires several sample preparation steps prior to analysis, including denaturation, reduction, alkylation, digestion, and release of glycan chains. Here we present a more straightforward, top-down approach that uses recent advances in Orbitrap mass spectrometry for the analysis of intact mAbs in native and denatured forms.

Methods

Sample Preparation

Herceptin® (trastuzumab) IgG mAb from Genentech (Figure 1) was buffer exchanged prior to mass spectrometric analysis into 100mM ammonium acetate using Micro Bio-Spin™ 6 columns (Bio-Rad). The antibody solution was analyzed at a concentration of 10µM in either 100mM aqueous ammonium acetate or in a mixture of acetonitrile:water, 1:1 with 0.1% FA.

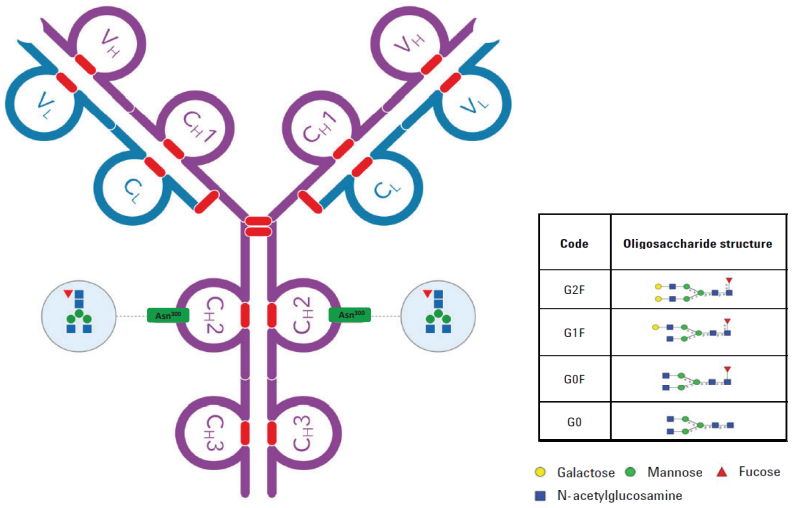
Mass Spectrometry

Top-down analysis of the intact antibody was carried out on modified Thermo Scientific™ Exactive™ Plus, Thermo Scientific™ Q Exactive™, and Thermo Scientific™ Orbitrap Elite™ instruments in direct infusion electrospray or static nanospray mode. Higher-energy collision dissociation (HCD) was employed for the first two instruments, and electron transfer dissociation (ETD), HCD, and electron transfer higher-energy collision dissociation (EThcD) were used on a modified Orbitrap Elite instrument.

Data Analysis

Data analysis was performed using Thermo Scientific™ Protein Deconvolution™ 2.0 software and ProSightPC™ 2.0 software.

FIGURE 1. Schematic representation of the structure and glycoforms for Herceptin IgG monoclonal antibody.



Results

Orbitrap MS Analysis of Herceptin Antibody in Denatured and Native Forms

In contrast to electrospray ionization (ESI) MS spectra of denatured antibody where observed charge states ranged from 34+ to 62+, in native ESI-MS spectra, the signal is spread over only a few charge-state peaks, primarily 23+ to 28+ (Figure 2). Different glycoforms of the antibody are clearly baseline-resolved, allowing their accurate assignment. For example, the peak observed at 148056.95 Da can be attributed to two G0F oligosaccharidic chains (Δm = 2.4ppm), whereas the remaining six glycoforms can be assigned to G0/G0, G0F/G0, G0F/G1F, G0F/G2F (or G1F/G1F), G1F/G2F and G2F/G2F glycoforms (Figure 3).

FIGURE 2. Orbitrap mass spectra of the Herceptin antibody in denatured and native forms. Spectra were acquired at a resolution setting of 17,500 using modified Q Exactive and Exactive Plus instruments.

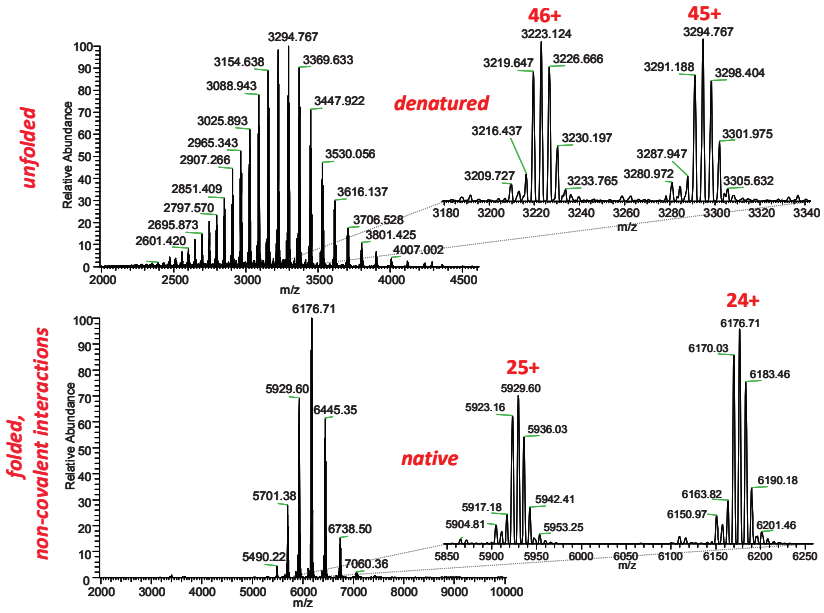
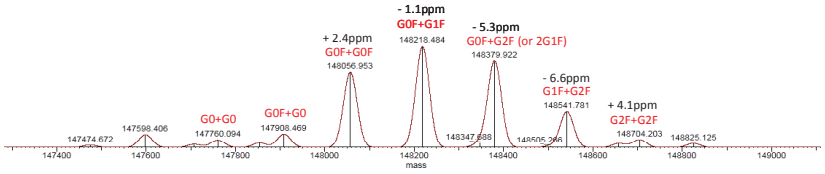
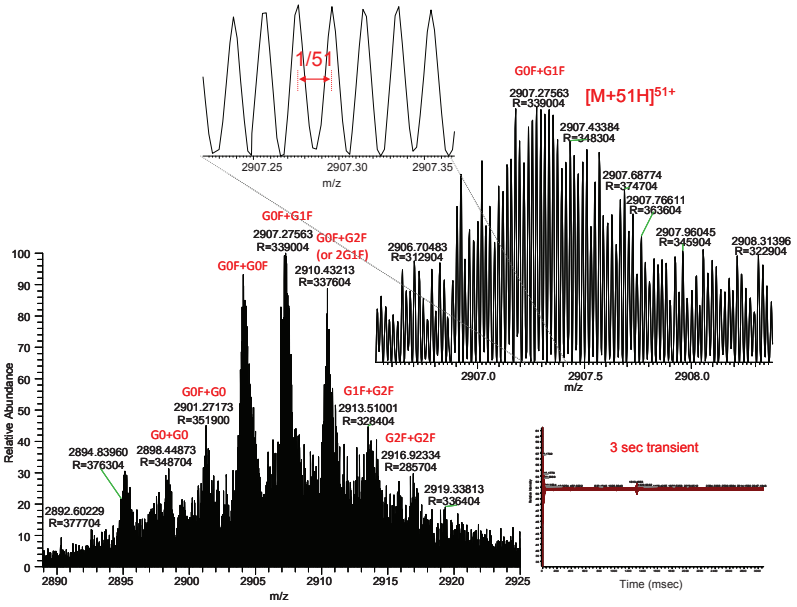


FIGURE 3. Deconvoluted Orbitrap mass spectrum of the intact Herceptin antibody. The deconvolution was performed using the ReSpect™ algorithm from Protein Deconvolution 2.0 software.



Orbitrap technology is capable of ultrahigh resolving power in excess of 1,000,000 when appropriate tolerance and tuning requirements are met [1]. Three-second long transients could be used for the analysis of intact Herceptin antibody wherein baseline separation of its isotopes has been demonstrated (Figure 4). Experiments were carried out on a modified Orbitrap Elite hybrid mass spectrometer equipped with a compact high-field Orbitrap mass analyzer specifically selected from a batch of serial assemblies. Software was custom modified to allow transients up to three seconds long to be processed using advanced signal processing [2]. Intact Herceptin 51+ ions were trapped in the HCD cell using He gas as described by Shaw *et al.* [3], and 500 transients were averaged to obtain the ultrahigh resolution spectrum shown in Figure 4.

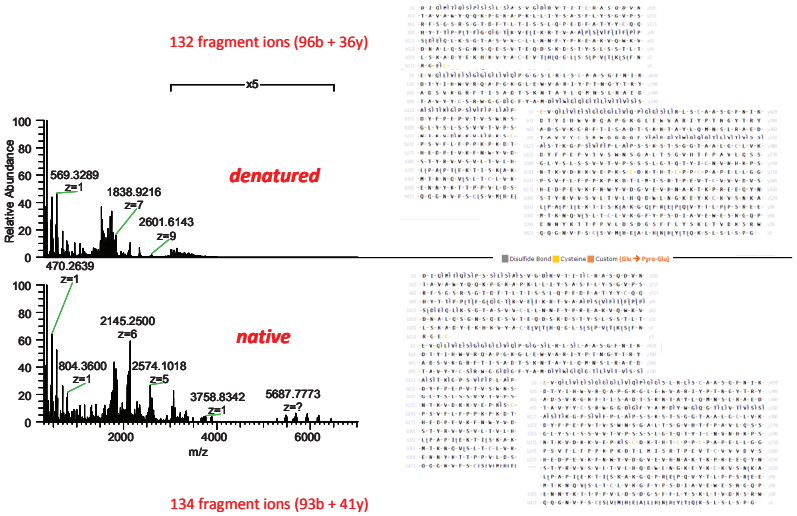
FIGURE 4. Ultrahigh resolution Orbitrap SIM scan showing baseline isotopically resolved $[M+51H]^{51+}$ ions of intact Herceptin antibody. 500 transients with a length of three seconds were averaged on an Orbitrap Elite instrument.



Orbitrap MS/MS Analysis of Herceptin Antibody in Denatured and Native Forms

Comparison of HCD data acquired in native vs. denatured conditions showed very high similarity in terms of location of the assigned cleavage sites and total number of b and y (134 vs. 132) fragment ions (Figure 5). In both cases most of the assigned cleavage sites are located in the disulfide-bond free regions. The central portion of the light chain has been well-sequenced, with at least six backbone cleavages confirmed by the identification of both b- and y-fragment ions. Good sequence coverage was also observed for the N-terminal part of the heavy chain where pyroglutamate formation at Glu1 residue was detected. The regions between variable and first constant domain, second and third constant domains and the C-terminal part of the heavy chain were also well covered. The large portion of light and heavy chains which was not sequenced with HCD could be explained by the presence of disulfide-bridges and secondary and tertiary structure of the antibody in native form which is most probably partially retained in denatured form as well.

FIGURE 5. HCD mass spectra of Herceptin antibody in denatured and native forms and ProSightPC software results. Spectra were acquired using modified Q Exactive and Exactive Plus instruments.



Based on work done by Horn *et al.* [4], we decided to acquire HCD spectra of the intact Herceptin mAb on the Orbitrap Elite mass spectrometer at three different resolution settings (120,000; 240,000; and 480,000 at *m/z* 400) to check if ultrahigh resolving power is required to resolve possible overlapping isotopic clusters. An example showing the need of ultrahigh resolving power for the top-down analysis of intact antibodies is illustrated in Figures 6 and 7. The HCD spectrum of the intact Herceptin mAb shows baseline resolved overlapping isotopic patterns of b_{115} (light chain) and b_{113} (heavy chain) 8+ fragment ions at a resolution setting of 480,000 (Developer's Kit only). At a lower resolution setting of 240,000, these two overlapping isotopic patterns with a delta of 22 mmu are partially resolved making still possible the unambiguous assignment of both fragment ions. Lowering the resolution further, to 120,000, it is not possible to resolve and unambiguously assign these two different fragment ions.

FIGURE 6. HCD mass spectrum of Herceptin antibody in denatured form showing baseline resolved overlapping isotopic patterns of b_{115}^{8+} (light chain) and b_{113}^{8+} (heavy chain) fragment ions at a resolution setting of 480,000.

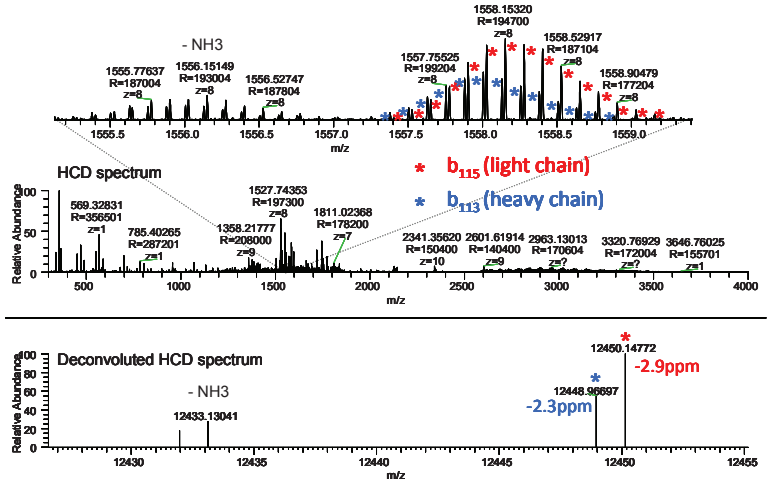
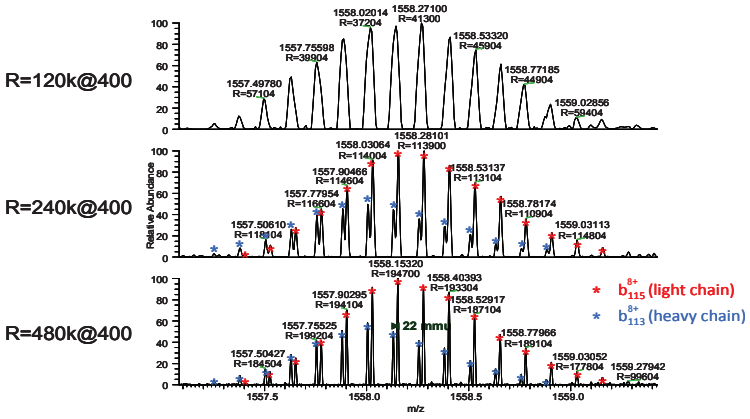


FIGURE 7. Overlapping isotopic patterns of b_{115}^{8+} (light chain) and b_{113}^{8+} (heavy chain) fragment ions measured on Orbitrap Elite mass spectrometer at three different resolution settings.



To further improve the sequence coverage, a combination of ETD and HCD (EThcD) was performed on the intact Herceptin antibody in denatured conditions (Figure 8 and Figure 9). Averaging 768 msec transients for 30 min (1350 uscans) in a direct infusion experiment resulted in identification of 125 fragment ions (62c + 29z, 25b + 9y) from the light chain and 138 fragment ions (35c + 52z, 19b + 32y) from the heavy chain. This corresponds to a sequence coverage of 38.5% for the light chain and 23.7% for heavy chain. The total sequence coverage was 28.4%.

FIGURE 8. EThcD mass spectrum of the intact Herceptin antibody in denatured form.

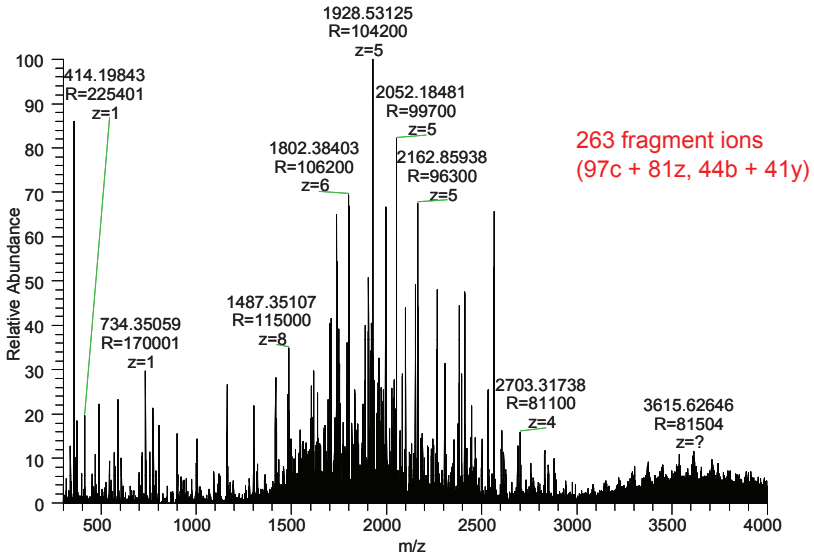
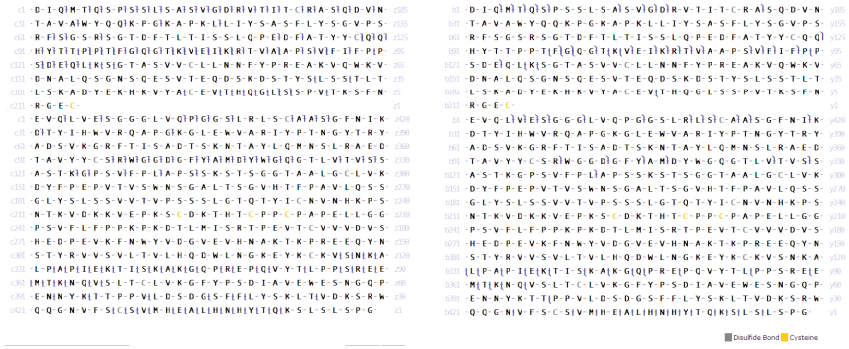


FIGURE 9. ProSightPC software results of the EThcD analysis of the intact Herceptin antibody in denatured form.



Conclusion

- Orbitrap top-down analysis can be used as a powerful tool for the characterization of therapeutic mAbs in both native and denatured forms.
- Ultrahigh resolving power achieved for 3-second detection time on a compact, high-field Orbitrap instrument, allowed to baseline isotopically resolve an 148 kDa intact antibody.
- Ultrahigh resolving power was used to resolve and unambiguously assign overlapping isotopic clusters from top-down analysis of an intact antibody.

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Rosati, S.; van den Bremer, E.; Schuurman, J.; Parren, P.; Kamerling, J.; Heck, A. J. R. In-depth qualitative and quantitative analysis of composite glycosylation profiles and other micro-heterogeneity on intact monoclonal antibodies by high-resolution native mass spectrometry using a modified Orbitrap. *mAbs* **2013**, *5* (6), 917-924. [Full Text]

Rosati, S.; Thompson, N. J.; Heck, A. J. R. Tackling the increasing complexity of therapeutic monoclonal antibodies with mass spectrometry. *TrAC Trends in Analytical Chemistry* **2013**, *48*, 72-80. [Abstract]

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Glycan and Glycopeptide Analysis

In the area of biotherapeutics, recombinant monoclonal antibodies (mAbs) have gained significant importance in diagnostic and therapeutic applications in recent years. The general structural features of mAbs, such as assembly of light and heavy chains via disulfide bridges, are commonly known. However, the heterogeneity of antibodies, mostly due to the variation of attached sugar moieties, requires thorough characterization. These molecules contain complex oligosaccharide moieties whose sites of attachment, presence or absence, and relative profiles can have a significant impact on therapeutic efficacy, pharmacokinetics, immunogenicity, folding, and stability of a biologic drug. Thorough characterization is necessary to verify the correctness of the overall molecule and provide a reproducible, safe, and effective biological drug compound.

Mass spectrometry (MS) has emerged as one of the most powerful tools for the structural elucidation of glycans. This is due to its sensitivity of detection and its ability to analyze complex mixtures. The complex branching and isomeric nature of glycans pose significant analytical challenges to the identification of these structures. In addition to the characterization of the sugar sequence, the analysis must elucidate branching, linkages between monosaccharide units, and the location of possible sulfate or phosphate groups. To ensure comprehensive structural elucidation and structural isomer differentiation, a combination of high mass resolution, accurate mass, sensitivity, multistage fragmentation, and multiple fragmentation techniques are required.

The high-resolution accurate mass (HRAM) of the Orbitrap family of mass spectrometers enables the rapid discovery of glycan and glycopeptide information that was previously difficult or impossible to capture. The Thermo Scientific™ Q Exactive™ MS enabled with multiple fragmentation techniques such as HCD and ETD is the most versatile MS/MS system in the modern laboratory today.
The combination of high resolution chromatography and new separation technologies specific for glycans, such as the Thermo Scientific™ GlycanPac™ AXH-1columns further drives a comprehensive LC-MS integrated workflow for glycan and glycopetide analysis.

Structural Analysis of Labeled *N*-Glycans from Proteins by LC-MS/MS Separated Using a Novel Mixed-Mode Stationary Phase

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Application Note 20786

Key Words
GlycanPac AXH-1, LC/MS, LC-MS/MS, HILIC, WAX, mixed-mode, labeled *N*-glycans, UHPLC, MS detection, Q Exactive, charge, SimGlycan software

Abstract
This application note describes the liquid chromatography-mass spectrometry (LC/MS) analysis of fluorescently labeled *N*-glycans released from proteins. The chromatographic separation is carried out with a novel Thermo Scientific™ GlycanPac™ AXH-1 (1.9 µm, 2.1 × 150 mm) column for high-resolution and high-throughput analysis of glycans. This column possesses unique selectivity that provides separation of glycans based on charge, size, and polarity. MS and MS/MS analyses are performed using a Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap™ mass spectrometer in negative ion mode to provide detailed structural information of *N*-glycans released from proteins.

Introduction

Glycans are involved in a wide range of biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development [1]. Glycans are commonly investigated as important species in therapeutic protein drug development because there is strong evidence that bioactivity and efficacy are affected by glycosylation [2]. Commonly, both the structure and types of glycans attached to the proteins are examined. Understanding, measuring, and controlling glycosylation in glycoprotein-based drugs, glycan content of glycoprotein products, as well as thorough characterization of biosimilars has become increasingly important.

The structures of glycans are highly diverse, complex, and heterogeneous due to post-translational modifications. This makes it challenging to comprehensively characterize glycan profiles and determine their structures [3]. It is therefore essential to separate all isomeric, charge, and branching glycan variations to understand the detailed structure of the glycans by LC-MS/MS methods.

Various HPLC separation modes have been used for the analysis of glycans, including normal phase (NP) or hydrophilic interaction (HILIC) chromatography, ion-exchange (IEX) chromatography, and reversed-phase (RP) chromatography. Because they are highly hydrophilic, polar substances, neutral glycans are commonly separated using amide HILIC columns, such as the Thermo Scientific™ Accucore™ 150-Amide-HILIC column [4], which separates glycans by hydrogen bonding, resulting in a size and composition-based separation. This type of column is particularly useful for the separation of glycans released from monoclonal antibodies, of which the majority are neutral [5].

Based on novel mixed-mode surface chemistry, the GlycanPac AXH-1 column combines both weak anion-exchange (WAX) and HILIC retention mechanisms for optimal selectivity and high resolving power [6]. The WAX functionality provides retention and selectivity for negatively charged glycans, while the HILIC mode facilitates the separation of glycans of the same charge according to their polarity and size. As a result, the GlycanPac AXH-1 column

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provides unparalleled capabilities for glycan separations. In addition, this column has the flexibility to be used in a purely ion-exchange, charge-based separation mode for the separation of various glycans without discrimination of size and polarity. This makes it a suitable tool for accurate quantification of glycans based on charge, which cannot be achieved with any other HPLC/UHPLC columns on the market. The GlycanPac AXH-1 column is designed for use with LC-fluorescence detection and LC/MS applications using volatile aqueous buffers (e.g., ammonium acetate or ammonium formate) and acetonitrile. The substrate of the GlycanPac AXH-1 column is based on high purity 1.9 µm and 3.0 µm spherical silica for UHPLC and standard HPLC applications, respectively.

In this application note we highlight the use of a GlycanPac AXH-1 column and Q Exactive hybrid quadrupole-Orbitrap mass spectrometer for the structural analysis of a 2-aminobenzamide (2AB) labeled *N*-glycans from bovine fetuin.

Experimental Details

Consumables	Part Number
GlycanPac AXH-1, 1.9 µm, analytical column (2.1 × 150 mm)	082472
Deionized (D.I.) water, 18.2 MΩ-cm resistivity	
Fisher Scientific™ HPLC grade acetonitrile (CH_3CN)	AC610010040
Fisher Scientific LC/MS grade formic acid	A117-50
Fisher Scientific ammonium formate	AC40115-2500
Thermo Scientific Premium 2 mL vial convenience kit	60180-600
New England Biolabs® PNGase F	P0705L
Bovine fetuin from a commercial source	
Thermo Scientific™ Hypercarb™ 6 mL cartridge	60106-403
Fisher Scientific trifluoracetic acid	28904
Fisher Scientific sodium cyanoborohydride	AC16855-0500
Fisher Scientific anthranilamide (2AB)	AC10490-5000
Fisher Scientific glacial acetic acid	AA36289AP

Equipment	Part Number
Thermo Scientific™ Dionex™ UltiMate™ 3000 Bio-RS system, consisting of LPG-3400RS pump, TCC-3000RS thermal compartment, WPS-3000TRS pulled-loop well plate autosampler, FLD3400RS fluorescence detector with Dual-PMT, and a 2 µL micro flow cell	6078.4330
Q Exactive hybrid quadrupole-Orbitrap mass spectrometer	
Thermo Scientific™ SpeedVac™ system	
Thermo Scientific Lyophilizer (Labconco® FreeZone® -105 °C 4.5 L benchtop freeze dry system)	16-080-207
Thermo Scientific 24-port SPE vacuum manifold	60104-233

Buffer Preparation

Ammonium formate (80 mM, pH 4.4): Dissolve 5.08 ± 0.05 g of ammonium formate (crystal) and 0.60 g of formic acid in 999.6 g of D.I. water. Sonicate the resulting solution for 5 minutes.

Sample Preparation

1. Native *N*-glycans are released from glycoproteins with PNGase F enzyme and purified by a Hypercarb cartridge (6 mL) with the help of 24-port SPE vacuum manifolds under vacuum. The released glycans are conjugated with a 2-amino benzamide (2AB) label group using the reported procedure of Bigge et al. [7].

2. Dissolve 2AB labeled *N*-glycan from fetuin (5,000 pmol) in 25 µL D.I. water in a 250 µL autosampler vial.

3. Add 75 µL acetonitrile to the same vial and mix till uniformity.

Note: Store the standard at -20 °C.

Separation Conditions					
Column:	GlycanPac AXH-1, 2.1 x 150 mm, 1.9 µm				082472
Mobile phase A:	Acetonitrile / water (80:20, v/v)				
Mobile phase B:	Ammonium formate (80 mM, pH 4.4)				
Column temperature:	30 °C				
Sample volume:	1 µL				
Gradient:	Time (min)	%A	%B	Flow Rate (mL/min)	Curve
	-10	97.5	2.5	0.4	5
	0	97.5	2.5	0.4	5
	30	87.5	12.5	0.4	5
	35	75.0	25.0	0.4	5
	40	62.5	37.5	0.4	5

MS Conditions	
MS instrument:	Q Exactive hybrid quadrupole-Orbitrap MS
Ionization mode:	Negative ion mode
MS scan range:	380-2000 *m/z*
Resolution:	70,000
AGC target	1×10^6
Max IT:	60 ms
dd-MS2 resolution:	17,500
MS/MS AGC target	2×10^5
MS/MS max IT:	1000 ms
Isolation window:	2 *m/z*
Dynamic exclusion:	90 s

Data Processing and Software	
Chromatographic software:	Thermo Scientific™ ChromQuest™ Chromatography Data System version 5.0
MS data acquisition:	Thermo Scientific™ Xcalibur™ software version 2.2 SP1.48
MS/MS data analysis:	SimGlycan® software (PREMIER Biosoft)

Results

Glycan Separation by Charge, Size and Polarity

Figure 1 shows the separation of neutral and acidic 2AB labeled *N*-glycans from bovine fetuin using a GlycanPac AXH-1 (1.9 µm, 2.1 × 150 mm) column. The glycan elution profile consists of a series of peaks grouped into several clusters in which the neutral glycans elute first, followed by monosialylated, disialylated, trisialylated, tetrasialylated, and finally pentasialylated species. Analytes in each cluster represent glycans of the same charge. Within each cluster, the glycans having the same charge are further separated according to their sizes and polarity by HILIC interaction. The structure of the glycans present in each peak was determined in an LC-MS/MS study as shown in the following section.

Structural elucidation

The 2AB labeled *N*-glycans from bovine fetuin were separated on the GlycanPac AXH-1 column based on the separation conditions using a two eluent system and analyzed on a Q Exactive benchtop mass spectrometer. The total ion chromatogram (TIC) is shown in Figure 1. For structural elucidation, data dependant MS/MS spectra were acquired on all precursor ions ($z \leq 2$) and SimGlycan software from PREMIER Biosoft was used for data analysis [8]. The detailed structural information obtained (Table 1) from the MS/MS data further validated the ability of GlycanPac AXH-1 columns to separate glycans based on charge, size, isomers, and polarity. These results also confirmed that the GlycanPac AXH-1 column would be ideal for MS use.

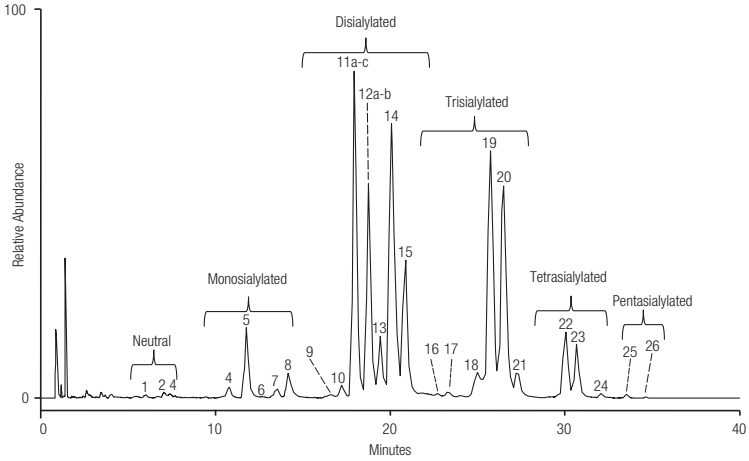

Figure 1: LC/MS analysis of 2AB labeled *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column

Peak	Compound Stucture (2AB labeling are not shown)
1	
2	
4	
5	
6	
7	
8	
9	
10	
11a	
11b	
11c	
12a	
12b	
13	
14	
15	
16	
17	
18	
19	
20	
21	
22	
23	
24	
25	
26	

N-acetyl glucosamine **(GlcNAc)**

Mannose **(Man)**

Galactose **(Gal)**

N-acetyl neuraminic acid **(Neu5Ac)**

N-glycolyl neuraminic acid **(Neu5Gc)**

L-fucose **(L-Fuc)**

Table 1: Structural characterization of glycans present in each peak by the separation of 2AB labeled *N*-glycans from bovine fetuin using a GlycanPac AXH-1 column

Conclusion

The GlycanPac AXH-1 column is a high-performance, silica-based HPLC column for simultaneous separation of glycans by charge, size, and polarity. It is designed for high-resolution and high-throughput analysis with unique selectivity for biologically important glycans. We have demonstrated that this column provides unique selectivity and excellent resolution for glycans released from fetuin.

LC-MS/MS analysis of 2AB labeled *N*-glycans derived from glycoproteins are demonstrated using GlycanPac AXH-1 columns. The Q Exactive hybrid quadrupole-Orbitrap instrument provides excellent MS/MS fragmentation information to enable characterization of glycan structures.

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Integrated LC/MS Workflow for the Analysis of Labeled and Native N-Glycans from Proteins Using a Novel Mixed-Mode Column and a Q Exactive Mass Spectrometer

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Application Note 595

Key Words
GlycanPac AXH-1, HILIC, WAX, glycomics, glycoproteins, glycopeptides, glycans, labeled *N*-glycans, Q Exactive, SimGlycan software

Goal
Develop a comprehensive method for the structural characterization of released glycans from proteins. The described integrated method covers sample preparation, separation, mass spectrometry data acquisition, and analysis.

Introduction
Glycans are widely distributed in biological systems in 'free state' as well as conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They play significant roles in many biological and physiological processes, including recognition and regulatory functions, cellular communication, gene expression, cellular immunity, growth, and development.[1] Glycans can affect efficacy and safety of protein based drugs. For example, recombinant proteins and monoclonal antibodies (mAb) are often dependent on the structure and types of glycans attached to the proteins.[2] The structures of glycans are diverse, complex, and heterogeneous due to post-translational modifications (PTMs) and physiological conditions. Minor changes in glycan structure can result in striking differences in biological functions and clinical applications. The structural characterization of glycans is essential in bio-therapeutics and bio-pharmaceutical projects.[3] In addition to the characterization of the sugar sequence, the analysis must elucidate linkages and separate all isomeric, charge, and branching variations of glycans.

Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans. Hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterionic-based packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. A limitation of this approach is that identification of the glycan charge state is not possible due to the fact that glycans of different charge states are intermingled in the separation envelope.

The Thermo Scientific™ GlycanPac™ AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural analysis of glycans, either labeled or native, by LC-fluorescence or LC/MS methods.The GlycanPac AXH-1 column is based on innovative mixed-mode surface chemistry combining both weak anion-exchange (WAX) and HILIC retention mechanisms. The WAX functionality provides retention and selectivity for negatively charged glycans, while the HILIC mode facilitates the separation of glycans according to their charge, polarity, and size. As a result, the GlycanPac AXH-1 column provides unparalleled separation capabilities for glycans.

LC-MS/MS analysis of glycans requires the processing of large sets of data. The incorporation of SimGlycan® software (PREMIER Biosoft) alleviates this issue, thus enabling the development of a true high-throughput workflow.

This application note presents a step-by-step method for the release, labeling, separation, and structural elucidation of *N*-glycans from proteins by LC-MS/MS.

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Experimental Conditions

Chemicals and Reagents

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific™, AC610010040)
- LC/MS grade formic acid (Fisher Scientific, A117-50)
- Ammonium formate (Fisher Scientific, AC40115-2500)
- Thermo Scientific Premium 2 mL vial convenience kit, 60180-600
- PNGase F (New England BioLab, P0705L)
- Bovine fetuin (Sigma-Aldrich®, F2379)
- Thermo Scientific™ Hypercarb™ cartridge, 6 mL, 60106-403
- Trifluoracetic acid (Fisher Scientific, 28904)
- Sodium cyanoborohydride (Fisher Scientific, AC16855-0500)
- Anthranilamide (2AB) (Fisher Scientific, AC10490-5000)
- Glacial acetic acid (Fisher Scientific, AA36289AP)
- Dimethylsulfoxide (DMSO) (Fisher Scientific, D128500LC)
- Sodium hydroxide (NaOH) (Fisher Scientific, S318-100)
- Ammonium acetate (Fisher Scientific, A637-500)
- SEC column, 0.9 x 50 cm Sephadex® (GE Healthcare, G-10-120)
- GlykoClean™ G Cartridges, Prozyme, GC250
- 2-mercaptoethanol (Fisher Scientific, O3446I-100)

Equipment

- Thermo Scientific™ Dionex™ UltiMate™ 3000 system, including pump: LPG-3400RS, thermal compartment: TCC-3000RS, pulled-loop well plate auto sampler: WPS-3000TRS, fluorescence detector with Dual-PMT: FLD3400RS, and 2µL micro flow cell: 6078.4330
- Q Exactive hybrid quadrupole-Orbitrap mass spectrometer
- Thermo Scientific™ SpeedVac™ Concentrator
- Thermo Scientific Lyophilizer (Labconco® FreeZone® -105 °C 4.5 L benchtop freeze dry system) 16-080-207
- Thermo Scientific 24-Port SPE vacuum manifold, 60104-233

Buffer Preparation

- Ammonium formate (80 mM, pH 4.4):
  Dissolve 5.08 ± 0.05 g of ammonium formate (crystal) and 0.60 g of formic acid in 999.6 g of DI water. Sonicate the resulting solution for 5 min.
- 0.1 M sodium phosphate buffer, pH 7.25:
  Add 102.24 mg of Na_2HPO_4 and 38.14 mg of NaH_2PO_4 to 10 mL of DI water. Vortex to mix the solid completely. Verify that the pH of the solution is 7.25 ± 0.02.

Release of *N*-Glycans from Proteins

1. Dissolve 1 mg of the bovine fetuin protein in 500 µL of 0.1 M sodium phosphate buffer, pH 7.2 ± 0.05, in an Eppendorf tube.
2. Add 0.5 µL of 2-mercaptoethanol to this solution.
3. Finally, add 50 U (units) of PNGase F and incubate total solution at 37 °C water bath for 18 h.
4. Cool to room temperature and purify the released glycans as described in the next section.

Purification of *N*-Glycans

Purify free glycans after digestion using a Hypercarb cartridge as follows:

1. Attach a single Hypercarb cartridge per reaction to a designated port in the SPE manifold.
2. Slowly, and with a consistent flow rate, pre-treat each cartridge with the following volumes of reagents in the order described: 15 mL of 1M NaOH, 15 mL of HPLC grade water, 15 mL of 30% acetic acid, 15 mL of HPLC grade water.
3. Prime the cartridge with 15 mL of 50% acetonitrile/0.1% trifluoroacetic acid (TFA), followed by 15 mL of 5% acetonitrile/0.1% TFA.
4. Load the entire sample volume into the cartridge and let it permeate into the resin by pulsing the vacuum on and off quickly.
5. Rinse the reaction tube with ~50 µL of HPLC grade water, transfer into the cartridge, and pulse the vacuum again.
6. Wash the cartridge with 15 mL of HPLC grade water, followed by 15 mL of 5% acetonitrile/0.1% TFA.
7. Elute the glycans with 4 x 2.5 mL of 50% acetonitrile/ 0.1% TFA into a labeled 15 mL conical tube.
8. Immediately freeze samples on dry ice and then lyophilize to dryness (16–24 h).
9. After lyophilization, dissolve the solid in 1 mL of water, dry the samples again in a 1.5 mL Eppendorf tube, and store at -20 °C.

2AB Labeling Reaction

Carry out the labeling reaction using a modified reported procedure.[4]

1. Prepare the 2AB labeling reagent (100 µL): Dissolve 2-aminobenzamide (4.6 mg) in 70 µL of DMSO.
2. Add 30 µL of glacial acetic acid (100%) to the mixture.
3. Transfer the complete solution to a black or light-protected, screw-cap, 1.5 mL Eppendorf tubes containing 6.4 mg of sodium cyanoborohydride.
4. Incubate the solution at 60 °C for 10 min to dissolve sodium cyanoborohydride completely. Occasionally vortex the solutions. When all the solids are completely dissolved, the 2AB labeling reagent is ready to use for the labeling reaction.
5. Add 20 µL of 2AB labeling reagent to 50 µg of free glycans and vortex to mix the solution. Then, incubate the mixture at 60 °C for 3 h.

Clean Up of Labeled Glycans

1. After completion of the 2AB reaction, add 250 µL of acetonitrile to the vial at room temperature.
2. Purify the samples using a GlykoClean G cartridge; pre-equilibrate the column with the following solutions in the order they appear: wash with 3 mL of deionized water, 3 mL acetonitrile, 3 mL of 96% acetonitrile.
3. Add the labeled glycans to the pre-equilibrated column.
4. Wash with 96% acetonitrile.
5. Elute the glycans with 5 mL of DI water.
6. Lyophilize the solution to dryness.
7. Upon dryness, dissolve the sample in 500 µL of water.
8. Further purify the labeled glycans using a size-exclusion chromatography (SEC) Sephadex® column to get highly pure labeled oligosaccharides.
9. Inject the samples onto an SEC column connected to a UV detector. Equilibrate the column with 10 mM ammonium acetate at a flow rate of 0.35 mL/min until a steady baseline of 205 nm is achieved.
10. Run the column with 10 mM ammonium acetate for 90 min and collect glycan containing fractions using UV detection at 205 nm.
11. Dry the combined fractions by lyophilization, re-suspend with 1 mL of DI water. Quantify the glycans[5] and then store the remaining sample at -20 °C for future use.
12. Ready for use as 2AB labeled *N*-glycan from fetuin.

Sample Preparation for Injection

1. Mix 25 µL of purified labeled glycans at 0.2 nmol/µL in DI water with 75 µL of acetonitrile.
2. Transfer the total solution to the auto sampler vial for analysis.

Note: Store the standard at -20 °C.

Separation Conditions

Column	GlycanPac AXH-1, 2.1 x 150 mm, 1.9 µm
Mobile phase	A: acetonitrile + water (80:20, v/v)
	B: ammonium formate (80 mM, pH 4.4)
Flow rate (µL/min)	400
Column temperature (°C)	30
Sample volume (injected) (µL)	1
Mobile phase gradient	Refer to Table 1

Table 1. Mobile phase gradient

Time (min)	% A	%B	Flow (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

MS Conditions

MS instrument	Q Exactive hybrid quadrupole-Orbitrap MS
Source	HESI-II probe
Ionization mode	Negative ion
Full MS	
MS scan range (*m/z*)	380–2000
Resolution	70,000
Microscans	1
AGC target	1×10^6
Max IT (ms)	60
dd-MS2	
dd-MS2 resolution	17,500
Microscans	3
MS/MS AGC target	2×10^5
MS/MS max IT (ms)	250–1000
Isolation window (*m/z*)	2
NCE	35
Stepped NCE	8%
Dynamic exclusion (s)	90

Source Conditions

Source position	C
Sheath gas flow rate (arb units)	20
Auxillary gas flow rate (arb units)	5
Sweep gas flow rate	0
Spray voltage (kV)	3.30
Capillary temperature (ºC)	275
S-lens RF level	50
Heater temperature (ºC)	300

Data Processing and Software

Chromatographic software	Thermo Scientific™ Chromquest™ v 5.0 Chromatography Data System
MS data acquisition	Thermo Scientific™ Xcalibur™ software v 2.2 SP1.48
MS/MS data analysis	SimGlycan software v 4.5

SimGlycan Search Parmeters

Ion mode	Negative
Adduct	H
Chemical derivatization	Underivatized
Match fragment ion for charge state	< Precursor *m/z* charge state
Precursor ion *m/z*	10 ppm
Fragment ion	0.05 Da
Modification	2AB
Class	Glycoprotein
Sub class	*N*-glycan (Intact Core)
Biological source	Bovine Fetuin
Pathway	Unknown
Search structure	All
Glycan type	All
% of evident glycosidic linkages	2
Fragmentation pattern	Specify Expected Fragments in the Spectra
Glycosidic	B: Yes; C: Yes; Y:Yes; Z:Yes
Cross-ring	A:Yes; X:Yes
Glycosidic/Glycosidic	Z/Z: Yes; Y/Y: Yes; B/Y or Y/B: Yes; C/Z or Z/C: No; Z/Y or Y/Z: No; B/Z or Z/B: No; C/Y or Y/C: Yes
Cross-ring/Glycosidic	A/Y or Y/A: Yes; A/Z or Z/A:Yes; X/Y or Y/X: Yes; X/Z or Z/X: No; X/B or B/X: Yes; X/C or C/X: Yes

Results and Discussion

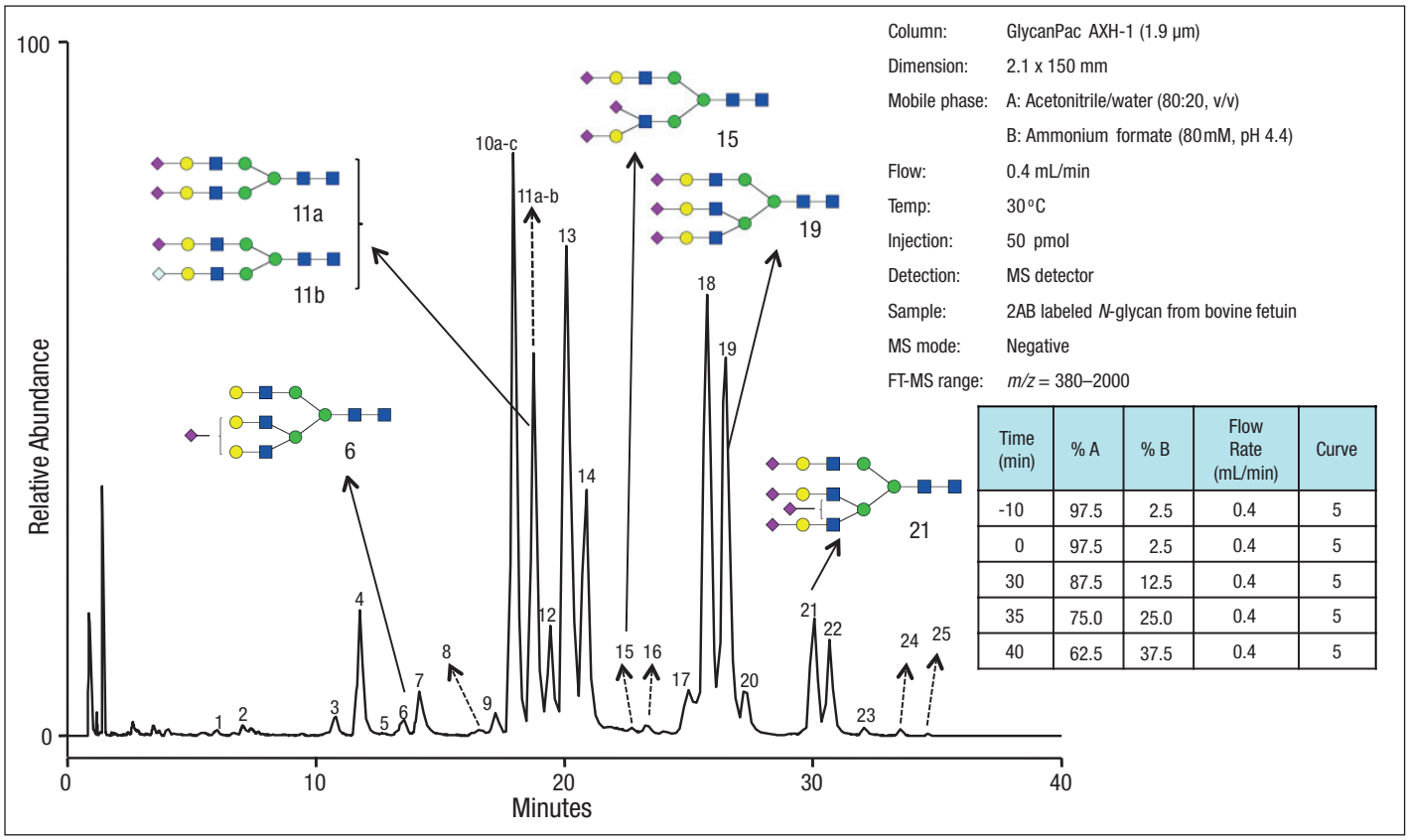
The protocol outlined in this application note yields detailed information on the set of glycans present in proteins including mAbs. The protocol describes a fully integrated workflow that combines novel column technology (GlycanPacAXH-1 column), mass spectrometry (Q Exactive mass spectrometer), and a bioinformatics tool (SimGlycan software). This fully integrated workflow is demonstrated for *N*-glycans released from bovine fetuin glycoprotein, but can be used for released *N*-glycans from any glycoprotein.

The GlycanPac AXH-1 column described in this application note can be used for qualitative and quantitative characterization of neutral and charged glycans present on proteins. The elution of glycans is based on charge: the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated to penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. Separation of glycans based on charge, size, and polarity–combined with MS–provides complete structural and quantitative information.

2AB labeled *N*-linked glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer (Figure 1). Data-dependant MS/MS spectra were acquired on all precursor ions ($z \geq 2$), and SimGlycan software was used for structural elucidation. A representative example of the analysis is shown in Figure 2. The Q Exactive mass spectrometer was selected for these experiments because of its 140,000 FWHM resolution at *m/z* 200, high scan speeds at all resolution settings, and sensitivity. All of these contribute to the detection of minor glycan species and generation of high-quality MS/MS spectra even for low-abundance glycans.

Additionally, the Q Exactive mass spectrometer has the ability to generate higher-energy collisional dissociation (HCD) with high-resolution, accurate-mass (HR/AM) fragment ions. This allows for differentiation of near-mass fragment ions, which were observed to be useful for correctly assigning branching and linkage. The variation of collision energy can provide different fragment ions within the mass spectrometer. To maximize both glycosidic and cross-ring fragments, normalized stepped collision energy (NSCE) was incorporated. This provided optimum conditions for generation of a maximum number of both cross-ring and glysodic cleavages in a single spectrum, thereby increasing confidence in the identification (Figure 2). The detailed structural information obtained from the MS/MS data shown in Table 2 further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity.

The use of LC-MS/MS for glycan analysis increases the complexity of data analysis due to the large number of MS/MS spectra generated. SimGlycan software was incorporated to simplify data analysis.[6,7] SimGlycan software predicts the structure of a glycan from the MS^n data. It accepts the raw MS^n files, matches them with its own database of theoretical fragmentation of over 22,000 glycans, and generates a list of potential glycan structures. Each proposed structure is assigned a score to reflect how closely it matches with the experimental data.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 1. LC-MS analysis of 2AB labeled *N*-glycans from bovine fetuin by GlycanPac AXH-1 column with MS detection

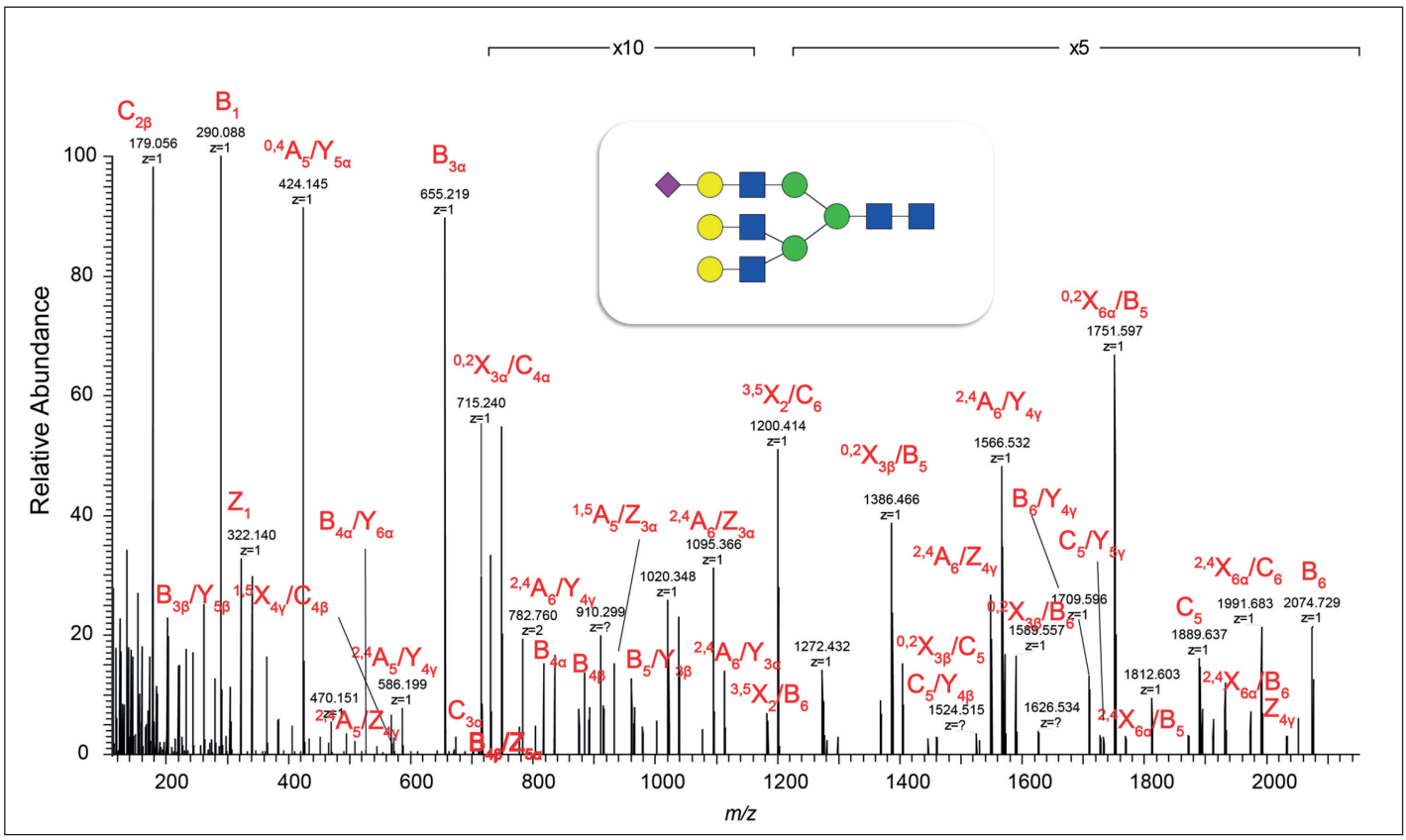
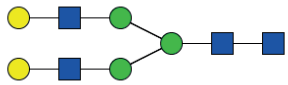
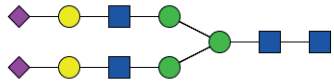
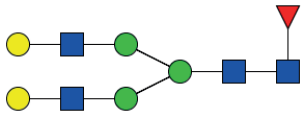
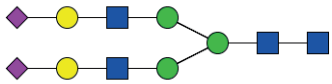
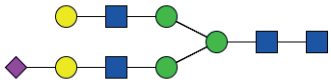
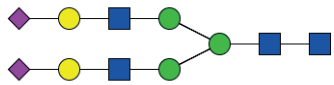
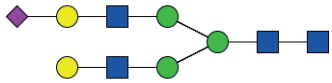
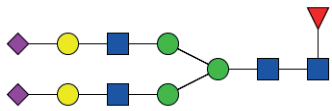
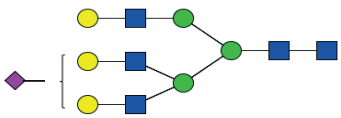
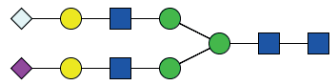
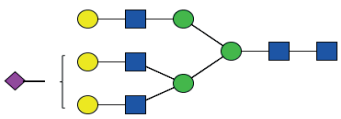
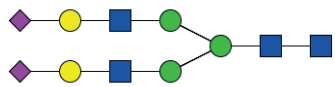
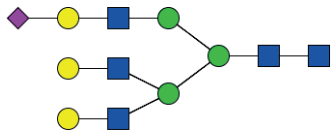
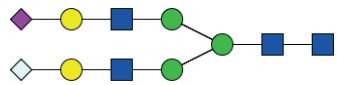
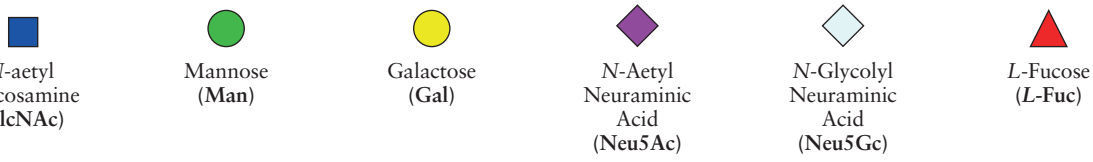
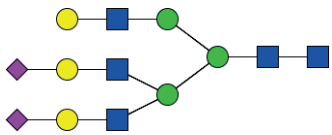
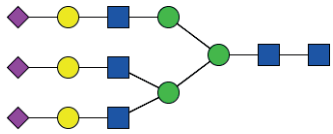
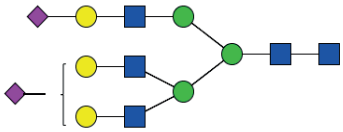
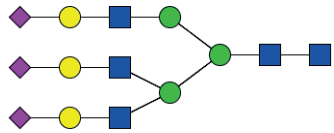
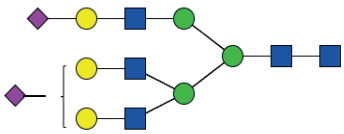
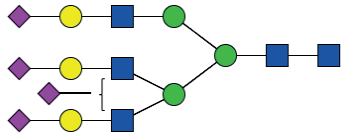
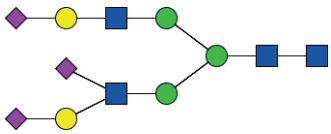
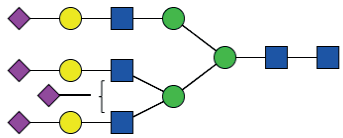
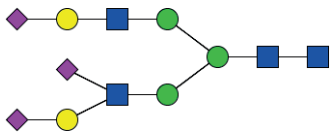
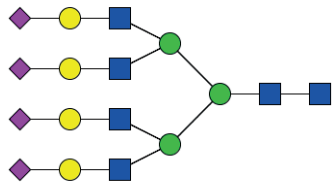
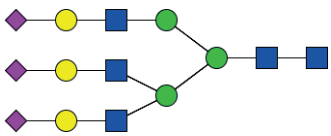
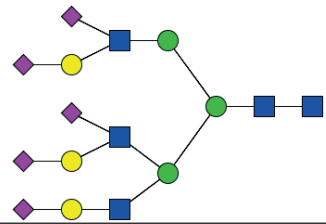
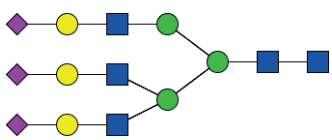
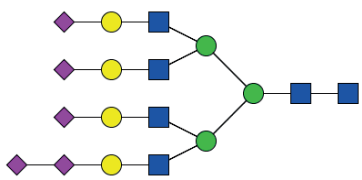


Figure 2. HCD MS/MS spectrum of a 2AB-labeled monosialylated triantennary *N*-glycan from bovine fetuin

Table 2. Structural identification of glycans present in each peak by the separation of 2AB labeled *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure (2AB labeling is not shown)	Peak (Figure 1)	Compound structure (2AB labeling is not shown)
1		8	
2		9	
3		10a	
4		10b	
5		10c	
6		11a	
7		11b	

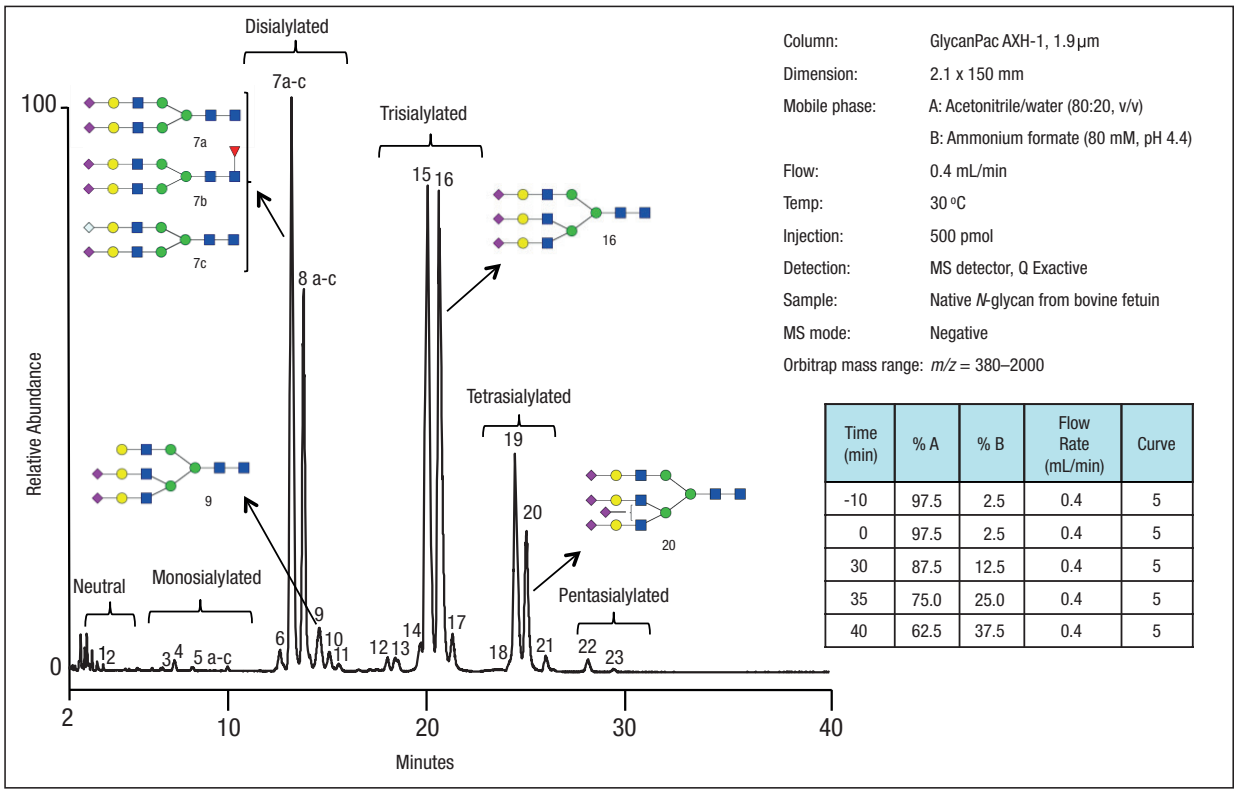


12		19	
13		20	
14		21	
15		22	
16		23	
17		24	
18		25	

LC-MS Analysis of Native *N*-Glycans Released from Proteins

The GlycanPac AXH-1 column is also suitable for analysis of native glycans. Analyzing unlabeled glycans not only eliminates the extra reaction step and cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Figure 3 shows the LC/MS analysis of native *N*-glycans from bovine fetuin using the GlycanPac AXH-1 column. Detailed information is in Table 3. A representative MS/MS spectrum for a trisialylated triantennary glycan is shown in Figure 4.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 3. LC/MS analysis of native *N*-glycan from bovine fetuin

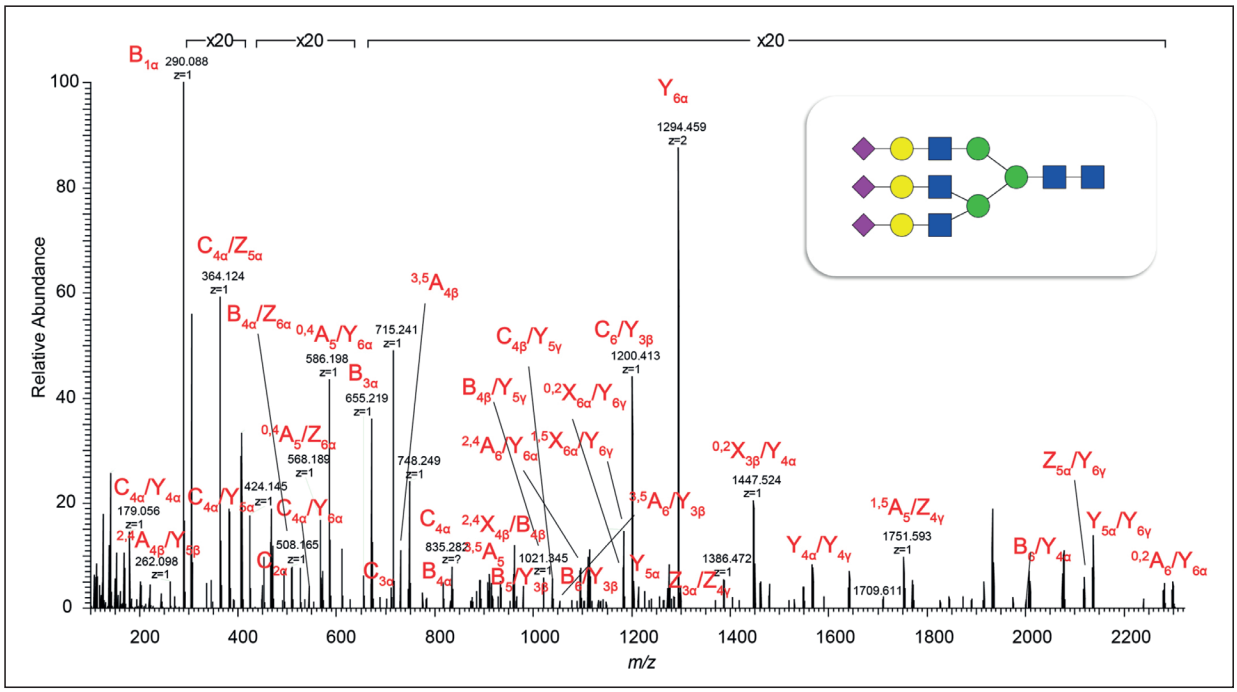
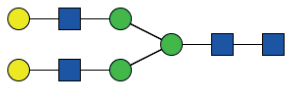
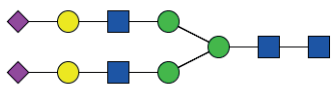
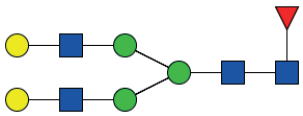
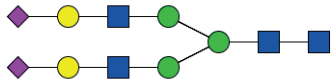
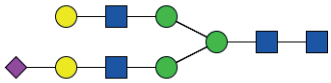
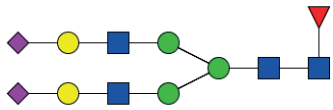
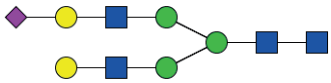
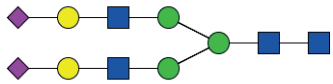
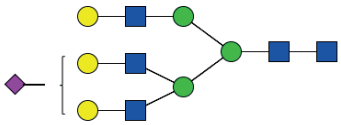
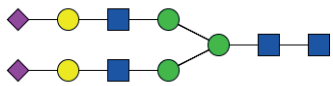
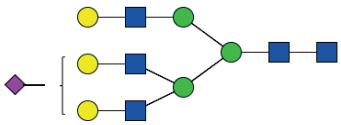
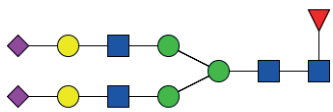
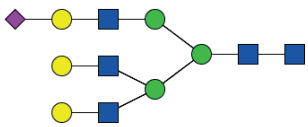
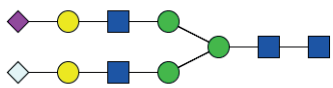
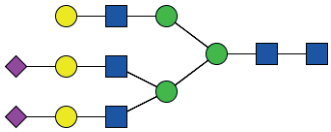
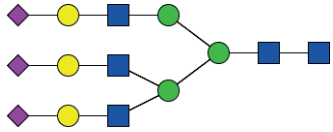
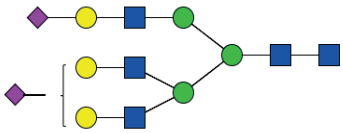
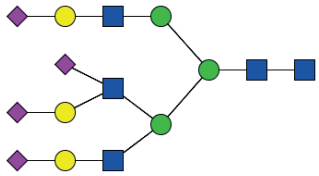
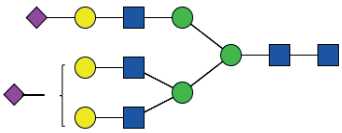
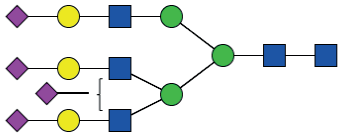
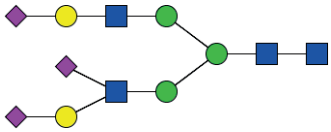
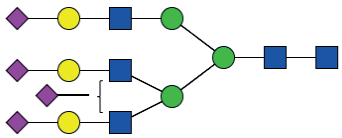
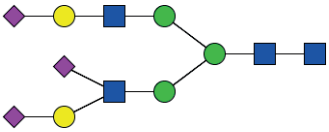
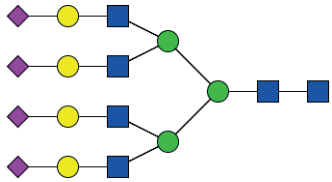
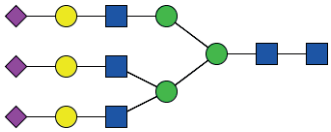
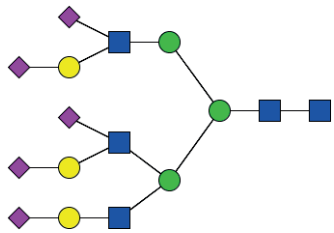
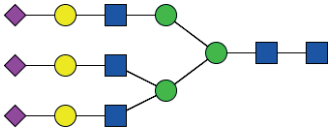
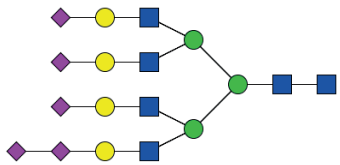
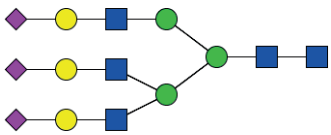


Figure 4. MS/MS spectra for a native trisialylated triantennary *N*-glycan released from bovine fetuin

Table 3. Structural identification of glycans present in each peak by the separation of native *N*-glycans from bovine fetuin using GlycanPac AXH-1 column and Q Exactive mass spectrometer

Peak (Figure 1)	Compound structure	Peak (Figure 1)	Compound structure
1		6	
2		7a	
3		7b	
4		7c	
5a		8a	
5b		8b	
5c		8c	

9		17	
10		18	
11		19	
12		20	
13		21	
14		22	
15		23	
16			

Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially for glycans containing multiple sialic acids (Figure 3). However, labeled glycans require smaller amounts (10 times) of samples for MS analysis as compared to native glycans. Thus, the GlycanPac AXH-1 column is useful for the analysis of biologically relevant glycans including glycans from antibodies, either labeled or native, by LC-fluorescence or LC-MS methods. If the amount of the sample is not extremely limited, analysis of unlabeled glycans using the GlycanPac AXH-1 is highly feasible.

Conclusion

- A fully integrated workflow for structural characterization of native and fluorescently labeled *N*-glycans released from proteins was demonstrated successfully.
- Novel GlycanPac AXH-1 column demonstrated excellent separation of released N-glycans especially forsilalylated species. It allowed for their sensitive detection by the Q Exactive mass spectrometer and identification by SimGlycan software.
- This LC-MS integrated technology is also useful for the separation and structural characterization of reduced *O*-linked glycans from proteins, mucins, and the analysis of charged and neutral glycosylaminoglycans and glycolipids.

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7. Saba, J.; Apte, A.; Meitei, N.S.; Viner, R., Thermo Scientific Application Note 516: Automated Glycan Structural Isomer Differentiation Using SimGlycan Software.

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Singapore +65 6289 1190
Spain +34 914 845 965
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Structure Characterization and Differentiation of Biosimilar and Reference Products Using Unique Combination of Complementary Fragmentation Mechanisms

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Overview

Purpose: To analyze difference of protein structure in biosimilar and reference products using Orbitrap LC-MS/MS

Methods: A unique data-dependent instrument method that utilizes two different fragmentation mechanisms was applied for peptide sequence and PTM identification and quantification using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer. While generating HCD MS/MS spectra on peptides in a data-dependent experiment, the method identifies glycopeptides on-the-fly using the diagnostic ions from glycan fragmentation. A subsequent ETD fragmentation is then triggered on the same peptide to produce information of amino acid sequence and site of glycosylation. The new Thermo Scientific™ PepFinder™ 1.0 software for peptide imaging, was used for data analysis.

Results: A LC-MS/MS workflow was developed for differentiating minor difference of protein structure in biosimilar and reference products using an Orbitrap Fusion LC-MS/MS and PepFinder 1.0 software. This new approach offers efficient, confident and comprehensive analysis, not only for biosimilar comparability study but also for lot-to-lot comparison of a same compound.

Introduction

Biosimilars are subsequent versions of innovator biopharmaceutical products created after the expiration of the patent on the innovator product. The approval of a biosimilar product by a regulatory agency requires thorough characterization that demonstrates comparability with a reference product in quality, safety and efficacy. High resolution mass spectrometry provides accurate characterization of various protein properties including primary structure, type and location of post-translational modifications (PTMs), and low abundant sequence variants or impurities. In this study, we developed a robust approach for comparability study of biosimilar and reference product. Minor differences in products including glycosylation were systematically compared using high resolution LC/MS/MS with complementary fragmentation methods and a new peptide mapping software package.

Methods

Samples

An original drug, a recombinant variant and its biosimilar product, TPA, I-TNK and G-TNK, were digested using trypsin after reduction and alkylation. Tenectelplase (TNK) is a recombinant TPA with the following minor sequence changes:

T103->N (Becomes N-glycosylation site)
N117->Q (Removes N-glycosylation site)
KHRR (296-299) -> AAAA

Liquid chromatography

Peptides were separated using with a Thermo Scientific™ EasySpray™ source setup containing 50-cm C_{18} column (2 µ particle size) and a high-pressure easy nanoLC (U-HPLC). The LC solvents were 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Flow rate was 250 µL/min. A 70 min gradient was used to elute peptides from the column.

Mass spectrometry

Samples were analyzed using an Fusion mass spectrometer with a Thermo Scientific™ EASY-ETD™ ion source. An instrument method designed for glycopeptide analysis was used for this study. This method primarily acquires HCD MS/MS spectra on peptides in a data-dependent top-ten experiment. However, if diagnostic sugar oxonium ions from glycan fragmentation are detected in the HCD MS/MS spectrum, a subsequent ETD fragmentation is then triggered on the same precursor peptide to produce amino acid backbone sequence information to identify the site of glycosylation. Therefore, for each glycopeptide, this HCD product-dependent ETD method (HCDpdETD) generates a pair of HCD and ETD spectra, producing information for the peptide sequence and the site of glycosylation as well as confirming glycan structure. Orbitrap MS spectra were acquired at 120,000 resolution (at *m/z* 200) with an AGC target of $4x10^5$. MS/MS spectra were acquired at 30,000 resolution (at *m/z* 200) with an AGC target of $5x10^4$. Capillary temperature was set to 275 °C and the S-lens level was set at 60. The priority for precursor selection for data-dependent MS/MS was for the highest charge state followed by the lowest *m/z*. HCD collision energy was 30 and ETD activation time was charge dependent based on the standard calibration.

Data analysis

Data was analyzed using PepFinder 1.0 software. This software provides automated analyses of liquid chromatography/tandem mass spectrometry data for large-scale identification and quantification of known and unknown modifications. Peptide identification is achieved by comparing the experimental fragmentation spectrum to the predicted spectrum of each native or modified peptide. Peak areas of related peptide ions under their selected-ion chromatograms (SIC) are used for relative quantification of modified peptides. A mass tolerance of 5 ppm was used to ensure accurate identification.

Results

1. Peptide identification and protein sequence coverage

The data was analyzed and the results were compared. Peptide mapping results indicated 100% sequence coverage for all of the data files. The relative abundance of each modified peptide forms was calculated and compared between files. A five order magnitude dynamic range for identified peptide abundance was achieved, which allowed identification of modified peptides with less than 0.01% in abundance of the unmodified versions (data not shown). Figure 1 shows an example of the sequence coverage view for one of the data files.

Figure 1. 100% sequence coverage of I-TNK

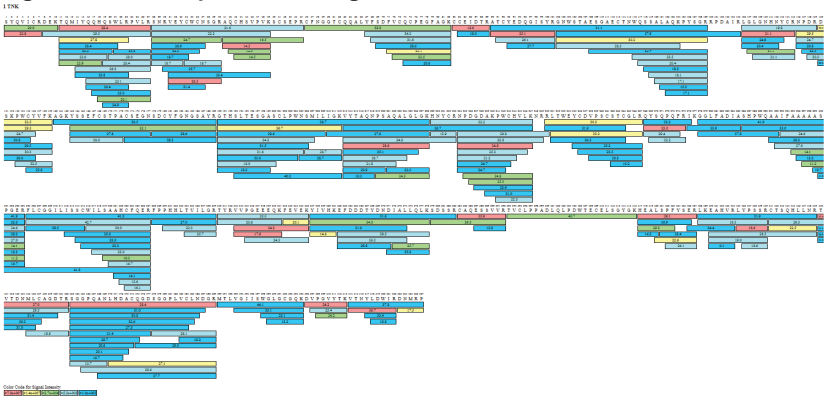
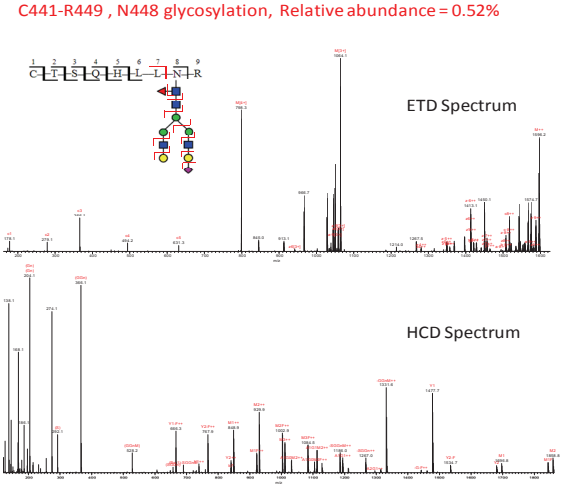


Table 1. Identified glycosylation sites, percentage of glycosylation and the number of glycoforms identified with high confidence

Site of glycosylation	Sample	# glycoforms	% glycosylation
N 103	I-TNK	18	>99
N 103	G-TNK	11	>99
N117	TPA	14	>99
N 184	I-TNK	12	19
N 448	TPA	44	>99
N 448	I-TNK	36	>99
N 448	G-TNK	47	>99

Figure 2. Characterization of glycopeptides using HCDpdETD. G-TNK peptide C441-R449 with glycosylation on N448. Top left is fragment ion coverage showing peptide backbone fragmentation from ETD (black, with glycan preserved) and fragmentation of peptide and glycan from HCD (red).



2. Glycosylation of TPA, I-TNK and G-TNK

A total of four glycosylation sites were identified, three of which are over 99% glycosylated. N448 was glycosylated in all three samples, while N103 was detected in I-TNK and G-TNK and N117 only in TPA. The forth glycosylation site, N184, was identified only in I-TNK and only 19% of this site is glycosylated (Table 1). I-TNK has an additional glycosylation site (N184) even though it shares the same amino acid sequence as G-TNK, suggesting a different manufacturing process. Examples of two identified glycopeptides are shown in Figure 2 and Figure 3.

Figure 3. Characterization of glycopeptides using HCDpdETD. I-TNK peptide G102-R129 with glycosylation on N103. The fragment ion coverage at the top of this figure shows peptide backbone fragmentation from ETD (black, with glycan preserved) and fragmentation of peptide and glycan from HCD (red).

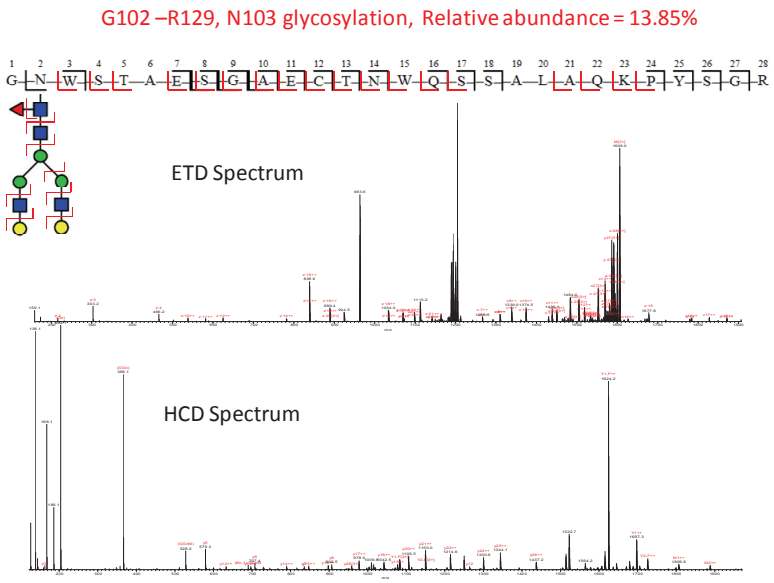


Table 2. Comparison of N448 glycoforms in the three samples. Only those with relative abundance higher than 1% in at least one of the samples are included. The five major glycoforms are highlighted in bold. Abbreviations for glycan structure: Antenna A, core fucose (Fuc) F, mannose (Man) M, galactose (Gal) G, *N-acetyl neuraminic acid (NANA) S, N-glycolyl neuraminc acid (NGNA) Sg*

N448 Glycoform	TPA	I-TNK	G-TNK
N448+A2G2F	**6.41%**	**5.40%**	**3.23%**
N448+A2S1G0	5.18%	2.57%	<1%
N448+A2S1G0F	<1%	<1%	1.79%
N448+A2S1G1F	**23.11%**	**16.86%**	**14.43%**
N448+A2S2F	**37.96%**	**35.34%**	**37.59%**
N448+A3G3F	<1%	1.29%	<1%
N448+A2Sg1S1F	1.32%	<1%	<1%
N448+A3S1G2F	1.59%	2.48%	<1%
N448+A3S2G0	1.43%	<1%	<1%
N448+A3S2G1F	**5.19%**	**7.00%**	**5.04%**
N448+A4S2G2F	<1%	<1%	2.20%
N448+A4S1G3F	<1%	1.16%	<1%
N448+A3S3F	**9.33%**	**11.61%**	**16.50%**
N448+A4S3G1F	1.17%	6.55%	2.62%
N448+A4S4F	1.67%	7.20%	6.51%

The type and relative abundance of gllycoforms were compared across the three samples and the following was observed:

1. The relative abundance and identity of the various glycoforms on N448 were consistent among all three samples (Table 2). Most of glycans on this site contain sialic acid.
2. The identity of the glycoforms on N103 are similar between I-TNK and G-TNK, but the relative abundance profiles are markedly different. Although the most abundant form, A2S1G1F, is the same in the two samples, the second and the third most abundant forms are not. For the top five most abundant forms, only two of them were common in the two samples (data not shown).
3. The glycoforms on N117 are primarily high mannose, which is very different from the glycans identified on any of the other sites (data not shown).
4. Glycosylation on N184 was only detected for the I-TNK sample (data not shown), with all of the glycans containing sialic acid.

3. Other identified and quantified modifications

Besides glycosylation, other covalent modifications that were indentified in these three samples included cysteine alkylation, deamidation, overalkyation, Cys+DTT, oxidation, formylation, and glycation. Figure 4 shows confident identification and localization of a low abundant double oxidation on W406. The relative abundance of the oxidized form is less than 0.1%.

A total of 12 N-deamidation sites were indentified with high confidence in the three samples. Deamidation on N140 was only identified in I-TNK and G-TNK, but not in TPA. Other sites of N-deamidation were consistent across all three samples (Table 3). Figure 5 shows examples of a peptide that were identified in 3 different forms: native and deamidated on two different Asp residues, respectively.

Figure 4. Identification of low abundant double oxidized peptide T393-K416 and localization of double oxidation to W406.

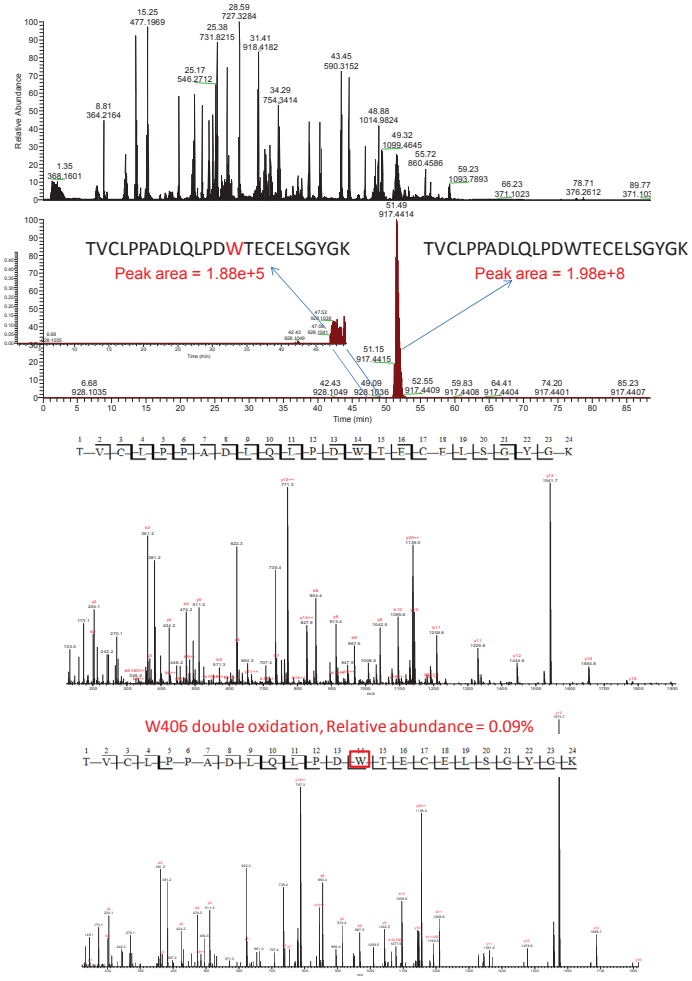


Figure 5. Identification and localization of two deamidation sites, N140 and N142 , on peptide L136-R145. High resolution HCD spectrum of this peptide in native form (top), with deamidation either on N142 (middle) or on N140 (bottom).

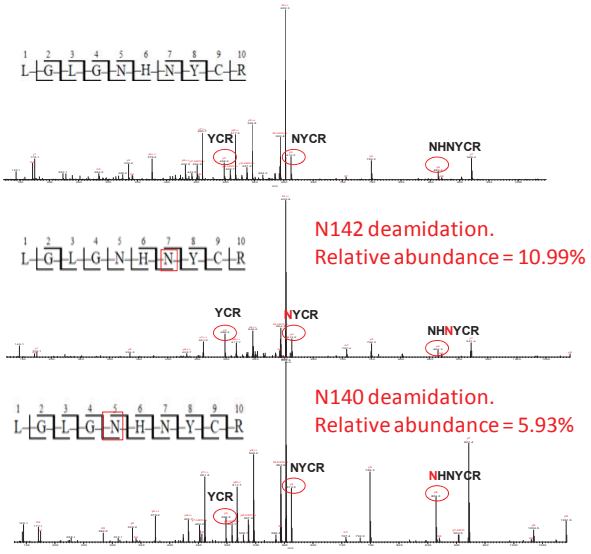


Table 3. Identified N-deamidation sites and relative abundance of deamidation

Location of N-deamidation	TPA	I-TNK	G-TNK
N140	ND	12.24%	10.21%
N142	3.68%	3.82%	2.70%
N205	2.08%	1.61%	<0.5%
N218	0.63%	<0.5%	<0.5%
N234	<0.5%	<0.5%	<0.5%
N37	29.83%	22.83%	19.64%
N370	8.24%	13.56%	<0.5%
N454	3.62%	2.71%	2.27%
N469	3.71%	2.05%	1.24%
N486	11.20%	10.80%	7.64%
N516	3.68%	2.87%	2.20%
N524	1.32%	<0.5%	1.80%

Conclusion

A LC-MS/MS workflow was developed to differentiate minor differences in protein structure for biosimilar and reference products using an Orbitrap Fusion instrument and new peptide mapping software, PepFinder 1.0. This workflow provides qualitative and quantitative biosimilar to reference product comparison.

1. 100% sequence coverage was obtained for all the nine data files analyzed .
2. The identified covalent modifications, both expected and un-expected, include cysteine alkylation, deamidation, overalkyation, Cys+DTT, oxidation, formylation, glycation and glycosylation. The relative abundance of the modified forms was calculated and compared between datasets. Confident identification and precise localization of low abundant PTMs was achieved.
3. Glycosylated peptides were characterized using the unique HCDpdETD method which generates information of peptide sequence, site of glycosylation as well as glycan structure. Comparison of glycosylation sites as well as the type and relative abundance of glycoforms indicated that there are significant differences in glycosylation between the three samples.

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Middle-down Analysis of Monoclonal Antibody Middle using Nano-flow Liquid Chromatography and a Novel Tribrid Orbitrap Mass Spectrometer

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Overview

Purpose: Sensitive analysis of monoclonal antibody using middle-down approach on a novel Thermo Scientific™ Orbitrap Fusion™ Tribrid™ mass spectrometer

Methods: A method combining nano-flow liquid chromatography and complementary higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD) fragmentation on an Orbitrap Fusion Mass Spectrometer was developed and implemented

Results: A monoclonal antibody was enzymatically and chemically cleaved into Fd', Fc/2 and light chain respectively. Intact masses of the monoclonal antibody and its proteolytic fragments were measured. The proteolytic fragments (Fd', Fc/2 and light chains) were directly fragmented using HCD and ETD. An average 50% amino acid coverage was achieved and glycosylation site was unambiguously identified

Introduction

Monoclonal antibodies (mAbs) are an increasingly important line of therapeutics for the biopharmaceutical industry. The demand to better understand the biochemical and biophysical properties of mAbs has become critical. Recent developments in high resolution mass spectrometry (MS) with multiple dissociation techniques have clearly shown its distinctive power for characterization of intact proteins and in particular its subunits. The mass spectrometry–based study of mAbs in middle-down approach provides a wealth of information to interpret its structural features. Here, we describe the use of an Orbitrap Fusion mass analyzer in combination with nano liquid chromatography (LC) for characterizing a mAb protein using different dissociation techniques.

Methods

Sample Preparation

The intact mAb protein was enzymatically cleaved below the hinge region into a F(ab')2 fragment and two Fc fragments. Aliquot of candidate NIST RM 8670 mAb lot #3F1b was treated with FabRICATOR® (Genovis, Sweden) at 37 ºC for 1 hour. The resulting F(ab')2 and Fc fragments were further denatured and reduced in 50mM dithiothreitol (Sigma, Saint Louis, MO) at 56 ºC for 1 hour. The proteolytically fragmented mAb was diluted to 1.5 µg/µL using 0.1% formic acid in water.

Liquid Chromatography

Fd' and Fc/2 fragments after reduction were chromatographically eluted from a Thermo Scientific™ PepSwift™ column, Monolithic easy spray column (200 µm x 25 cm, Thermo Fisher Scientific, Amsterdam, the Netherlands). One µL of the stock was loaded per injection. Nano flow reverse phase chromatography was performed with a 800 nl/minute gradient of 5-60% in 32minutes using the Thermo Scientific™ EASY-nLC™ 1000 system. The proteins were directly detected by a standard Orbitrap fusion mass spectrometer. Liquid chromatography solvents used include the aqueous as 0.1% formic acid in water (Fisher Scientific, Fair Lawn, New Jersey) and the organic as 0.1% formic acid (Fisher Scientific, Fair Lawn, New Jersey) in acetronitirile (Fisher Scientific, Fair Lawn, New Jersey).

Mass Spectrometry

The Fd', Fc/2 and light chain eluted were directly detected by a standard Orbitrap Fusion MS under both full scan mode and tandem scan mode using higher-energy collisional dissociation (HCD) and electron transfer dissociation (ETD). Full mass spectra of mAb fragments were acquired at 240,000 resolution at *m/z* 200 with mass range *m/z* 400-2000. AGC setting for full MS spectrum was at 1e5 with 100ms maximum injection. Ion transferring temperature was set at 300 ºC. Full mass spectra of intact mAb were acquired at 15,000 resolution at *m/z* 200 with mass range *m/z* 1000-6000.

Data Analysis

The full mass spectra were analyzed with Thermo Scientific™ Protein Deconvolution™ 3.0 software for molecular mass determination . The tandem mass spectra of Fd', Fc/2 and light chain were deconvoluted by Xtract. The deconvoluted spectra were processed by ProSightPC 3.0 software for middle-down data interpretation.

Results

Full Mass Spectrum of Intact mAb

Intact mAb (candidate NIST RM 8670 mAb lot #3F1b) was surveyed by LC-MS. Data shown below was acquired at 15,000 resolution on Orbitrap Fusion MS with source CID at 70. The application of 40-80 source CID is beneficial for most mAb proteins to help remove the adducts and thus promote a cleaner signal. The optimal SID setting is protein dependent.

FIGURE 1. Full MS spectrum of the intact mAb (candidate NIST RM 8670 mAb lot #3F1b) obtained from LC-MS analysis.

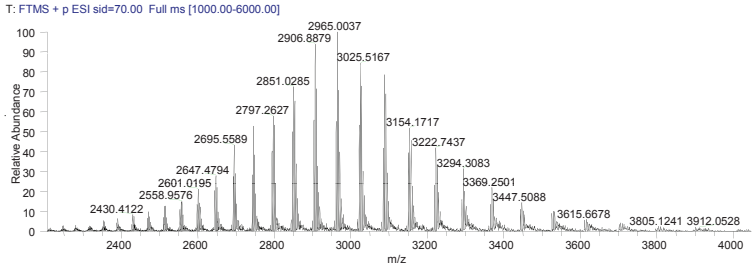
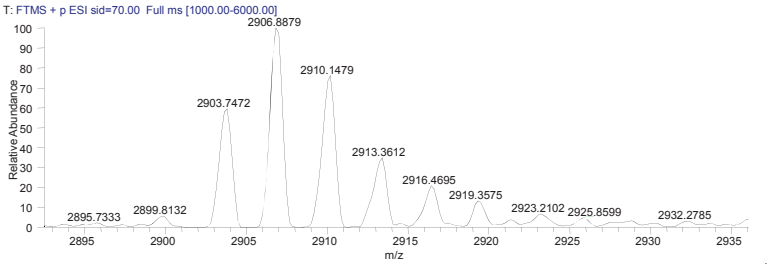


FIGURE 2. Expanded view of full MS spectrum of the intact mAb. Different glycoforms at charge +51 was shown.



Intact LC-MS Analysis of Proteolytic Fragmented mAb

A mixture including approximately 20 pmol of Fd', Fc/2 and light chain, respectively, was eluted at 800 nl/min and directly analyzed at 240,000 resolution on Orbitrap Fusion MS. The mAb fragments were separated by PepSwift monolithic column. For a 800 nl/min gradient of 5-60% in 32 minutes, different glycoforms of Fc/2 were first eluted at 20.67minute, followed by light chain at 22.34minute. Eluted last was Fd' chain at 24.76min.

Full MS spectra acquired at 240,000 resolution provides base line resolution of the isotope distribution of the ~25,000Da mAb fragments. The isotopically resolved spectra were deconvoluted for monoisotopic masses. Figure 6 shows the deconvoluted monoisotopic mass of the light chain is 23113.3568Da, which suggests a mass accuracy of 2.2ppm comparing to its theoretical monoisotopic mass at 23113.3041Da.

FIGURE 3. Total Ion Current Chromatogram of MAb Fragments Eluted from a PepSwift Monolithic Nano Column.

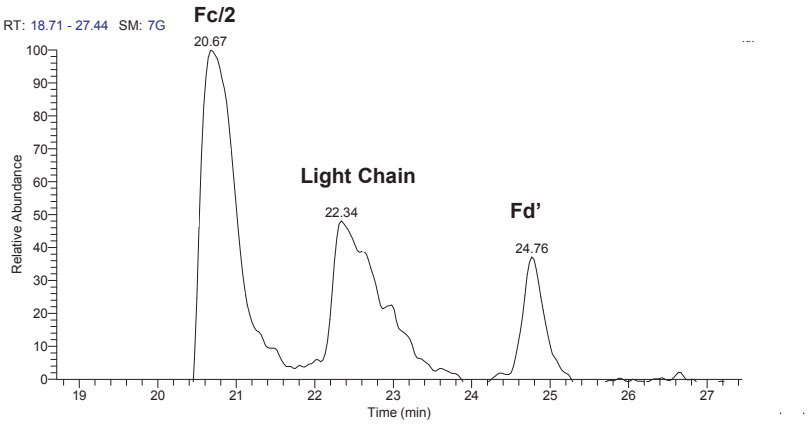


FIGURE 4. Full MS spectrum of mAb fragments at 240,000 resolution. (A) Full MS spectrum of Fc/2; (B) Full MS spectrum of light chain; (C) Full MS spectrum of Fd'.

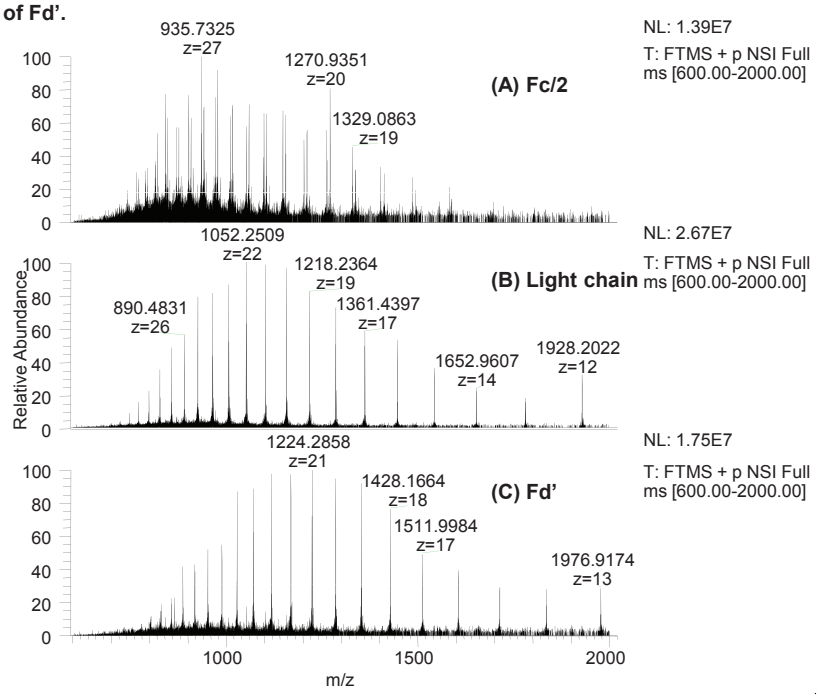


FIGURE 5. Isotopically resolved mAb fragments at 240,000 resolution. (A) Fc/2+G1F, charge +28; (B) Light Chain, charge 21; (C) Fd', charge 21.

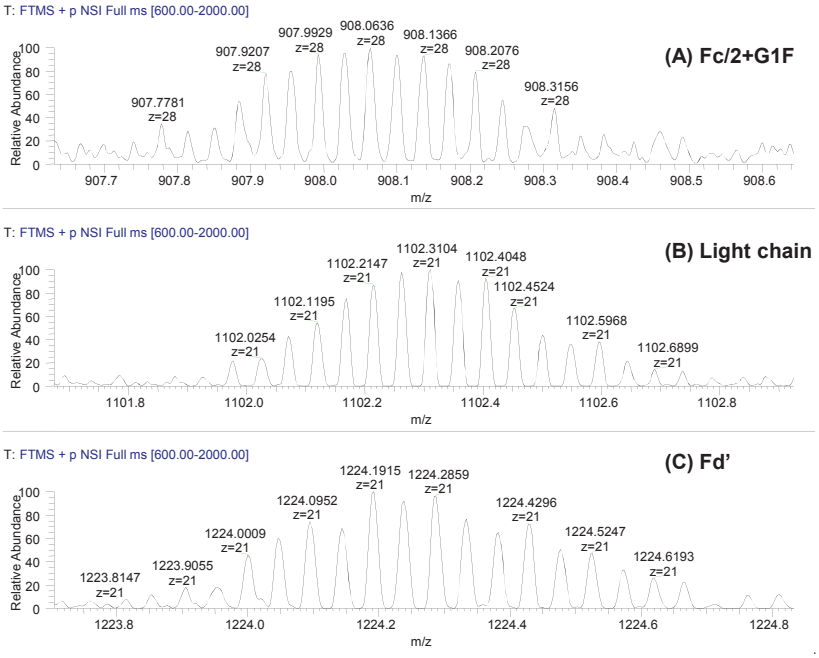
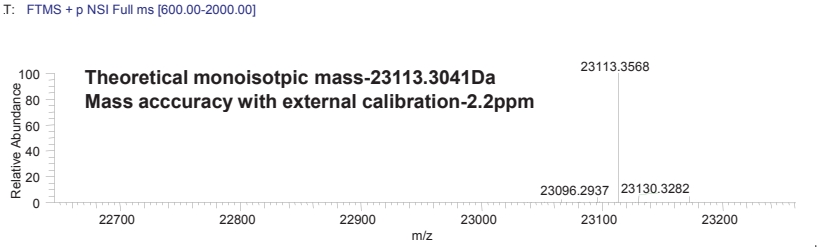


FIGURE 6. Deconvoluted monoisotopic mass of light chain.



Top Down Analysis of Proteolytic Fragmented MAb

Middle down analysis of mAb protein was performed by LC-MS analysis of the proteolytic fragments. Precursor ions at *m/z* 964.65 of light chain, *m/z* 1117.87 of Fd' and *m/z* 902.20 of the G0F glycoform of Fc/2 were selected respectively for ETD and HCD MS/MS at 120,000 resolution. Reaction time of 3-5 ms was applied for ETD fragmentation. The normalized collision energy for HCD was 15-25 %. As shown in Figure 7, tandem spectra generated contain well resolved, multiply charged fragment ions. Interpretation of these ions based on the mAb protein sequence was performed using ProSightPC 3.0. The combined results of ETD and HCD suggest 50% sequence coverage for light chain, 52% coverage for Fc/2 with G0F and 32% coverage for Fd' respectively (Figure 8, 10, 11). N-terminal modification of pyroglutamate of Fd'chain was confirmed based on fragment ions from both ETD and HCD. Both intact and tandem spectra identified the Lys loss at C-terminus of Fc/2.

ETD is widely known for its advantage in preferentially fragmenting the peptide back bone and keeping the labile modifications attached. It has been recognized as the method of choice for locating the sites of such labile PTMs including glycosylation. Multiple identified ETD fragments between Asn61 and Asn79 unambiguously located the glycan G0F on Asn61 of Fc/2 chain. In the highly complex tandem spectrum, the high resolution and accurate mass allows and is also necessary for confident identification of low abundance glycan-containing fragment ions in the presence of interference. For example, shown in Figure 7 insert is the identification of the +9 charged C_{61} ion with G0F; Figure 9 presents the unambiguous identification of C_{57} without G0F. Although not all the isotopic peaks of this c ion were observed due to background interference, all seven isotopes identified were within 3 ppm mass error (external mass calibration). Both ETD ions, C_{61} and C_{57}, have played critical roles in identification and localization of G0F on Asn61 residue.

FIGURE 7. ETD spectrum of Fc/2 with G0F with precursor ion as *m/z* 902.20, charge +28. The insert is an expanded view of the spectrum covering C61 ion at charge +9 with 3ppm mass accuracy, which confirms the addition of G0F glycan.

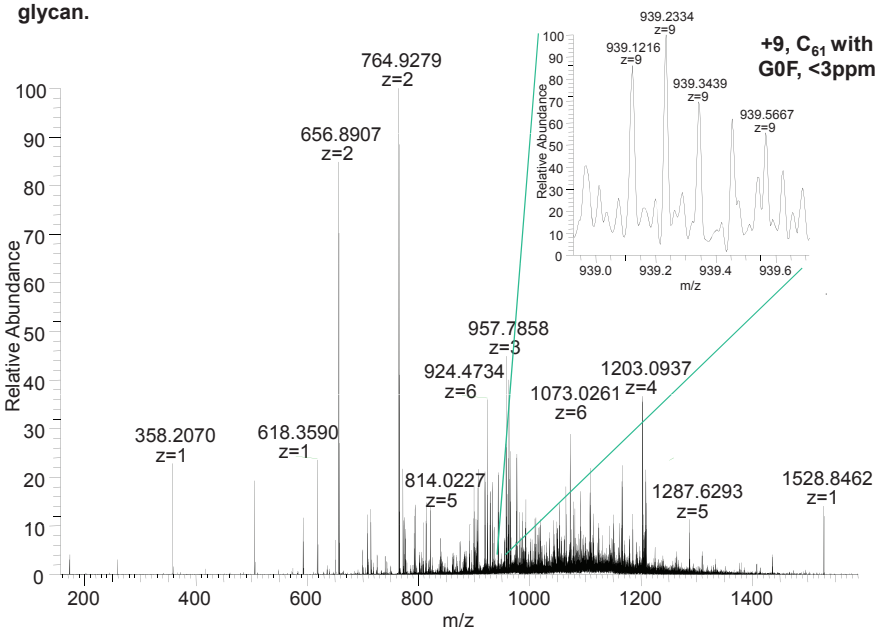


FIGURE 8. ETD (blue) and HCD (red) coverage of Fc/2+G0F chain. Asn61 was highlighted for addition of G0F.

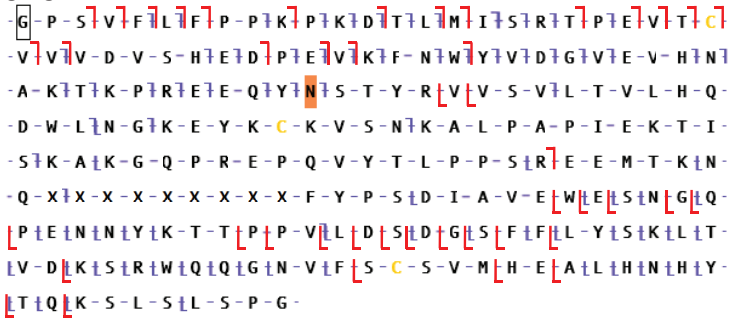


FIGURE 9. Comparison of experimental C_{57} ion of Fc/2+G0F chain to theoretically predicted isotope distribution. This ion is critical for identifying and localizing G0F on Asn61 residue.

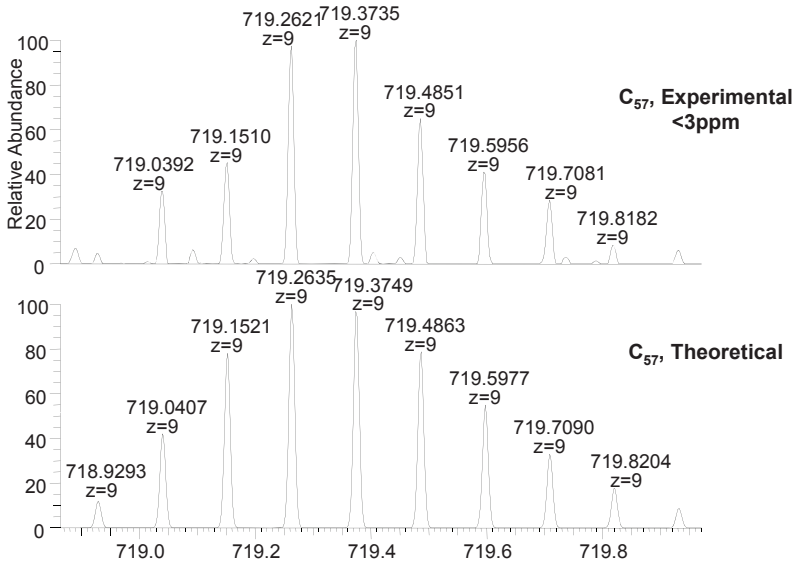


FIGURE 10. ETD (blue) and HCD (red) coverage of light chain.

FIGURE 11. ETD (blue) and HCD (red) coverage of Fd' chain.

Conclusion

The middle-down study indicated that complementary fragmentation mechanisms allow extensive sequence coverage as well as identification and localization of PTMs including labile glycosylation. The middle-down approach produced detailed structure information deep into the middle of the mAb chains especially in the epitope region.

Acknowledgements

We would like to thank National Institute of Standards and Technology for providing the intact mAb protein sample (candidate NIST RM 8670 mAb lot #3F1b).

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Novel Glycan Column Technology for the LC-MS Analysis of Labeled and Native *N*-Glycans Released from Proteins and Antibodies

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Overview

Purpose: Development of novel high-performance liquid chromatography (HPLC) and ultra high-performance liquid chromatography (UHPLC) columns for high-resolution separation and structural characterization of native and fluorescently labeled *N*-glycans released from various proteins, including antibodies.

Methods: UHPLC with fluorescence detection (FLD) for the chromatographic analysis of labeled *N*-glycans. LC with mass spectrometry (MS) and LC-MS/MS analysis for structural characterization of both labeled and native *N*-glycans from proteins by MS detection.

Results: We have developed a high-performance, silica-based HPLC/UHPLC column (Thermo Scientific™ GlycanPac™ AXH-1) specifically designed for simultaneous separation of glycans by charge, size, and polarity. It is designed for high-resolution, and high-throughput analysis, with unique selectivity for biologically relevant glycans, either labeled or native, by LC-FLD and LC-MS methods.

Introduction

Glycans are widely distributed in biological systems in 'free state' and conjugated forms such as glycoproteins, glycolipids, and proteoglycans. They are involved in a wide range of biological and physiological processes.[1] The functions, including efficacy and safety of protein-based drugs such as recombinant proteins and monoclonal antibodies (MAbs), are often dependent on the structure and types of glycans attached to the proteins.[2] The structures of glycans are quite diverse, complex, and heterogeneous due to post-translational modifications and physiological conditions. The structural characterization and quantitative estimation of glycans is highly essential in biotherapeutics and biopharmaceutical projects.[3] However, it is tremendously challenging to comprehensively characterize glycan profiles and determine the structures of glycans.

The GlycanPac AXH-1 columns are high-performance HPLC/UHPLC columns specifically designed for structural, qualitative, and quantitative analysis of glycans. They are designed for high-resolution and high-throughput analysis, with unique selectivity for biologically relevant glycans, including glycans from antibodies—either labeled or native—by LC-fluorescence or LC-MS methods. Because glycans are highly hydrophilic and polar substances, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine, or zwitterion packing materials are often used for glycan analysis. These HILIC columns separate glycans mainly by hydrogen bonding, resulting in size- and composition-based separation. However, identification of the glycan charge state is not possible with these types of columns because glycans of different charge states are intermingled in the separation envelope, making this approach limited. The GlycanPac AXH-1 column, which is based on advanced mixed-mode chromatography technology, overcomes these limitations and can separate glycans based on charge, size, and polarity configuration. In addition, each glycan charge state can be quantified. The GlycanPac AXH-1 column provides both greater selectivity and higher resolution, along with faster quantitative analysis.

Methods

Sample Preparation

Release native glycans from glycoproteins with PNGase F enzyme. Conjugate the released glycans with 2-amino benzamide (2AB) label group using the reported procedure of Bigge et al.[4] Here, 2-AB A1 (P/N GKSB 311), 2-AB A2 (P/N GKSB 312), and 2-AB A3 (P/N GKSB 314) were purchased from Prozyme® (Hayward, CA). Prior to analysis, dissolve samples in 100% buffer (100 mM ammonium formate, pH = 4.4) and dilute further with acetonitrile to make 30% buffer and 70% acetonitrile.

Liquid Chromatography

All the glycans were separated using a Thermo Scientific™ Dionex™ UltiMate™ 3000 BioRS system and either a Thermo Scientific™ Dionex™ UltiMate™ FLD-3400RS Rapid Separation Fluorescence Detector or MS detector.

Mass Spectrometry

MS analysis was performed with a Thermo Scientific™ Q Exactive™ Benchtop LC-MS/MS in negative ion mode at the following settings: MS scan range 380–2000. FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of $1e^6$; and DDA MS2 acquired at 17,500 resolution at *m/z* 200 with AGC target of $2e^5$.

Data Analysis

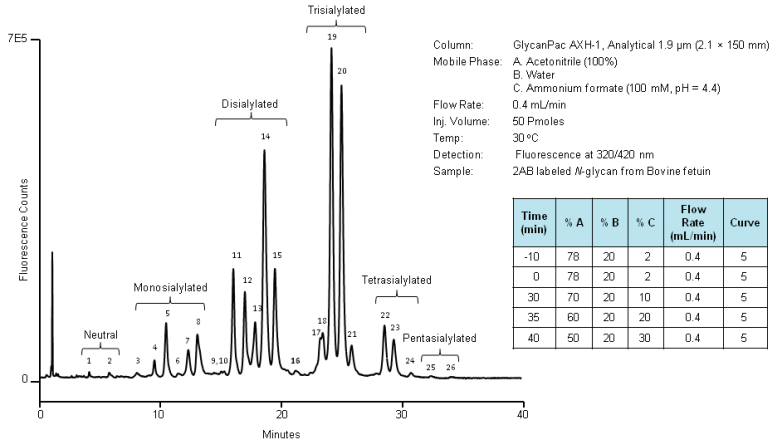
SimGlycan® software (PREMIER Biosoft) was used for glycan identification and structural elucidation data analysis.[5] SimGlycan software accepts raw data files from Thermo Scientific™ mass spectrometers and elucidates the associated glycan structure by database searching and scoring techniques.

Results

Separation of Labeled Glycans Based on Charge, Size, and Polarity

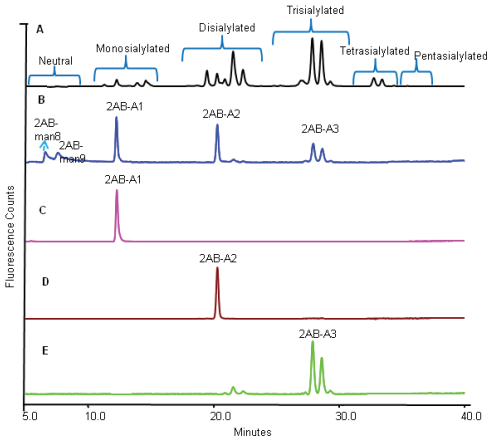
The GlycanPac AXH-1 column can be used for qualitative, quantitative, and structural analysis as well as characterization of uncharged (neutral) and charged glycans present in proteins. Figure 1 shows bovine fetuin on the GlycanPac AXH-1 (1.9 µm, 2.1 ×150 mm) column using fluorescence detection. The separation and elution of glycans are based on charge: the neutral glycans elute first, followed by the separation of acidic 2AB labeled *N*-glycans from monosialylated, disialylated, trisialylated, tetrasialylated and finally pentasialylated species. Glycans of each charge state are further separated based on their size and polarity. The retention time of each glycan charge state was confirmed using 2AB labeled glycan standards (as shown in Figure 2). Separation of glycans is based on charge, size, and polarity, which provides significant structural and quantitative information. The chromatographic profiles shown in Figures 1 and 2, detected by fluorescence detection, provide qualitative information about the separation of *N*-glycans**.** The structure of glycans present in each peak was determined from the LC-MS study using the GlycanPac AXH-1 (1.9 µm) column as shown in the following section.

FIGURE 1. Separation of 2AB labeled *N*-glycans from Bovine fetuin by charge, size and polarity.



Time (min)	% A	% B	% C	Flow Rate (mL/min)	Curve
-10	78	20	2	0.4	5
0	78	20	2	0.4	5
30	70	20	10	0.4	5
35	60	20	20	0.4	5
40	50	20	30	0.4	5

FIGURE 2. Comparison of 2AB labeled *N*-glycans standards and 2AB-*N*-glycans from fetuin.

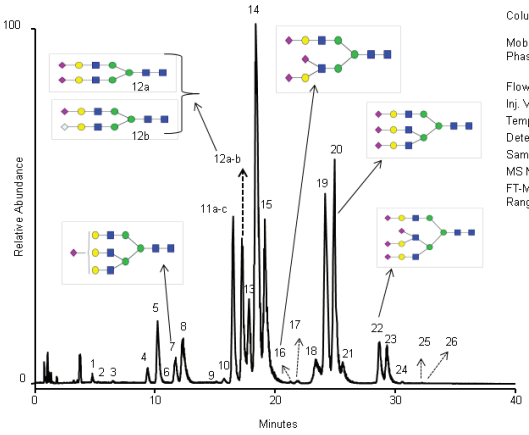


Time (min)	% A	% B	% C	Flow Rate (mL/min)	Curve
-10	78	20	2	0.4	5
0	78	20	2	0.4	5
30	70	20	10	0.4	5
35	60	20	20	0.4	5
40	50	20	30	0.4	5

LC-MS and LC-MS/MS Analysis of 2AB Labeled *N*-Glycan Using GlycanPac AXH-1 Column

The coupling of the GlycanPac AXH-1 column to MS was also explored. This is particularly attractive as MS, with it's ability to provide structural information, enables in-depth analysis of complex glycans. 2AB labeled *N*-glycans from bovine fetuin were separated on the GlycanPac AXH-1 column and analyzed on a Q Exactive mass spectrometer. Data-dependant MS/MS spectra were acquired on all precursor ions ($z \leq 2$) and SimGlycan software was used for glycan structural elucidation. A representative example of the analysis is shown in Figure 3**.** The detailed structural information obtained from the MS/MS data further validated the ability of the GlycanPac AXH-1 column to separate glycans based on charge, size, and polarity. However, coelution of different charge state glycans (Figure 4) is common with other commercially available HILIC columns.

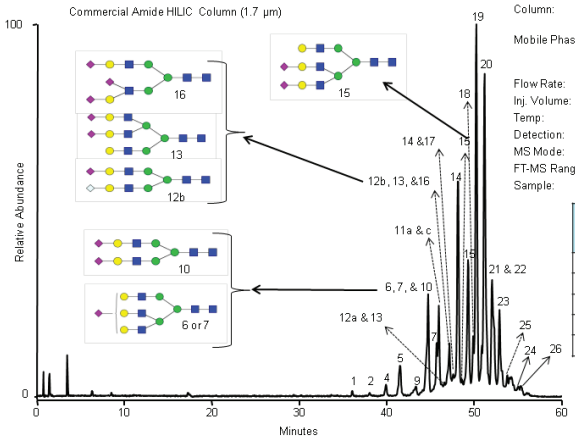
FIGURE 3. LC-MS analysis of 2AB labeled *N*-glycans from Bovine fetuin by the GlycanPac AXH-1 (1.9 µm) column with MS detection.



Column: GlycanPac AXH-1, Analytical 1.9 µm (2.1 × 150 mm)
Mobile Phase: A. Acetonitrile (80%) + water (20%) B. Ammonium formate (80 mM, pH = 4.4)
Flow Rate: 0.4 mL/min
Inj. Volume: 50 Pmoles
Temp: 30 °C
Detection: MS (Q Exactive)
Sample: 2AB labeled *N*-glycan from Bovine fetuin
MS Mode: Negative
FT-MS Range: 380-2000 *m/z*

Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

FIGURE 4. LC-MS analysis of 2AB labeled *N*-glycans from Bovine fetuin by a commercial amide HILIC column (1.7 µm) with MS detection.



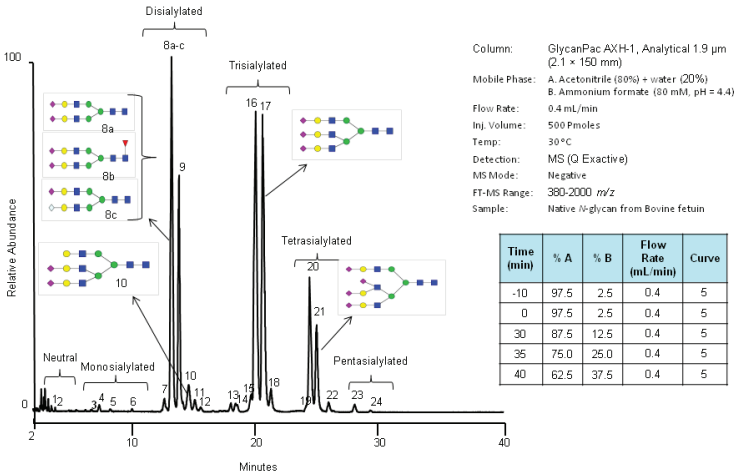
Column: Commercial amide HILIC, 1.7 µm (2.1 × 150 mm)
Mobile Phase: A. Acetonitrile (100%) B. Ammonium formate (100 mM, pH = 4.4)
Flow Rate: 0.4 mL/min
Inj. Volume: 50 Pmoles
Temp: 30 °C
Detection: MS (Q Exactive)
MS Mode: Negative
FT-MS Range: 380–2000 *m/z*
Sample: 2AB labeled *N*-glycan from Bovine fetuin

Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	75.0	25.0	0.4	5
0	75.0	25.0	0.4	5
5	75.0	25.0	0.4	6
55	55.0	45.0	0.4	6
63	50.0	50.0	0.4	6

LC-MS Analysis of Native Glycans Released from Proteins

The GlycanPac AXH-1 column is well suited for high-performance LC/MS separation and analysis of native glycans from MAbs and other proteins. Analyzing unlabeled glycans not only eliminates the extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction. Figure 5 shows the LC/MS analysis of native *N*-glycans from Bovine fetuin using the GlycaPac AXH-1 column (1.9 µm). The native glycans were separated based on charge, size, and polarity. Using an ammonium formate/acetonitrile gradient highly compatible with MS detection, the separation enables excellent MS and MS/MS fragmentation data for accurate confirmation of the glycan structure of each chromatographic peak. Native glycan profiles are significantly different from the profile of fluorescently labeled glycans, especially higher sialic acid glycans. However, fluorescently labeled glycans generally provide better and more MS/MS fragmentation peaks. The GlycanPac AXH-1 column is useful for the analysis of both native and labeled *N*-glycans, depending on the amount of sample available. If the amount of the sample is not extremely limited, analysis of unlabeled glycans using the GlycanPac AXH-1 is highly feasible.

FIGURE 5. LC-MS analysis of native *N*-glycans from Bovine fetuin. All the peaks are detected by MS detection in negative ion mode.

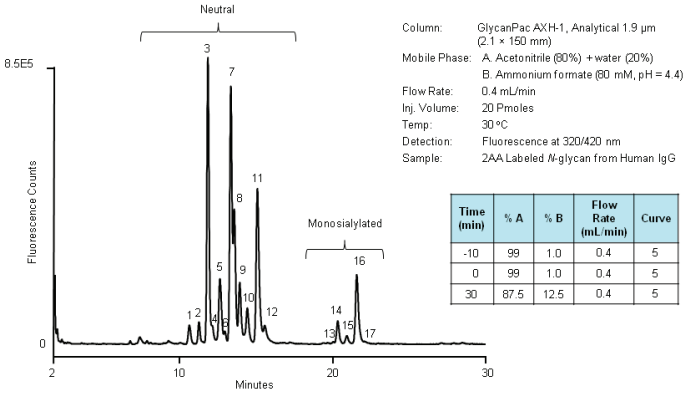


Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Structural Analysis of *N*-Glycans from Antibodies by LC-MS Using GlycanPac AXH-1 Column

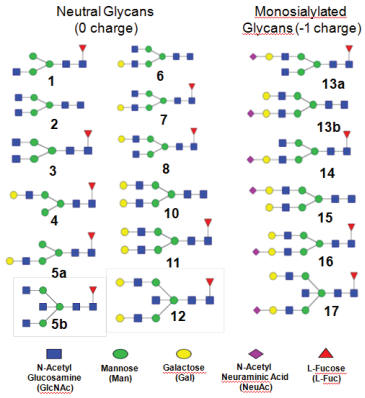
Antibody research has gained significant interest as a part of the development of protein biotherapeutics. Glycosylation of antibodies is a prime source of product heterogeneity with respect to both structure and function. Variation in glycosylation is one of the main factors in product batch-to-batch variation,[2-3] affecting product stability *in vivo* and significantly influencing Fc effector functions *in vivo.* A representative example of the chromatographic separation of antibody glycans is shown in Figure 6, where 2AA labeled *N*-glycans from IgG were separated using the GlycanPac AXH-1 column (1.9 µm). Characterization of glycans in each peak was performed by LC-MS/MS and results are shown in Figure 7. Three different glycan charge states were found in this human IgG; the majority of glycans are neutral or monosialylated, with minor amounts of disialylated glycans. Separation of glycans based on charge provides advantages compared to other commercially available HILIC columns.

FIGURE 6. Analysis of 2AA labeled *N*-glycans from human IgG.



Time (min)	% A	% B	Flow Rate (mL/min)	Curve
-10	99	1.0	0.4	5
0	99	1.0	0.4	5
30	87.5	12.5	0.4	5

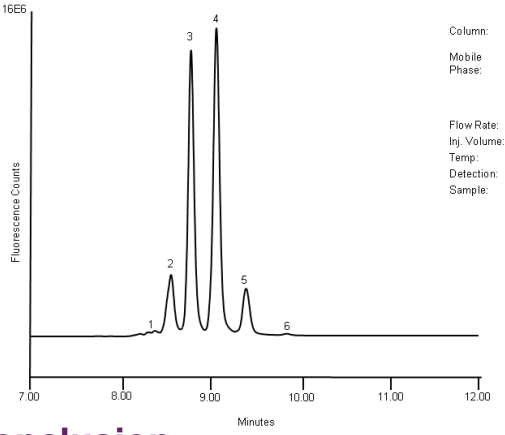
FIGURE 7. Mass spectroscopic characterization of glycans in each Figure 6 peak.



Quantitative Determination of Glycans Based on Charge

Quantitative analysis of each glycan is essential for quick assessment of glycan variation in protein batch comparisons and for comparison of diseased to normal cell glycosylation profiles. In addition, quantitative analysis of glycans separated based on charge state also provide a tool for calculating the relative amounts of different sialic acid linkages after enzymatic digestion with silidase S and sialidase A. Figure 8 shows the quantitative analysis of 2AB labeled *N*-glycans based on charge the using GlycanPac AXH-1 column (1.9 µm) with fluorescence detection. The relative amount of each charge state glycan was estimated using a standard curve. A standards curve was drawn using the data from the chromatographic analysis of 2AB labeled A2 glycan standard, with the injection of different amount of samples starting from 0.1 to 5 pmole).

FIGURE 8: Quantitative estimation of each charge state glycan in 2AB labeled *N*-glycan from Fetuin



Column: GlycanPac AXH-1, Analytical 1.9 µm (2.1 × 150 mm)
Mobile Phase: 0–6 min in 10% Ammonium formate (50 mM, pH =4.4) 90% Acetonitrile and 6–12 min in 20% Ammonium formate (50 mM, pH = 4.4) + 30% water +50% CH_3CN
Flow Rate: 0.4 mL/min
Inj. Volume: 40 Pmoles
Temp: 30 C°
Detection: Fluorescence at 320/420 nm
Sample: 2AB labeled *N*-glycan from Bovine fetuin

Peak No.	Peak Type	% AUC from Total
1	Neutral	0.4
2	Monosialic	8.6
3	Disialic	38.4
4	Trisialic	45.4
5	Tetrasialic	7.0
6	Pentasialic	0.2

Conclusion

- The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size, and polarity not possible with commercial HILIC columns.
- LC-ESI-FTMS or FT-MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.
- The GlycanPac AXH-1 column is useful for both high-resolution charge-based separation and easy quantification of glycans.
- The GlycanPac AXH-1 columns are compatible with various MS instruments.
- These new columns have high-chromatographic efficiency and excellent column stability.
- The GlycanPac AXH-1 column is also useful for the separation of reduced *O*-glycans from proteins and mucins.
- The GlycanPac AXH-1 column is useful for the analysis of charged and neutral glycosylaminoglycans and glycolipids.

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Targeted Quantification of *O*-Linked Glycosylation Site for Glycan Distribution Analysis

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Overview

Purpose: Develop an automated workflow for identification and quantification of *O*-linked glycopeptides and corresponding glycoforms per targeted protein.

Methods: Combine IP-MS using MSIA extraction with unbiased HR/AM LC-MS and MS/MS data acquisition. Perform a novel iterative searching strategy based on retention time correlation of precursor and product ion accurate mass values creating a targeted list to perform qualitative and quantitative analysis across samples.

Results: Incorporation of MSIA IP enrichment strategy resulted in ca. 1,000-fold increase in measured area under the curve (AUC) values per Apo CIII peptide compared to the measured AUC values extracted from whole serum digest. The increase in sensitivity facilitated the detection, characterization, and quantification of key *O*-linked glycosylation region of Apo CIII and enabled direct comparison to the unmodified form of the peptides.[1]

Introduction

The detection of glycoproteins, characterization of various glycoforms, and identification of the modified region of a protein can be challenging due to the complexity of the background, dilution of the precursor ion signal, and lack of common PTM mass shift. Our approach to increase the throughput for identification and quantification for *O*-linked glycopeptides and corresponding glycoforms is to perform targeted data extraction from protein/peptide sequences. Multi-level scoring attributes are implemented to automate data reduction, resulting in a refined list of highly confident peptides. Incorporation of unbiased data acquisition of high-resolution, accurate-mass (HR/AM) mass spectrometry (MS) and tandem-MS dramatically increases the scoring routine.[2] The final report provides a score and integrated peak area across biological samples for modified and unmodified peptides attributed to the targeted protein(s).

Methods

Sample Preparation

To perform initial testing, serum samples were collected from normal and stroke patients. Each sample was divided into two equal aliquots. One set of samples were reduced, alkylated, and digested with trypsin and used without further preparation. The second set of samples were aspirated into an MS immunoassay (MSIA) direct analysis in real time (D.A.R.T.) tip[3] covalently loaded with an anti-Apo CIII antibody. Following MSIA extraction, each sample was washed then reduced, alkylated, and digested using a similar protocol as that for the serum samples with the ratio of analyte:enzyme held constant. Prior to analysis, a constant amount of the PRTC kit was spiked as an internal standard.

Liquid Chromatography

LC separation was performed using a Thermo Scientific™ Hypersil GOLD™ 100 × 0.075 mm column with 3 μm particle size and a binary solvent system comprised of: A) 0.1% formic acid and B) 0.1% formic aid in MeCN. A linear gradient of 5–45% B was performed over 40 minutes prior to column washing and re-equilibration.

Mass Spectrometry

All experiments were performed on a Thermo Scientific™ Q Exactive™ mass spectrometer operated in data-dependent/dynamic-exclusion mode using a Top10 acquisition scheme. Full-scan MS spectra were acquired using a resolution setting of 70K and all HCD product ion spectra were acquired using 15K. All data was acquired using internal lock mass.

Data Analysis

Initial unbiased data searching was performed using Thermo Scientific™ Proteome Discoverer™ 1.4 software to identify all proteins and corresponding peptides. The initial search strategy was performed with typical variable modifications of phosphorylation, oxidation, and Cys alkylation. The Apo CIII protein sequence and initial set of identified peptides from step 1 was transferred to prototype version of Thermo Scientific™ Pinpoint™ 1.4 software to search for *O*-linked glycopeptides. The screening tool incorporates base peptide sequence information with individual glycan information to calculate the composite chemical formula facilitating HR/AM MS data extraction resulting in a list of putative glycopeptides and corresponding glycoforms. The list was used to perform higher level data extraction, verification/scoring, and relative quantification using HR/AM MS data. Relative quantification was compared between the glycopeptides and unmodified peptides originating from Apo CIII.

Results

A common stock of serum sample from two different patient types was divided into two equal aliquots. The only difference was the introduction of an automated IP extraction using MSIA D.A.R.T. tips loaded with anti-Apo CIII antibody. The histogram for an Apo CIII tryptic peptide is presented in Figure 1, comparing the measured AUC values with and without MSIA extraction prior to digestion and LC-MS analysis.

The Pinpoint screening tool utilizes *in-silico* sequence generation to create a list of unmodified peptides (Figure 2). The list of unmodified peptides are also subjected to all possible glycan additions (*N*- and/or *O*-linked modifications) to create a master list of peptides and *m/z* values used to extract data. The resulting table lists the peptide sequences and modifications as well as all corresponding LC and MS information such as retention times, precursor charge states, mass errors, and protein sequence location. The results from the screening tool are directly exported to the Pinpoint main workbook integrated analysis.

FIGURE 1. Representation of the comparative sample preparation workflow to evaluate *O*-linked glycopeptide detection and quantification

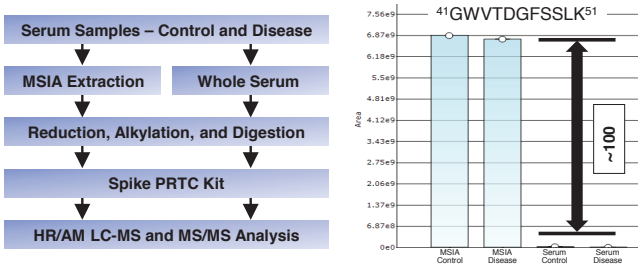
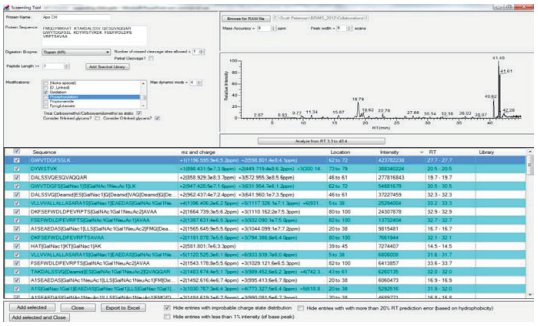


FIGURE 2. Screen capture showing the Pinpoint screening tool used to identify putative modified and unmodified peptide sequences from a RAW file



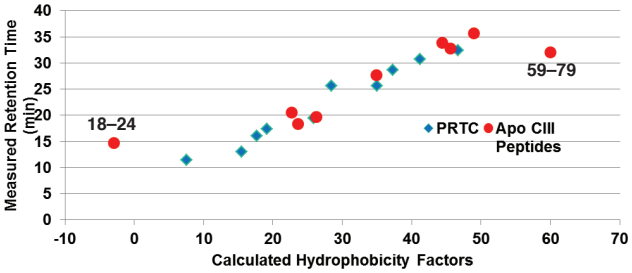
The information generated in the screening provides significant data reduction to eliminate peptide candidates in the unmodified as well as modified forms. Even for a small protein such as Apo CIII, which has only 79 residues (omitting the first 20 *N*-terminal residues identified as the signaling chain), the use of common *in-silico* processing parameters (common PTMs and 1 missed cleavage) generates over 8,000 possible sequences. The screening tool reduces this list down to 16 peptides in less than one minute. Enabling the possibility of *O*-linked glycopeptides increases the number of possible sequences to over 100,000 and provides a list of 92 putative peptides. The list is further reduced based on the presence of additional retention time and MS information to a final list that is further evaluated, scored, and quantified in an automated routine. Table 1 lists the identified set of peptides unmodified or modified with non *O*-linked glycans. Each peptide has an MS/MS spectrum acquired under the elution peak profile. The AUC ratios for the list of peptides are close to 1:1 except for those peptides with missed cleavage sites. Three of the peptides covering residues 22–40, 59–79, and 61–79 show greater response for the “disease” sample compared to the “normal.” Each of the missed cleavage peptides also shows greater variance than the tryptic peptides.

The incorporation of the PRTC kit into the mix helps to map the measured RT values to the expected times. Figure 3 shows the overlap of the PRTC peptides to those of the Apo CIII peptides. All of the tryptic peptides align with the PRTC peptides except peptides with missed cleavage sites. The goal is to incorporate additional scoring metrics to each peptide. In addition, the establishment of a known RT for unmodified peptides provides landmarks for the *O*-linked glycosylated forms as they are expected to elute under similar times.

Table 1. List of peptides attributed to the tryptic digestion of Apo CIII with and without standard modifications. The hydrophobicity factors (HF) were calculated using Krokhin's SSRCalc algorithm and the dot product correlation coefficient was calculated based on precursor isotopic distribution overlap of experimental to theoretical values. AUC values were determined based on the MS peak profile.

Position	Targeted Peptide	HF	%CV	RT (min)	AUC Serum		AUC MSIA		AUC Ratio	Dot-Product
					Control	Disease	Control	Disease		
1–17	SEAEDASLLSFM[Oxid]Q [Deamid]GYMKHATK		26	24.33	3.7E+03	N/A	1.6E+07	9.2E+06	0.59	0.99
1–17	SEAEDASLLSFMQGYMK	48.99	0.5	35.68	1.2E+06	1.0E+05	3.1E+09	3.1E+09	1.01	1.00
1–17	SEAEDASLLSFM[Oxid]QGYMK		3	32.3	9.5E+05	N/A	1.2E+09	1.3E+09	1.07	1.00
1–17	SEAEDASLLSFM[Oxid]QGYM [Oxid]K		9	26.24	9.4E+05	2.7E+05	4.5E+08	5.3E+08	1.19	1.00
18–24	HATKTAK	–2.90	9	14.7	9.3E+03	1.3E+04	3.5E+06	2.9E+06	0.84	1.00
22–40	TAKDALSSVQESQVAQQAR	23.61	15	18.29	N/A	N/A	1.4E+07	1.8E+07	1.35	0.98
24–40	DALSSVQESQVAQQAR	26.27	2	19.7	4.9 E+07	2.7E+07	1.1E+10	1.1E+10	0.97	1.00
41–51	GWVTDGFSSLK	34.94	1	27.65	2.0E+07	7.0E+06	6.9E+09	6.7E+09	0.98	1.00
41–60	GWVTDGFSSLKDYWSTVK	44.42	1	33.85	1.8E+04	1.9E+05	2.0E+07	2.0E+07	1.02	1.00
52–58	DYWSTVK	22.67	4	20.53	2.4E+07	1.1E+07	6.1E+09	5.6E+09	0.92	0.89
59–79	DKFSEFWDLDPEVRPTSAVAA	60	18	32.08	6.8E+05	8.0E+04	1.7E+08	2.5E+08	1.45	1.00
61–79	FSEFWDLDPEVRPTSAVAA	45.63	16	32.8	N/A	N/A	6.5E+07	9.1E+07	1.38	0.99

FIGURE 3. Plot of measured RT values as a function of HF values for the PRTC peptides and the targeted Apo CIII peptides from Table 1.



In addition to the peptides listed in Table 1, *O*-linked glycopeptides were also identified for the peptides covering residues 22–40, 59–79, and 61–79. An example of the MS-level data extraction for the 59–79 peptide modified with GalNAc1Gal1NeuAc1 is shown in Figure 4. The isotopic distribution profiles for the MSIA extracted samples match the theoretical distribution with correlation coefficients >0.98. The isotopic overlap for the serum digest samples shows little response. Figure 4c shows the relative AUC values for the Control vs. Disease samples. The AUC ratio for the *O*-linked glycopeptide 1.3 matches that for the unmodified peptide. Three additional *O*-linked glycoforms were identified for the 59–71peptide and five *O*-linked glycoforms were identified for the fully tryptic peptide 61–71. Figure 5 shows the results of the initial screening based on HR/AM MS data and retention time correlation. The group of *O*-linked glycopeptides and corresponding glycoforms should elute in proximity (<20%) of the unmodified peptide when using a C18-based column. The isotopic distribution analysis was also calculated for each glycoform. The goodness of fit for the relative AUC values per isotope are directly dependent on the mass accuracy of the predicted chemical composition of the peptide and glycan as well as the overall measured intensities.

FIGURE 4. MS-level data analysis of the *O*-linked glycopeptide DKFSEFWDLDPEVRPTSAVAA[GalNAc1Gal1NeuAc1]. Figure 4a shows the overlaid XIC trace for the six isotopes of the +3 charge state. Figure 4b shows comparative isotopic distribution analysis across each sample – MSIA extracted as well as serum digests and the corresponding histogram comparing the integrated peak areas across each sample.

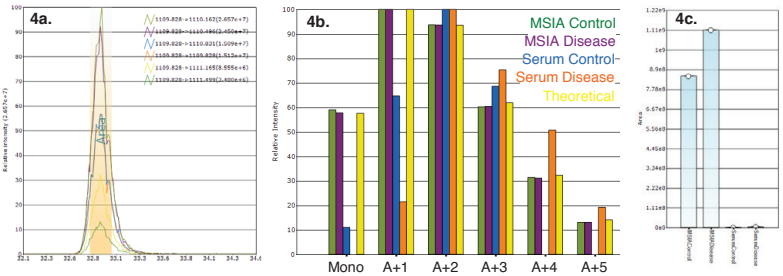
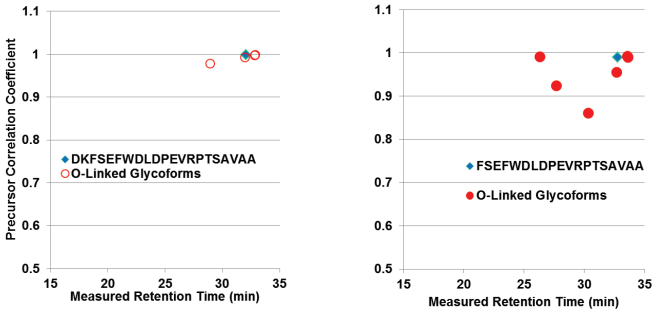


FIGURE 5. Comparative plots of the dot-product correlation coefficients as a function of measured retention times for the identified glycoforms for DKFSEFWDLDPEVRPTSAVAA and FSEFWDLDPEVRPTSAVAA.



In addition to MS data, product ion data was evaluated to provide additional confidence to the assigned glycopeptide sequence. Figure 6 shows an example of automated product ion determination used to distinguish each base peptide and glycan composition. Acquiring the product ion spectra in the Thermo Scientific™ Orbitrap™ instrument facilitated product ion charge state and accurate *m/z* value determination, which significantly increased product ion assignment based on the peptide sequence and proposed glycan composition identified from the screening tool. The key fragments used to confirm the base peptide sequence was *m/z* 2137 for the 61–79 peptide and *m/z* 2381 fragment for the 59–79 peptides assigned as the base peptide sequence. The mass errors calculated for each fragment ion was less than 5 ppm. Due to the degree of sequence overlap attributed to the missed cleavage site, additional product ions were used to further confirm the sequence, specifically the b-type ions. Similar data analysis was completed for each glycoform. The results for the *O*-glycoform distribution are presented in Figure 7. The unmodified forms of the peptide (with and without missed cleavage site) showed a lower relative response compared to the modified forms.

Incorporation of HR/AM MS HCD data acquisition facilitates peptide sequence determination and glycan composition, and for those *O*-linked glycopeptides modified at only one residue, site determination. For glycoforms modified at multiple sites, specific site determination generally requires electron transfer dissociation (ETD) product ion data collection. The workflow presented here can automatically create a secondary experimental method for targeted ETD data acquisition.

FIGURE 6. Comparative product ion spectra for the *O*-linked glycopeptides FSEFWDLDPEVRPTSAVAA and DKFSEFWDLDPEVRPTSAVAA with the same glycan modification. Both HR/AM HCD spectra were acquired under the precursor elution profiles.

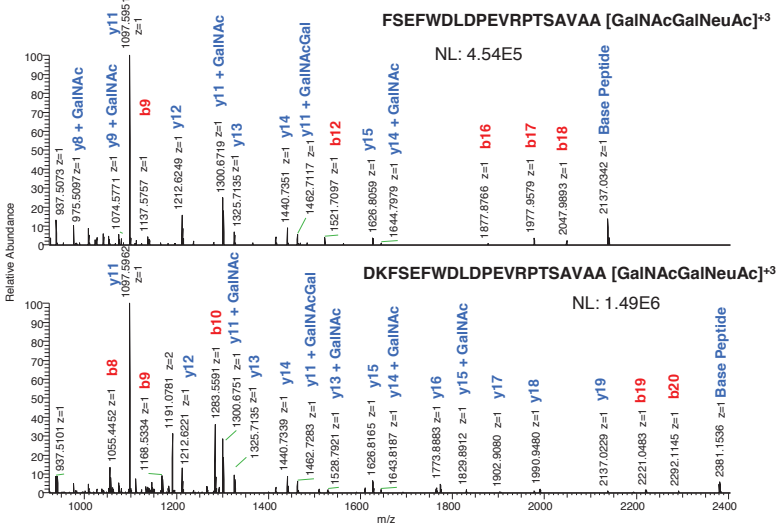
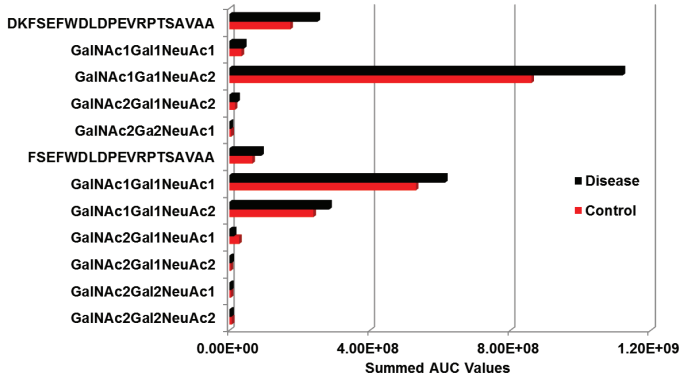


FIGURE 7. Comparative distribution of unmodified and *O*-linked glycopeptides from Apo CIII. Integrated peak areas from HR/AM MS data were used for the comparison of glycoform distribution as well as the relative amounts across each sample.



Conclusion

The targeted protein workflow presented facilitated detection, verification, and quantification of Apo CIII across biological samples. Targeting at the protein level provides significant advantages of evaluating unmodified peptides to provide landmarks for modified peptide confirmation.

- Incorporation of MSIA enrichment increased sensitivity ca. 1000-fold compared to whole serum digest analysis
- Unbiased HR/AM MS and data-dependent/dynamic-exclusion acquisition facilitates post-acquisition data processing workflow
- The Pinpoint screening tool generates a list of highly confident set of modified and unmodified targeted peptides from 100,000s of sequences
- Pinpoint data processing incorporates multiple scoring levels, significantly increasing confidence in the final relative quantification results

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A Complete Workflow Solution for Monoclonal Antibody Glycoform Characterization Combining a Novel Glycan Column Technology and Bench-Top Orbitrap LC-MS/MS

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Overview

Purpose: To develop a complete workflow solution for monoclonal antibody (mAb) glycoform characterization using a unique glycan column technology and a Thermo Scientific™ bench-top Orbitrap™ LC-MS/MS.

Methods: Glycans are separated using a recently developed high-performance HPLC/UHPLC column, a Thermo Scientific™ GlycanPac™ AXH-1 column. A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans.

Results: The GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity. A complete workflow solution was developed for glycan profiling combining the unique column technology and a bench-top Orbitrap LC-MS/MS (Figure 1). This workflow was applied to antibody glycoform characterization. Confident identification and structural confirmation were achieved for released glycans from a standard glycoprotein and a monoclonal antibody.

Introduction

Because glycosylation is critical to the efficacy of antibody therapeutics, the FDA requires that a consistent human-type glycosylation be maintained for recombinant monoclonal antibodies (mAb), irrespective of the system in which they are produced. The complex branching and isomeric nature of glycans pose significant analytical challenges for their identification and characterization. Liquid chromatography (LC) coupled to mass spectrometry (MS) has emerged as one of the most powerful tools for the structural characterization of glycans.

The recently developed GlycanPac AXH-1 column is a high-performance HPLC/UHPLC column specifically designed for structural, qualitative and quantitative analysis of glycans. It has a unique selectivity for biologically relevant glycans including glycans from antibodies, either labeled or native and is designed for high-resolution, high-throughput analysis by LC-fluorescence or LC-MS methods. Because glycans are very hydrophilic and polar, hydrophilic interaction liquid chromatography (HILIC) columns based on amide, amine or zwitterionic packing materials are often used for their analysis. HILIC columns separate glycans mainly by hydrogen bonding, resulting in size and composition-based separation. Identification of the glycan charge state is not possible by HILIC. The GlycanPac AXH-1 column overcomes these limitations and can separate glycans based on charge, size and polarity configuration. It provides both greater selectivity and higher resolution. In this study, we characterized N-linked glycans released from a glycoprotein standard and a monoclonal antibody by LC-MS/MS methods using the new column technology and high-resolution Orbitrap mass spectrometry.

Methods

Sample preparation

Native glycans are released from glycoproteins or mAb with PNGase F enzyme. The released glycans are conjugated with 2-amino benzamide (2-AB) label group with reported procedure of Bigge *et. al.*[1]

Liquid chromatography

All the glycans are separated using a recently developed high-performance HPLC/UHPLC column, GlycanPac AXH-1, on a Thermo Scientific™ Dionex™ Ultimate 3000 UHPLC with either s fluorescence or MS detector.

For intact antibody, a Thermo Scientific™ ProSwift RP-10R monolithic column (1 x 50 mm) was used for desalting. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Column was heated to 80 °C during analysis. Flow rate was 60 µL/min. After injection of 1 µg mAb, a 15 min gradient was used to elute mAbs from the column (0.0 min, 20%B; 1.0 min, 35%B; 3.0 min, 55%B; 4.0 min, 98%B; 7.0 min, 98% B; 7.1 min, 20%B; 15.0 min, 20%B).

Mass spectrometry

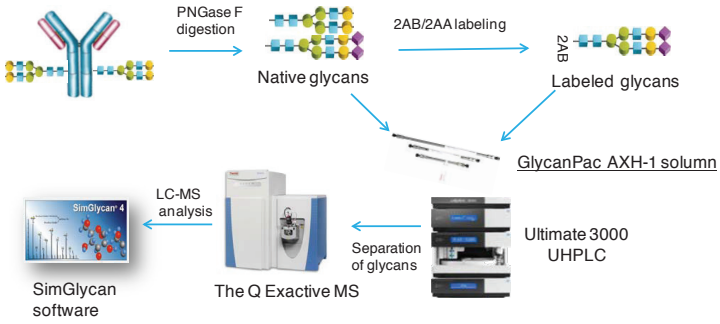
A data-dependent high-energy collision dissociation (HCD) method was performed in negative ion mode to analyze the glycans. The following MS and MS/MS settings were used: MS scan range 380-2000 *m/z*. FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of $1x10^6$ and DDA MS2 acquired at 17,500 resolution at *m/z* 200 with AGC target of $2x10^5$. Intact mAbs were analyzed by ESI-MS for intact molecular mass. The spray voltage was 4kV. Sheath gas flow rate was set at 10. Auxiliary gas flow rate was set at 5. Capillary temperature was 275 °C. S-lens level was set at 55. In-source CID was set at 45 eV. For full MS, resolution was 17,500 for intact mAb. The AGC target was set at $3x10^6$. Maximum IT was set at 250 ms.

Data analysis

SimGlycan® software from PREMIER Biosoft was used for glycan identification and structural elucidation[2]. SimGlycan software accepts raw data files from Thermo Scientific mass spectrometers and elucidates the associated glycan structure by database searching and scoring techniques.

Full MS spectra of mAb were analyzed using Thermo Scientific™ Protein Deconvolution™ 2.0 software. Mass spectra for deconvolution were produced by averaging spectra across the most abundant portion of the elution profile for the mAb. A minimum of at least 8 consecutive charge states from the input *m/z* spectrum were used to produce a deconvoluted peak. To identify glycoforms, the masses were compared to the expected masses of various combinations of commonly found glycoforms

Figure 1. A complete LC-MS/MS workflow solution for monoclonal antibody glycan profiling

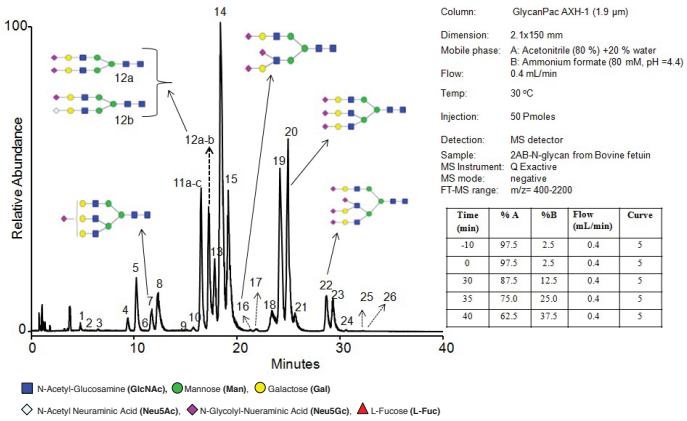


Results

Separation of Glycans Based on Charge, Size and Polarity

The GlycanPac AXH-1 column can be used for qualitative, quantitative, structural analysis and characterization of uncharged (neutral) and charged glycans present in proteins. The separation and elution of glycans are based on charge; the neutral glycans elute first, followed by the separation of acidic glycans from mono-sialylated, di-sialylated, tri-sialylated, tetra-sialylated and finally penta-sialylated species. Glycans of each charge state are further separated based on their size and polarity. In this study, the structure of glycans present in each peak was determined using high resolution LC-MS/MS. As shown in Figure 2, the detailed structural information obtained from the MS/MS data validated the ability of GlycanPac AXH-1 column to separate labeled N-glycans based on charge, size and polarity. However, co-elution of different charge state glycans is common with other commercially available HILIC column as shown in Figure 3.

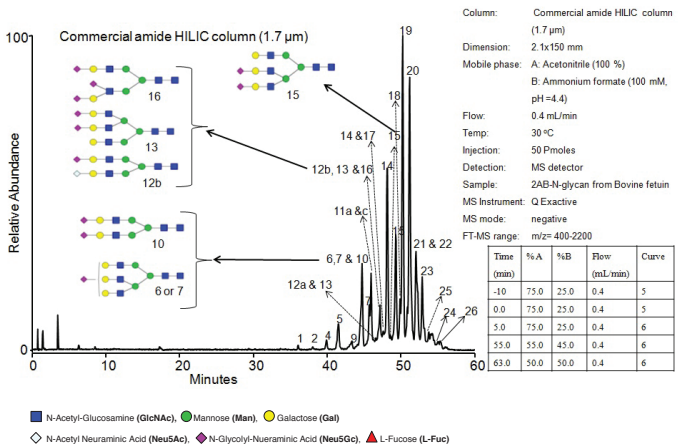
Figure 2. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by GlycanPac AXH-1 (1.9 µm) column with MS detection.



Column: GlycanPac AXH-1 (1.9 µm)
Dimension: 2.1x150 mm
Mobile phase: A: Acetonitrile (80 %) +20 % water
B: Ammonium formate (80 mM, pH =4.4)
Flow: 0.4 mL/min
Temp: 30 °C
Injection: 50 Pmoles
Detection: MS detector
Sample: 2AB-N-glycan from Bovine fetuin
MS Instrument: Q Exactive
MS mode: negative
FT-MS range: m/z= 400-2200

Time (min)	% A	%B	Flow (mL/min)	Curve
-10	97.5	2.5	0.4	5
0	97.5	2.5	0.4	5
30	87.5	12.5	0.4	5
35	75.0	25.0	0.4	5
40	62.5	37.5	0.4	5

Figure 3. LC-MS analysis of 2-AB labeled N-glycans from bovine fetuin by a commercial amide HILIC column (1.7 *µ*m) with MS detection.



The GlycanPac AXH-1 column is also well suited for high performance LC/MS separation and analysis of native glycans from proteins (data not shown). Analyzing unlabeled glycans not only eliminates the extra reaction step and cumbersome cleanup methods during labeling, but also retains the original glycan profile without adding further ambiguity imposed by the labeling reaction.

Monoclonal antibody (mAb) glycan profiling using GlycanPac AXH-1 column and high resolution LC-MS/MS

Intact mass measurement of a monoclonal antibody identified glycoforms derived from the combination of any two of the three N-glycans, G0F, G1F and G2F. However, the mass errors for some of the intact glycoforms of this antibody ranged from 20-60 ppm (Figure 4A) which is larger than the <10 ppm observed for other samples (data not shown). Furthermore, the intact mass error for the deglycosylated form of this antibody was within 10 ppm (Figure 4B), suggesting that some minor glycosylation forms of this molecule that were not detected at the intact level had interfered with the observed intact mass of the major glycoforms. To further characterize this antibody, released glycans from this protein were separated using the GlycanPac AXH-1column. The separation and elution of glycans from GlycanPac AXH-1 column are based on charge with neutral glycans eluting first, followed by the acidic sialylated species. Glycans of each charge state are further separated based on their size and polarity (**Figure 5**).

Figure 4. Observed molecular mass of glycosylated (A) and deglycosylated (B) forms of a intact monoclonal antibody. Some of the intact antibody major glycoforms have an observed mass error larger than expected. There are also two potentially double fucosylated peaks that need to be confirmed.

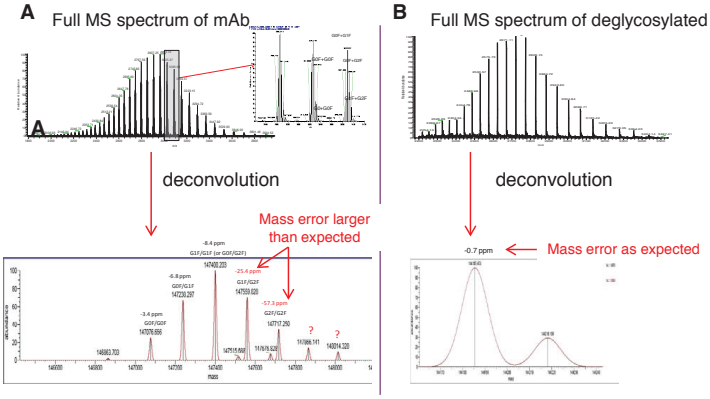
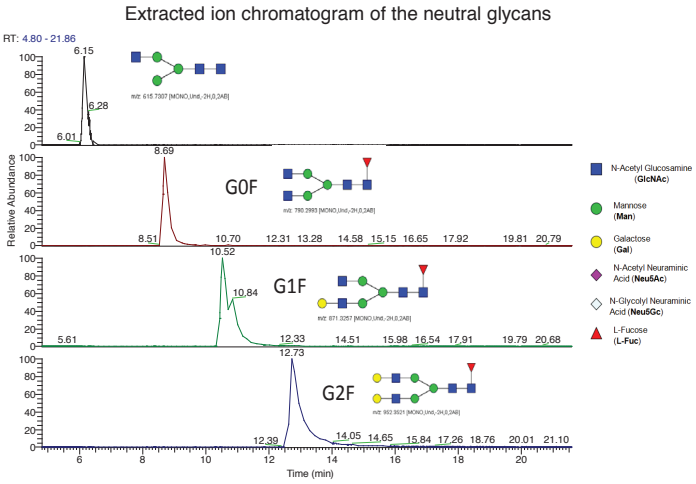
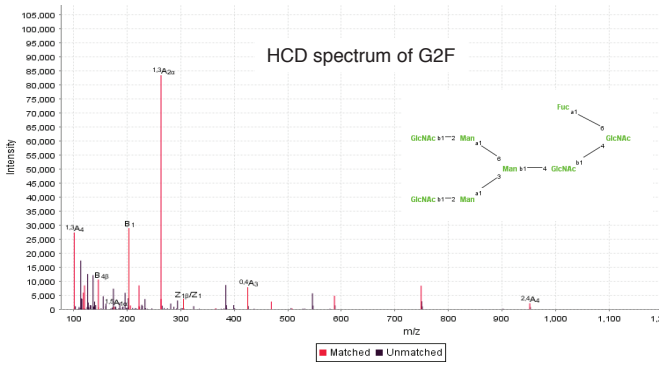


Figure 5. Separation of the major, neutral N-glycans on GlycanPac AXH-1 column



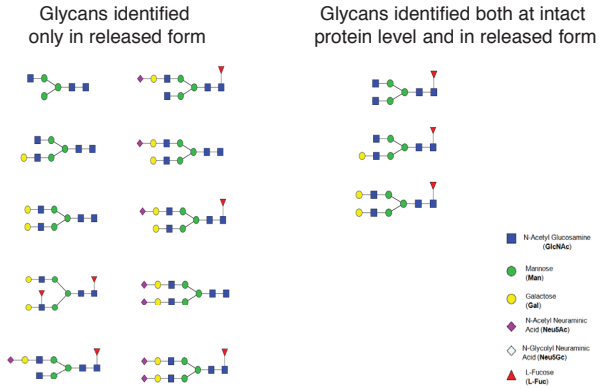
Characterization of glycans in each peak was performed by Full MS and data dependent MS/MS using HCD. The information-rich HCD spectra contain fragment ions that were generated from both cross-ring and glycosidic bond fragmentations (Figure 6). Three different types of glycans were found from this monoclonal antibody, the majority of glycans identified were neutral, including G0F, G1F and G2F which were also the major glycoforms identified at the intact protein level for this antibody (Figure 4A). Also identified were less abundant, non-fucosylated forms of G1 and G2, minor amounts of mono-sialylated and di-sialylated species with and without fucosylation, as well as double fucosylated species that were not identified at the intact protein level (Figure 7).

Figure 6. Identification and structural confirmation of released glycan using high resolution HCD MS/MS



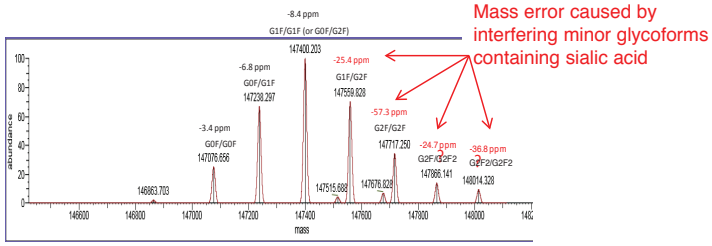
Fragment ion type	Percentage match (%) of theoretical fragments
Single glycosidic	32.14
Glycosidic/glycosidic	30.95
Single cross ring	20.21
Cross ring/glycosidic	14.95

Figure 7. Identified glycans from monoclonal antibody



These results explain that the unexpected mass error observed previously is due to the interfering minor glycoforms that have a molecular mass close to the major ones. In the deconvoluted MS spectrum, the base of the antibody major glycoform peaks covers a mass range of about 40 Da due to the distribution of the unresolved isotopic peaks of a large protein of this size. Therefore any interfering species within 20 Da of mass difference would cause a mass shift of the major glycoform peaks, rather than forming a separate peak. For example, in this case, the replacement of a Fuc and a Gal by Neu5Ac, which would have a mass difference of -17Da, could cause the negative mass shift observed in this study, especially when the interfering species is relatively low in abundance (Figure 8). Results in this study indicate that rapid and sensitive antibody glycan profiling can be achieved using GlycanPac AXH-1 column and HR/AM Orbitrap LC-MS/MS.

Figure 8 Annotated glycoforms of a monoclonal antibody



Conclusion

- GlycanPac AXH-1 column separates glycans with unique selectivity based on charge, size and polarity.
- The GlycanPac AXH-1 columns are compatible with MS instruments. LC-ESI-FTMS or FT-MS/MS analysis of both native and labeled glycans from proteins and antibodies were carried out successfully using GlycanPac AXH-1 columns.
- Confident identification and structural confirmation of glycans can be achieved using high-resolution HCD MS/MS which produces an informative spectrum containing glycosidic and cross ring fragment ions.
- A complete workflow solution was developed for glycan profiling combining the unique GlycanPac AXH-1 column technology and a bench-top Orbitrap LC-MS/MS.
- This workflow was applied to characterize a monoclonal antibody glycoforms. Confident identification and structural confirmation was achieved for released glycans from the monoclonal antibody.

References

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Improving Intact Antibody Characterization by Orbitrap Mass Spectrometry

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Introduction

Recombinant monoclonal antibodies have gained significant importance in diagnostic and therapeutic applications over the past years. In order to verify the correctness of the overall molecule to provide a reproducible, safe and effective biological drug compound, the correct protein sequence, as well as the presence and relative abundance of different glycoforms have to be confirmed.

Here we present an approach to analyze an intact monoclonal antibody in non-reduced and reduced condition by LC-MS using the Thermo Scientific™ Orbitrap Elite™ mass spectrometer. The intact antibody and the separated light and heavy chains were analyzed in Full MS experiments as well as with top-down experiments using in-source CID (SID), CID, HCD and ETD fragmentation techniques making use of the ultrahigh resolution of the mass spectrometer. For data evaluation ProSight software and Thermo Scientific™ Protein Deconvolution™ software version 1.0 packages were used.

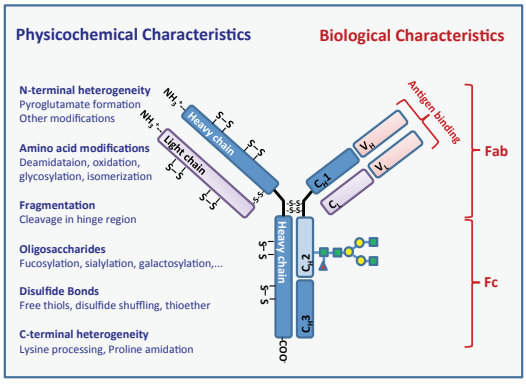


FIGURE 1: General structure of mAbs and their biological and physico-chemical characteristics.

Methods

Sample Preparation

AbbVie™ HUMIRA™ (adalimumab, Figure 2) [1]: The intact antibody (144 kDa) was dissolved in 0.1 % FA to 1 µg/µL; 5 µg HUMIRA were loaded onto the column.

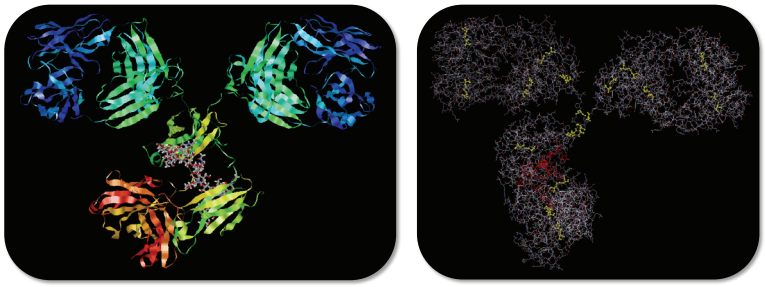


FIGURE 2: 3D structure of HUMIRA highlighting the attached glycans and cystein residues forming inter- and intra-chain disulfide bridges.

For analyzing HUMIRA light chain (24 kDa) and heavy chain (51 kDa) separately, 50 µg HUMIRA was reduced with DTT (20-fold molar excess, 56°C for 1 h) and alkylated with iodoacetamide (50-fold molar excess, room temperature for 30 min in the dark).

Instrument

A Thermo Scientific™ Surveyor™ MS Pump Plus was coupled to an Orbitrap Elite mass spectrometer that was equipped with ETD (Figure 3).

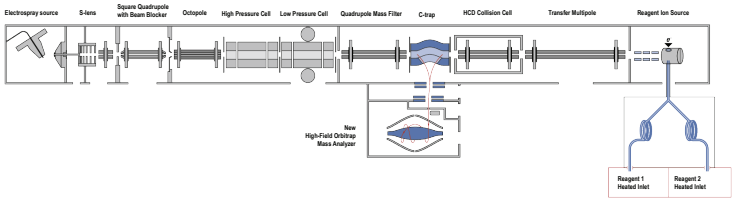


FIGURE 3: Schematics of the Orbitrap Elite hybrid ion trap-Orbitrap mass spectrometer equipped with an ETD source.

Samples were purified on a Thermo Scientific™ BioBasic™ C4 column (150 x 1 mm, 5 µm particles), solvent A: 0.1 % FA, 2 % ACN in H_2O, solvent B: 0.1 % FA in ACN. The LC gradient was 7 min 20–40 % B, 3 min 40–80 % B at a flow rate of 100 µL/min.

Data analysis was done using Protein Deconvolution and ProSight software packages.

Results

The analysis of large proteins of the size of intact anti-bodies (~150 kDa) using Orbitrap mass spectrometers has been significantly improved over the past few years. Large molecules like mAbs show only very short transient life-times due to their relatively big cross section. Thus, the method of choice for intact antibodies is to use the shortest transient duration (48 ms) available on the Orbitrap Elite MS (Figure 4).

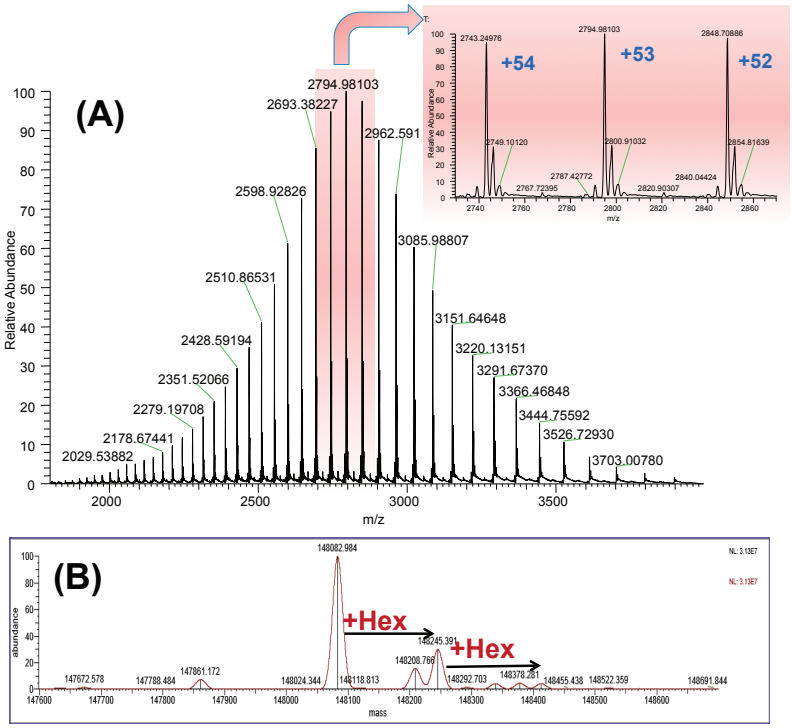


FIGURE 4: (A) Full MS spectrum of intact HUMIRA. The insert shows a zoom into the three most abundant charge states z=52,53,54. (B) Spectrum after deconvolution.

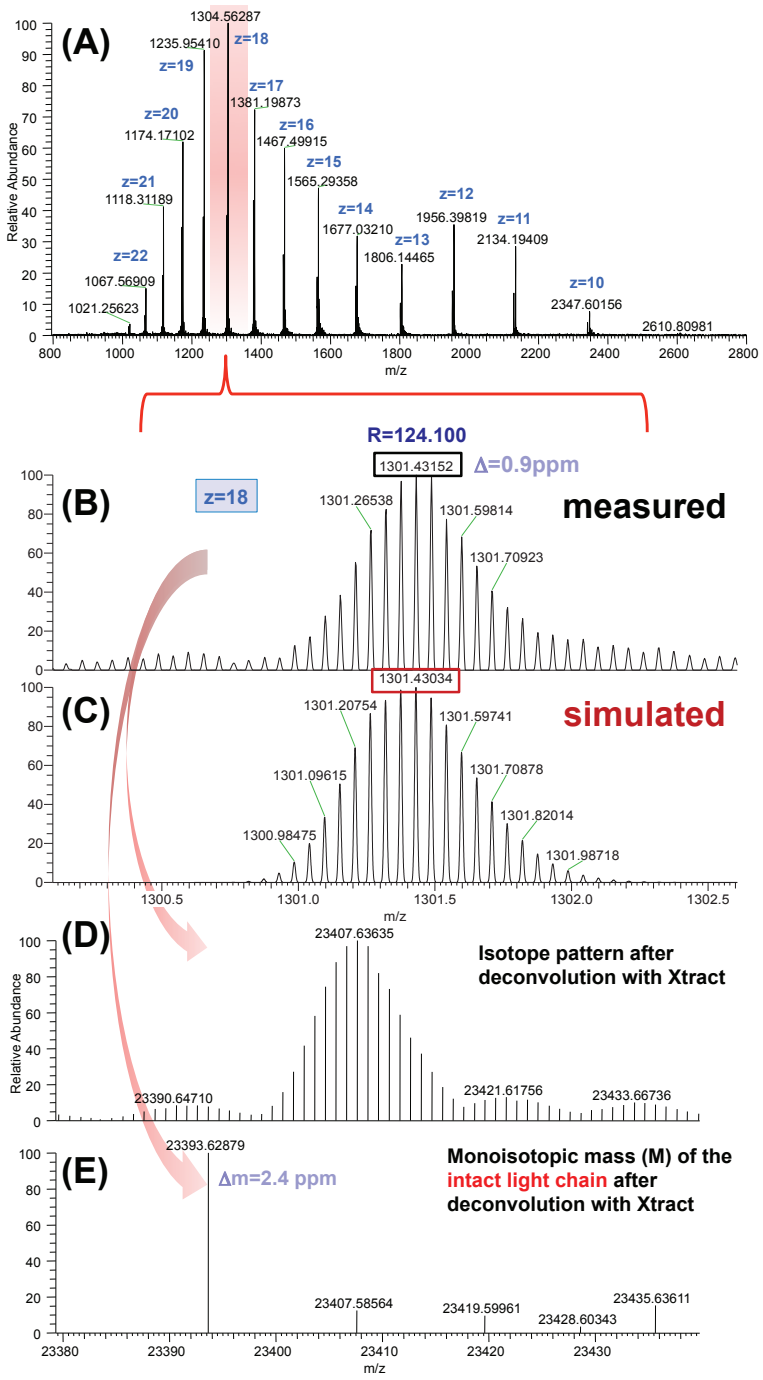


FIGURE 5. (A) Full MS spectrum of intact light chain of HUMIRA. (B) Zoom into +18 charge state of intact light chain. (C) Simulation of isotope pattern of +18 charge state. (D) Isotope pattern of intact light chain after deconvolution. (E) Monoisotopic mass (M) of the measured light chain of HUMIRA obtained after deconvolution with Xtract.

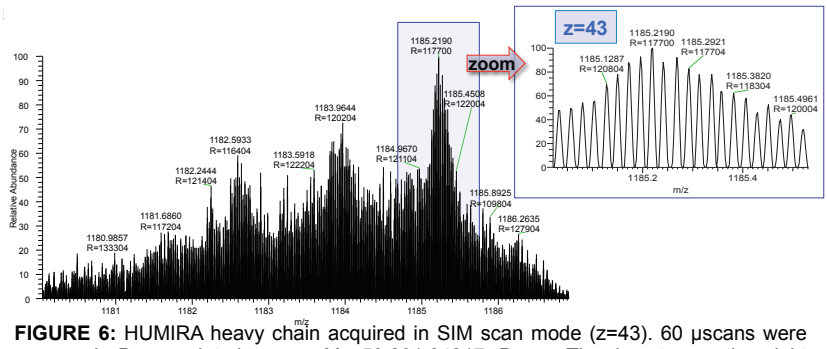


FIGURE 6: HUMIRA heavy chain acquired in SIM scan mode (z=43). 60 µscans were averaged. Deconvoluted mass: Mr 50,891.04317 Da. The inserts on the right demonstrate isotopic resolution of that charge state detected at m/z 1185 and masses obtained after deconvolution using Xtract.

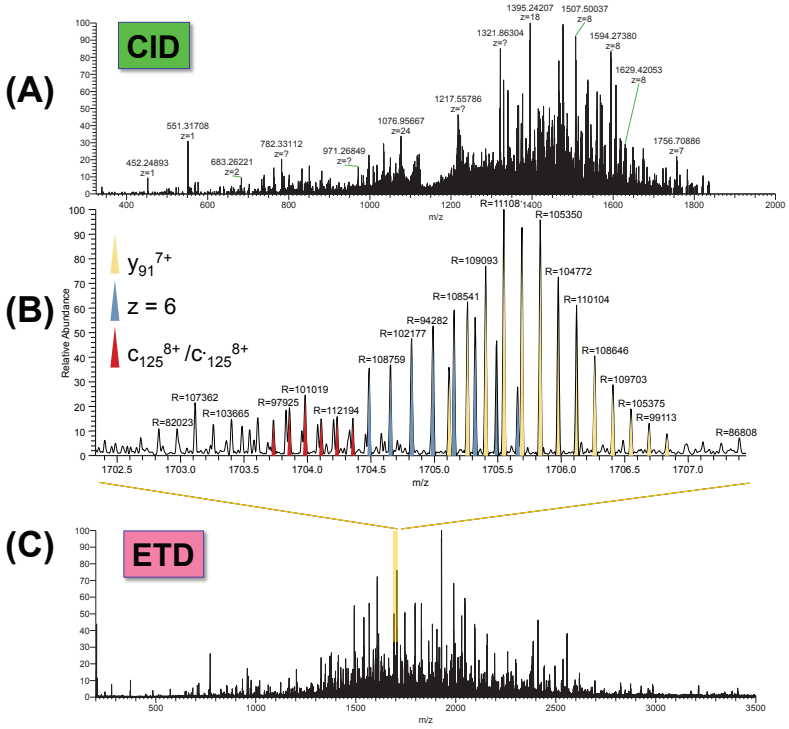


FIGURE 7: (A) CID spectrum and (C) ETD spectrum of **intact** HUMIRA antibody. (B) Zoom in into the ETD fragment ion spectrum of **intact** HUMIRA showing the need for highest resolution possible.

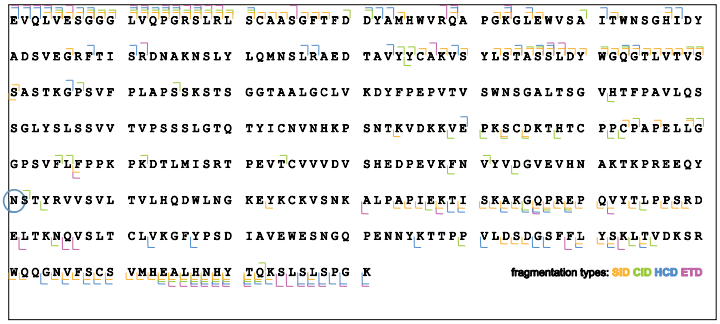
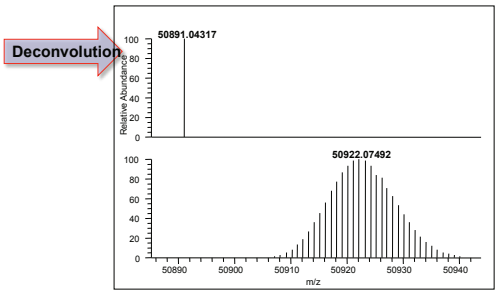


FIGURE 8: Summarized sequence coverage of the HUMIRA heavy chain using fragmentation techniques SID, CID, HCD, and ETD. Optimized conditions: trapping under high pressure settings. N: Putative glycosylation site.



Monoisotopic mass (M) of the intact heavy chain after deconvolution with Xtract

Isotope pattern and average mass of the intact heavy chain after deconvolution with Xtract

Conclusion

- The analysis of intact and reduced antibodies on the Orbitrap Elite mass spectrometer provides the accurate molecular weight, as well as valuable information about the presence and abundance of glycoforms.
- Analysis of the reduced antibody provides isotopically resolved mass spectra for both light and heavy chain.
- The combination of multiple fragmentation techniques in top-down analysis (SID, CID, HCD and ETD) generates comprehensive sequence coverage and enables fast localization of modifications with minimum sample preparation.
- For measurements of intact light and heavy chain as well as for the detection of fragment ion spectra from top-down experiments ultra-high resolution as provided by the Orbitrap Elite mass spectrometer is essential.

Abbreviations

ACN, acetonitrile; CID, collision-induced dissociation; C-trap, curved linear trap; DTT, dithiothreitol; ETD, electron transfer dissociation; FA, formic acid; HCD, higher energy collision-induced dissociation; mAb, monoclonal antibody; µS, micro-scan; SID, *in-source* decay; SIM, single ion monitoring.

References

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Glycan and Glycopeptide Analysis

Peer Review Journal Articles:

Rosati, S.; Rose, R. J.; Thompson, N. J.; van Duijn, E.; Damoc, E.; Denisov, E.; Makarov, A.; Heck, A. J. R. Exploring an Orbitrap Analyzer for the Characterization of Intact Antibodies by Native Mass Spectrometry. *Angew. Chem. Int. Ed.* **2012**, *51*, 12992-12996. DOI: 10.1002/anie.201206745

Peptide Mapping and Analysis

Peptide mapping is a critical tool for biopharmaceuticals, and has become a widely used method for comprehensive characterization of proteins, providing information on post-translational modifications (PTMs), sequence, degradation products and stability. Typical peptide mapping workflows involve enzymatic digestion followed by LC/UV, LC/MS, CE-MS or direction infusion MS analysis.

LC/UV peptide mapping workflows provide a comparative analysis that can be used to confirm the primary structure, consistency and stability of biopharmaceutical products, and has traditionally applied to late stage development and quality control (QC). For more in-depth protein mapping characterization, high-resolution, accurate mass MS is needed to clearly resolve the large number of peptides and peptide variants in such workflows, as well to provide confident assignment of peptide identifications.

The high-resolution, accurate mass of the Orbitrap family of mass spectrometers, combined with various fragmentation capabilities and the selectivity of MS/MS, provides a superior range of MS options for in-depth peptide mapping characterization applications. High speed automated peptide mapping can be performed using Thermo Scientific™ PepFinder™ software, providing in-depth profiling of biotheraputics. Numerous Thermo Scientific chromatography systems, columns and sample prep products offer a complete range of solutions for all types of biopharmaceutical peptide mapping needs as demonstrated in the following papers.

Application Note 1014

Concanavalin A Column for Analysis of Glycoproteins and Their Tryptic Glycopeptides

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Key Words
Off-Line 2D HPLC, Con A Enrichment, Reversed-Phase Peptide Mapping, UltiMate 3000 ×2 Dual Biocompatible Analytical LC System, Lectin Affinity Chromatography

Introduction

Glycosylation, one of the most common and complex post-translational modifications, is of great interest for its role in many biological processes such as molecular recognition, cell signaling, and immune defense. Additionally, glycosylation has profound effects on the properties of a glycoprotein including solubility, immunogenicity, circulatory half-life, and thermostability. These protein properties are important in today's biotechnology industry because proteins are produced for human therapeutics.

For in-depth research of a glycoprotein, peptide mapping is necessary. Several factors make glycopeptide research by mass spectrometry (MS) challenging. First, glycopeptides usually constitute a minor part of the glycoprotein's total peptides. Second, the MS signal intensities of the glycopeptides are lower relative to nonglycosylated peptides due to lower ionization efficiency and heterogeneous glycan structures on the same glycosylation site. Third, a glycopeptide's MS signal can be suppressed by nonglycosylated peptides. Therefore, enrichment of glycopeptides from a complex peptide mixture is desired and lectin affinity capture technology has been developed to fulfill this purpose.

Among all lectins, Concanavalin A (Con A) is the most well characterized and widely used. It can capture glycoproteins containing asparagine-linked (*N*-linked) high-mannose type glycans, *N*-linked hybrid type glycans, and some *N*-linked biantennary complex type glycans.

The Thermo Scientific ProSwift ConA-1S Affinity Column was designed to isolate glycans, glycopeptides, or glycoproteins from complex samples. But unlike other Con A columns, it is built upon a monolithic support and is designed to be used on a high-performance liquid chromatography (HPLC) system. HPLC compatibility and the monolithic support deliver multiple advantages: faster separation, better sample recovery, high peak efficiency, and high-throughput capability.

This approach also enables an automated configuration to identify glycopeptides using the ProSwift™ ConA-1S Affinity column to extract the glycopeptides from a peptide mixture. The isolated glycopeptides can then be separated by reversed-phase HPLC, and their identity confirmed by selected ion monitoring (SIM) of diagnostic sugar oxonium ions (e.g., *m/z* 204, 366, and 163). This method requires only a single quadrupole mass spectrometer.

Goal

The goals of this work are to:

- Use the ProSwift ConA-1S Affinity column to purify glycoproteins from commercial preparations of those glycoproteins.
- Extract glycopeptides from the tryptic digests of purified horseradish peroxidase (HRP), ovalbumin, and ribonuclease B glycoproteins.
- Identify individual glycopeptides of a tryptic digest after glycopeptide extraction using reversed-phase separation.
- Use a Thermo Scientific Dionex UltiMate 3000 ×2 Dual Biocompatible Analytical LC system to automate the experiment.

Thermo SCIENTIFIC

Equipment

- UltiMate™ 3000 ×2 Dual Biocompatible Analytical LC system, including:
  - DGP-3600BM Biocompatible Dual-Gradient Micro Pump
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
  - TCC-3000SD Thermostatted Column Compartment
  - DAD-3000 Diode Array Detector with 13 µL flow cell
- Thermo Scientific MSQ Plus Mass Spectrometer with electrospray ionization (ESI) source
- Thermo Scientific Dionex Chromeleon Chromatography Data System software version 6.80, SR9 or higher

Reagents and Standards

- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific P/N AC610010040)
- Formic acid, ~98% (Fluka P/N 94318, Sigma-Aldrich®)
- Albumin from chicken egg white (Ovalbumin), ≥98% (Sigma-Aldrich P/N A5503)
- Peroxidase from horseradish (HRP)(Sigma-Aldrich P/N P6782)
- Ribonuclease B (Worthington Biochemical P/N LS005710)
- Trypsin from bovine pancreas (Sigma-Aldrich P/N T1426)
- Endo H, 500,000 units/mL (New England BioLabs P/N P0702S)
- DL-Dithiothreitol, ≥99.0% (RT) (Fluka P/N 43819, Sigma-Aldrich)
- Iodoacetamide, ≥99% (HPLC) (Sigma-Aldrich P/N I6125

Conditions	
Columns:	ProSwift ConA-1S Affinity (5 × 50 mm, P/N 074148) Thermo Scientific Acclaim Polar Advantage II (PA2), 3 µm Analytical (3.0 × 150 mm, P/N 063705)
Mobile Phase:	ProSwift Column: A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH 5.3 B: 100 mM α-methyl mannoside in mobile phase A Acclaim™ PA2 Column: A: Water with 0.05% formic acid B: Acetonitrile with 0.04% formic acid
Gradient:	ProSwift Column: 0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B Acclaim PA2 Column: 0–5.0 min, 0% B; 5–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate:	ProSwift Column: 0.5 mL/min Acclaim PA2 Column: 0.425 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV absorbance at 214 nm MS SIM mode at *m/z* 163, 204, and 366
Sample Preparation:	Protein Samples: Dilute protein stock solution (2 mg/mL in DI water) in mobile phase A to 1 mg/mL before injection Peptide Samples: After tryptic digestion, dilute the peptide sample in mobile phase A to 1 mg/mL before injection

MSQ Plus™ Mass Spectrometer Conditions	
Ionization Mode:	ESI
Operating Mode:	Positive Scan
Probe Temperature:	400 °C
Needle Voltage:	3.5 kV
Detection Mode:	SIM at *m/z* 163, 204, and 366
Dwell Time:	0.5 sec
Cone Voltage:	140 V
Nebulizer Gas:	Nitrogen at 75 psi

Preparation of Solutions

Buffers for Tryptic Digestion

For detailed methods for preparing buffers for tryptic digestion, refer to Dionex (now part of Thermo Scientific) Application Update (AU) 183.[1]

Buffers for Deglycosylation

The buffers for deglycosylation are provided by New England Biolabs, the Endo H manufacturer. The buffer compositions are as follows: 10X Denaturation Buffer [5% sodium dodecyl sulfate (SDS), 0.4 M dithiothreitol] 5X Reaction Buffer [0.5M sodium phosphate, pH 5.5].

Protein Digestion Procedure

Tryptic Digestion

Reduce, alkylate, and dialyze HRP extensively against 50 mM sodium bicarbonate. Digest the resulting HRP with trypsin overnight. For detailed procedures, refer to AU 183.

Deglycosylation

1. Add 40 µg of glycoprotein to an Eppendorf tube. Prepare a 2 mg/mL solution by adding 20 µL DI water.
2. Add 2 µL 10X denaturation solution to the tube and heat at 100 °C for 10 min.
3. Cool, then add 2 µL 5X reaction buffer to the tube.
4. Add 2 µL of Endo H to the reaction. Incubate overnight at 37 °C.

Results and Discussion

Separation of a Glycoprotein from Its Nonglycosylated Counterpart and/or Nonglycosylated Impurities

Ovalbumin, ribonuclease B, and HRP are analyzed here as glycoprotein models. Figure 1A shows that roughly 80% of the commercial ovalbumin can be captured by the ProSwift ConA-1S Affinity column. A literature search shows that ovalbumin has one *N*-linked glycosylation site with approximately equal amounts of hybrid- and high-mannose type oligosaccharides that can be recognized by Con A. The other 20% of ovalbumin unbound to Con A can likely be attributed to the contaminant glycoproteins in ovalbumin that mainly have complex type glycan structures.[2]

Approximately 50% of ribonuclease B can be captured by the ProSwift ConA-1S Affinity column (Figure 1B). Ribonuclease B is reported to have a single glycosylation site with high-mannose type oligosaccharide chains. The ribonuclease B used in this study was labeled by the manufacturer to be "a mixture of ribonuclease A and ribonuclease B". This result indicates that the commercial ribonuclease B has roughly equal amounts of nonglycosylated ribonuclease A and glycosylated ribonuclease B. As shown in Figure 1C, most if not all of the HRP was captured by the ProSwift ConA-1S Affinity column. This observation agrees with the fact that HRP has nine potential glycosylation sites of which at least eight sites are occupied by heterogeneous high-mannose type oligosaccharides.[3]

Interestingly, the eluted fraction (glycoprotein fraction) peak of HRP was much sharper than the peak of ovalbumin or ribonuclease B. This observation suggests that HRP has a few dominant glycan structures with similar affinities to Con A.

Column:	ProSwift ConA-1S Affinity (5 × 50 mm)
Mobile Phase:	A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH at 5.3
	B: 100 mM α-methyl mannoside in mobile phase A
Gradient:	0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Samples:	**A**. Ovalbumin; **B**. Ribonuclease B; **C**. HRP
Sample Preparation:	1 mg/mL ovalbumin, ribonuclease B, or HRP in water/mobile phase A
Peaks:	1. Nonretained protein
	2. Retained protein (nominally glycosylated)
	3. Partially retained protein

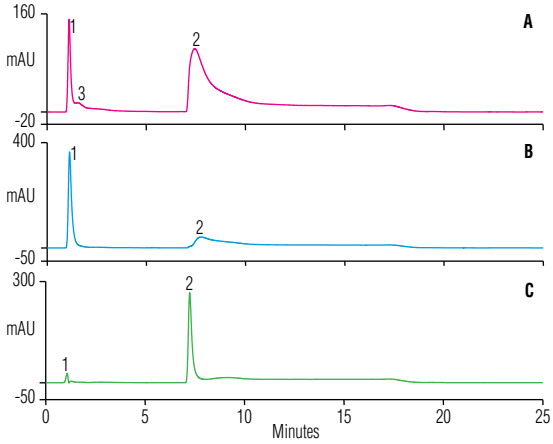

Figure 1. Glycosylated protein enrichment on the ProSwift ConA-1S Affinity column.

Separation of Glycopeptides from a Peptide Mixture

The glycoproteins discussed here were each digested with trypsin and used to test the affinity of the ProSwift ConA-1S Affinity column for glycopeptides. The separation of nonglycosylated peptides and glycopeptides by the ProSwift ConA-1S Affinity column and reanalysis of the collected fractions by reversed-phase chromatography were automated in an off-line 2D mode. The peptide fractions were collected in a 96-well plate using the fraction collector function of the WPS-3000TBFC Autosampler. The collected fractions were then loaded to an Acclaim PA2 column for peptide mapping.

Ovalbumin and ribonuclease B have only one glycosylation site, so a small fraction of their tryptic peptides will be bound to the ProSwift ConA-1S Affinity column. In contrast, HRP's multiple glycosylation sites, combined with glycan microheterogeneity on each site, predict that it will have a larger fraction of its tryptic peptides retained, which is confirmed in Figure 2C.

Figure 3 shows the peptide mapping of HRP tryptic peptides, its Con A flow-through fraction (nonglycosylated peptides), and its Con A captured fraction (glycopeptides). The UV chromatogram of the glycopeptide fraction of HRP digest shows approximately nine peaks, which may correspond to its nine glycosylation sites. Attachment of different glycans to the same glycosylation site (microheterogeneity) will have minor effects on the retention time of the peptide.[4] Therefore, a peptide with different glycans attached may be shown as a single peak in a reversed-phase chromatogram, albeit wider than a nonglycosylated peptide.

Column:	ProSwift ConA-1S Affinity (5 × 50 mm)
Mobile Phase:	A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH 5.3 B: 100 mM α-methyl mannoside in mobile phase A
Gradient:	0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Samples:	**A**. Ovalbumin tryptic peptides **B**. Ribonuclease B tryptic peptides **C**. HRP tryptic peptides
Sample Preparation:	Tryptic peptide samples diluted with mobile phase A, 1 mg/mL solution
Peaks:	1. Nonretained peptides 2. Retained peptides (nominally glycosylated) 3., 4. Peptides with weak interaction with Con A

The insets are enlargements of the first 10 min of each separation

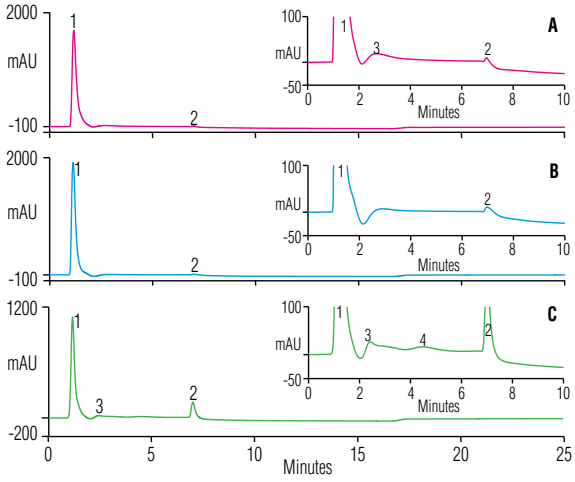

Figure 2. Glycosylated tryptic peptides enrichment on the ProSwift ConA-1S Affinity column.

Column:	Acclaim PA2, 3 µm (3.0 × 150 mm)
Mobile Phase:	A: Water with 0.05% formic acid B: Acetonitrile with 0.04% formic acid
Gradient:	0–5.0 min, 0% B; 5.0–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate:	0.425 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Sample Preparation:	HRP tryptic peptides diluted with mobile phase A,1 mg/mL solution

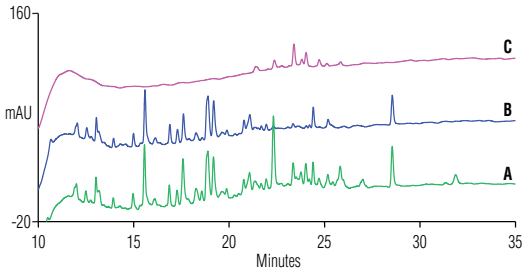

Figure 3. Peptide mapping of (A) HRP tryptic peptides, (B) HRP tryptic peptides ProSwift ConA-1S Affinity column flow-through fraction, and (C) HRP tryptic peptides ProSwift ConA-1S Affinity column eluted fraction.

Selective Monitoring of Glycopeptides by Monitoring Oxonium Ions

SIM scanning of glycan diagnostic oxonium ions and precursor ion scanning are two frequently used methods for selective detection of peptides with a post-translational modification such as glycosylation and phosphorylation.[4] Without the ability to do precursor ion scanning, scanning oxonium ions is the choice when using a single quadrupole mass spectrometer. It is reported that *m/z* 163, 204, 292, and 366 are marker ions for glycosylation.[4] Production of marker ions is controlled by the extent of collisional excitation, which depends on the voltage applied to the sampling cone. Maximum yield of marker for glycosylation is reportedly generated at a cone voltage of 140 V, which was applied in this study.

As Figure 4 shows, peak number, shape, and retention time in the mass spectroscopy traces for *m/z* 204 and 366 are equivalent to the UV chromatogram of the Con A captured fraction of the HRP tryptic peptides, offering further evidence that they are indeed glycopeptides. The sensitivity of peaks in the ion chromatogram is much higher than shown in the UV chromatogram. The extracted trace of ion *m/z* 163 can serve as a glycopeptide diagnostic ion in a less sensitive way.

In this work, the SIM trace of ion *m/z* 292 is a poor match for the UV trace (data not shown). It is known that oxonium ion 292 is from sialic acids (NeuAc+); therefore, the observation that *m/z* 292 is a poor diagnostic ion for these experiments may indicate lack of sialic acid containing oligosaccharide structures in the captured HRP tryptic glycopeptides.

The cone voltage is critical because diagnostic ion peaks under lower cone voltage, such as 100 V and 65 V in mass spectrum, do not match the UV chromatogram. Although *m/z* 204 or 366 can be generated from nonspecific fragmentation of peptide backbone, simultaneous detection of both *m/z* 204 and 366 provides strong evidence that the peptide is glycosylated. When comparing MS traces for *m/z* 204 and 366 with the UV chromatogram of the Con A captured fraction, the peptides in the captured fraction can be identified as glycopeptides.

Figure 5 shows that scanning for diagnostic oxonium ions is a selective and sensitive method to monitor glycopeptides in a peptide mixture that has not been passed through the ProSwift ConA-1S Affinity column. Major peaks in the *m/z* 204 SIM spectrum of unseparated HRP tryptic digest fit with peaks in the spectrum of the Con A captured fraction. Notice that some peaks shown in HRP tryptic digest cannot be found in the Con A captured fraction. These peaks may be lost in minor peaks (very wide peaks such as peaks 3 and 4, shown in Figure 2, with retention times of ~2.5 min and ~3.5 min) that elute just after the flow-through peak. This fraction probably has glycan structures that are not recognized by Con A. Published literature shows that HRP does have a minor glycan structure, Fuc(1-3)GlcNAc-, that could bind to Con A, though very weakly.[5]

Column:	Acclaim PA2, 3 µm (3.0 × 150 mm)
Mobile Phase:	A: Water with 0.05% formic acid B: Acetonitrile with 0.04% formic acid
Gradient:	0–5.0 min, 0% B; 5–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate:	0.425 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	**A**. UV at 214 nm **C**. *m/z* 204 in SIM mode **B**. *m/z* 366 in SIM mode **D**. *m/z* 163 in SIM mode
Sample Preparation:	Tryptic peptides diluted with mobile phase A, 1 mg/mL solution

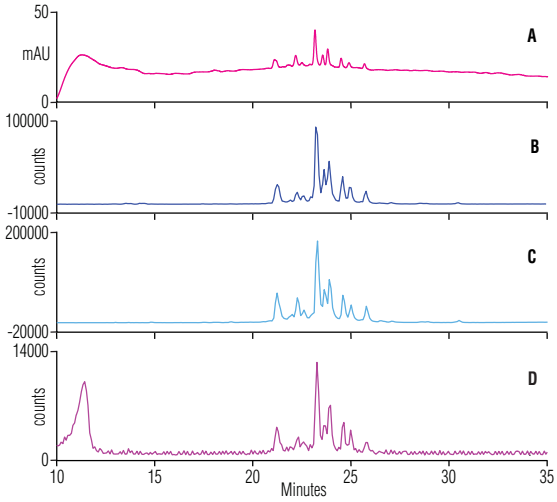

Figure 4. Peptide mapping of Con A captured fraction from the HRP tryptic digest detection by UV and MS in SIM mode.

Column:	Acclaim PA2, 3 µm (3.0 × 150 mm)
Mobile Phase:	A: Water with 0.05% formic acid B: Acetonitrile with 0.04% formic acid
Gradient:	0–5.0 min, 0% B; 5–35.0 min, 0–50% B; 35.5–45.0 min, 90% B
Flow Rate:	0.425 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	*m/z* 204 in SIM mode
Samples:	**A**. HRP tryptic peptides **B**. Con A captured fraction of HRP tryptic peptides **C**. Con A flow-through fraction of HRP tryptic peptides

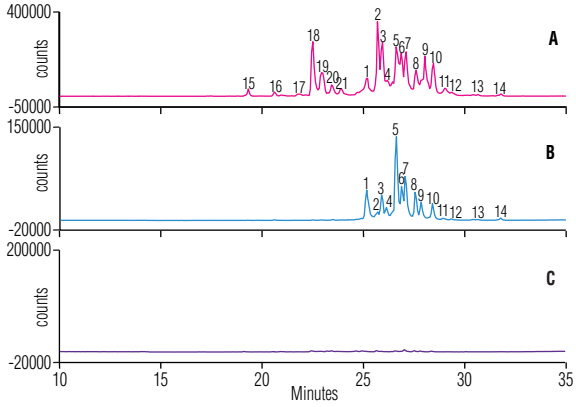

Figure 5. Peptide mapping of (A) HRP tryptic peptides, (B) Con A captured fraction of HRP tryptic peptides, and (C) Con A flow-through fraction of HRP tryptic peptides.

Endo H Digestion Eliminates the Con A Binding Ability of HRP

Endo H has been reported to cleave within the chitobiose core of high-mannose type and some hybrid type oligosaccharides from *N*-linked glycoproteins. Figure 6 shows that HRP cannot be retained by the ProSwift ConA-1S Affinity column after Endo H treatment. This observation confirms that main glycosylation types of HRP are high mannose and/or hybrid. Peak 3 in Figure 6 is postulated to be either the released oligosaccharides—because it is not found in the Endo H control (no HRP added)—or more likely, the fraction of glycosylated HRP that is not susceptible to Endo H (i.e., all Endo H-susceptible structures have been removed and only the nonsusceptible structures remain on the HRP). It is more likely a non-Endo H-susceptible fraction because oligosaccharides have little or no absorbance at 210 nm.

Column:	ProSwift ConA-1S Affinity (5 × 50 mm)
Mobile Phase:	A: 50 mM sodium acetate, 200 mM sodium chloride, 1 mM calcium chloride, pH 5.3
	B: 100 mM α-methyl mannoside in mobile phase A
Gradient:	0–5.0 min, 0% B; 5.0–5.5 min, 0–100% B; 5.5–15 min, 100% B
Flow Rate:	0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV at 214 nm
Sample Preparation:	1 mg/mL HRP in water/mobile phase A
Samples:	**A**. Before Endo H treatment
	B. After Endo H treatment
Peaks:	1. HRP
	2., 3. Deglycosylated HRP

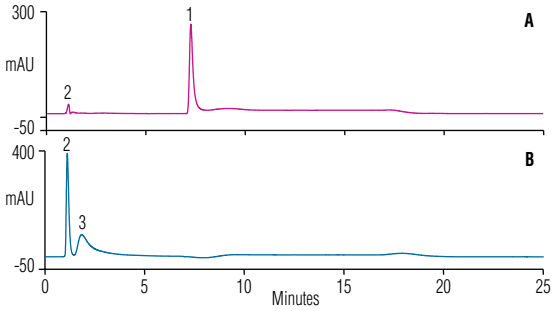

Figure 6. Glycosylated HRP enrichment on the ProSwift ConA-1S Affinity column (A) before and (B) after Endo H treatment.

Conclusion

This work shows that the HPLC-compatible ProSwift ConA-1S Affinity column can capture glycoproteins and glycopeptides efficiently. The UltiMate 3000 ×2 Dual Biocompatible Analytical LC system can automate the entire off-line 2D process from ProSwift ConA-1S Affinity column sample enrichment and fraction collection to automatic reanalysis of collected sample by peptide mapping. Monitoring oxonium ions (e.g., *m/z* 204, 366, and 163) in a peptide mixture with a single quadrupole mass spectrometer is a selective, sensitive, and reliable method that also confirms the identity of glycopeptides captured by the ProSwift ConA-1S Affinity column.

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France +33 1 39 30 01 10
Germany +49 6126 991 0
India +91 22 2764 2735
Ireland +353 1 644 0064
Italy +39 02 51 62 1267
Japan +81 6 6885 1213
Korea +82 2 3420 8600
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Application Update 188

High-Resolution Cation-Exchange Alternative to Peptide Mapping for Protein ID and QA/QC

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Key Words
Ion Exchange, ProPac SCX-10 Column, MabPac SCX-10 Column, Fast Separation

Goal
To develop an efficient and high-resolution peptide mapping method in cation-exchange mode as an alternative to reversed-phase separation

Introduction
Peptide mapping is commonly used to demonstrate protein identity. In later phases of pharmaceutical development and in quality assurance/control (QA/QC), peptide mapping with UV detection of the protein drug serves as a primary protein QC method. Although a reversed-phase separation is the typical choice for separating peptides, high-resolution ion-exchange chromatography is an alternate method that provides additional information and a different selectivity. In proteomics applications, strong cation-exchange chromatography is routinely the choice for the first dimension separation of peptides.[1]

The Thermo Scientific ProPac SCX-10 and MabPac SCX-10 Analytical Columns are constructed by grafting cation-exchange groups onto a nonporous polymeric particle that has a hydrophilic coating. This construction results in high-efficiency peaks and high-resolution separations of protein variants.[2] The work shown here demonstrates that strong cation-exchange chromatography can provide good resolution for peptide mapping, and thereby serve as an alternate or supplementary method for separating peptides.

Equipment
- Thermo Scientific Dionex UltiMate 3000 ×2 Dual Biocompatible Analytical Liquid Chromatography (LC) System, including:
  - DGP-3600BM Biocompatible Dual-Gradient Micro Pump with SRD-3600 Integrated Solvent and Degasser Rack
  - WPS-3000TBFC Thermostatted Biocompatible Pulled-Loop Well Plate Autosampler with Integrated Fraction Collection
  - TCC-3000SD Thermostatted Column Compartment
  - DAD-3000 Diode Array Detector
- Thermo Scientific Dionex Chromeleon Chromatography Data System software version 6.80, SR9 or higher
- Thermo Scientific Orion 2-Star Benchtop pH Meter

Reagents and Standards
- Deionized (DI) water, 18.2 MΩ-cm resistivity
- Acetonitrile (CH_3CN), HPLC grade (Fisher Scientific P/N AC610010040)
- Formic acid, analytical grade, SCRC, China
- Sodium perchlorate, analytical grade, SCRC, China
- Triethylamine, HPLC grade, CNW Technologies GmbH, Germany
- Trypsin from bovine pancreas (Sigma-Aldrich® P/N T1426)
- Myoglobin from equine heart (Sigma-Aldrich P/N M1882)
- DL-Dithiothreitol, ≥99% (Fluka P/N 43819, Sigma-Aldrich)
- Iodoacetamide, ≥99% (Sigma-Aldrich P/N I6125)

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Preparation of Solutions

Buffers for Tryptic Digestion

For detailed methods of preparing buffers for tryptic digestion, refer to Dionex (now part of Thermo Scientific) Application Update (AU) 183.[3]

Mobile Phases with Triethylamine Phosphate (TEAP)

Prepare a 20 mM triethylamine (TEA) solution, then adjust the pH of the solution to 2.0 or 3.9 using phosphoric acid. Add acetonitrile and sodium perchlorate to make mobile phase A or mobile phase B for the ion-exchange separation. For example, mix the pH 2 TEAP solution 1:1 with acetonitrile to prepare mobile phase A shown in Figure 1. To prepare mobile phase B shown in Figure 1, prepare 1 L of mobile phase A and dissolve 14.05 g of sodium perchlorate in that solution.

Sample Preparation

Reduce, alkylate, and dialyze myoglobin extensively against 50 mM sodium bicarbonate. Then digest the resulting reduced and alkylated myoglobin with trypsin overnight. For detailed procedures, refer to AU 183.

Results and Discussion

Use of a TEAP/sodium perchlorate mobile phase with manipulation of the pH and organic solvent modifier concentration allowed high-resolution separation of an equine heart myoglobin tryptic digest (Figure 1). This separation was easily accelerated without compromising resolution by simply increasing the flow rate. This method can also be applied to a synthetic peptide. Figure 2 shows similar high resolution and fast separation of a synthetic peptide and its byproducts. In contrast to the myoglobin tryptic digest separation, better separation was observed at pH 3.9 than at 2.0. This may be due to the basic nature of the synthetic peptide.

Separation on the cation-exchange column is primarily determined by analyte charge, but hydrophilic and hydrophobic interactions also play a role. Organic solvent modifiers such as acetonitrile improve separation by changing solubility, hydrophilic interaction, and possibly peptide conformation. Sodium perchlorate was used to elute peptides due to its better solubility in acetonitrile and its stronger elution power compared to the commonly used sodium chloride.

Column:	ProPac™ SCX-10, 10 µm (4 × 250 mm, P/N 075725)
Mobile Phase:	A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A
Gradient:	**1**. 0–40 min, 0–100% B, at 0.7 mL/min **2**. 0–28 min, 0–100% B, at 1.0 mL/min **3**. 0–20 min, 0–100% B, at 1.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

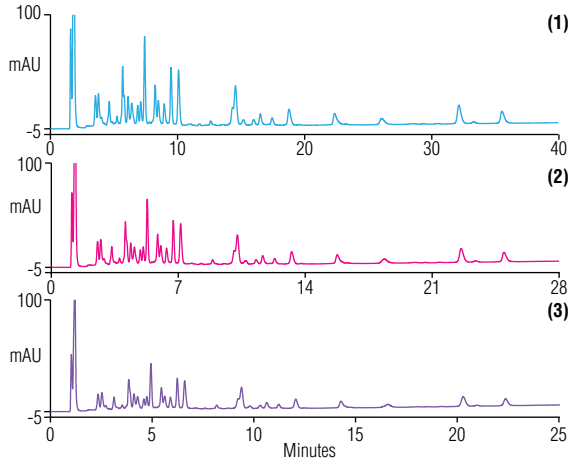

Figure 1. Chromatograms of a myoglobin tryptic digest with different flow rates.

Column:	ProPac SCX-10 10 µm (4 × 250 mm)
Mobile Phase:	**1**. A: 20 mM TEAP, pH 3.9, 50% acetonitrile B: 100 mM perchlorate in A **2**. A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM perchlorate in A
Gradient:	0–10 min, 0–50% B
Flow Rate:	1.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	A synthetic peptide and its byproducts The sequence of the peptide: (Ac-)YNIQKESTLPLVLRLRGG (–$CONH_2$) Calculated pI of the peptide: 11.4

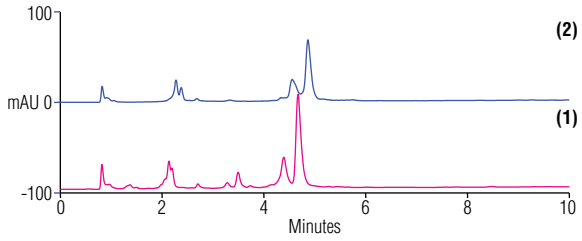

Figure 2. Chromatograms of a synthetic peptide and its byproducts at different mobile phase pH values.

Figure 3 shows that the ProPac SCX-10 column can provide high resolution comparable to the reversed-phase separations that use formic acid as a mobile phase modifier. This is in contrast to many ion-exchange columns that yield incomplete resolution and poor peak shape in peptide mapping. Figure 4 shows that a reversed-phase separation with trifluoroacetic acid (TFA) can be better than the cation-exchange separation.

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. Acclaim™ PA2, 3 µm (3.0 × 150 mm, P/N 063705) **3**. Acclaim 300, C18, 3 µm (3.0 × 150 mm, P/N 063684)
Mobile Phase:	**1**. A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A **2**. A: 98% water, 2% acetonitrile, 0.1% formic acid B: 98% acetonitrile, 2%water, 0.08% formic acid **3**. Same as 2
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.6 mL/min **3**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

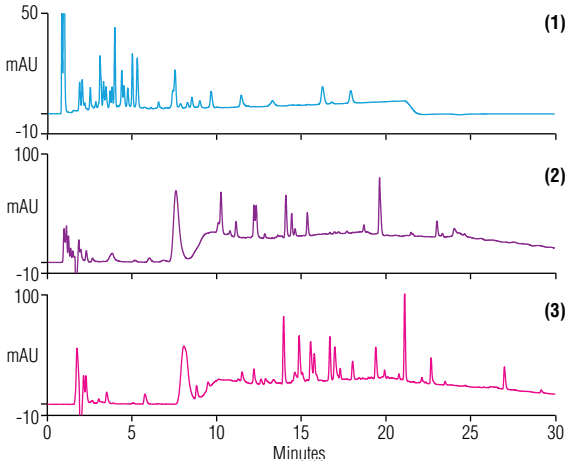

Figure 3. Chromatograms of a myoglobin tryptic digest separated by 1) a ProPac SCX-10 column, 2) a Thermo Scientific Acclaim PolarAdvantage II (PA2) column, and 3) an Acclaim 300 C18 column with formic acid as the mobile phase modifier of reversed-phase separation.

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. Acclaim PA2, 3 µm (3.0 × 150 mm, P/N 063705) **3**. Acclaim 300, C18, 3 µm (3.0 × 150 mm, P/N 063684)
Mobile Phase:	**1**. A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A **2**. A: 98% water, 2% acetonitrile, 0.04% TFA B: 98% acetonitrile, 2%water, 0.03% TFA **3**. Same as 2
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.6 mL/min **3**. 0–5 min, 0% B; 5–28 min, 0–100% B, at 0.5 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

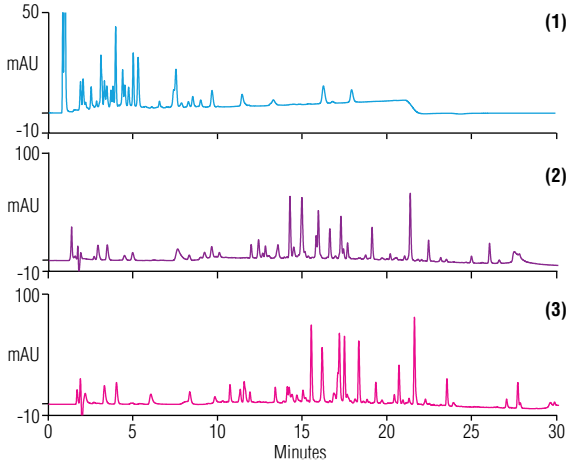

Figure 4. Chromatograms of a myoglobin tryptic digest separated by 1) a ProPac SCX-10 column, 2) an Acclaim PA2 column, and 3) an Acclaim C18 column with TFA as the mobile phase modifier of reversed-phase separation.

Table 1 summarizes peak capacity of an ion-exchange column, the Dionex ProPac SCX-10, and two reversed-phase columns, the Acclaim PA2 and Acclaim 300. Peak capacity(n_C) was calculated by the equation:

$$n_C = 1 + \frac{t_G}{W}$$

where t_G is the gradient time and W the peak width measured at 4σ (13.4% of the peak height). For detailed information, refer to Dionex (now part of Thermo Scientific) Technical Note (TN) 74.[4]

Table 1. Peak capacity comparison of three columns.

	ProPac SCX-10 (10 µm)	Acclaim PA2 (3 µm)	Acclaim 300 C18 (3 µm)
Formic acid as the mobile phase modifier in a reversed-phase separation	73	79	140
TFA as the mobile phase modifier in a reversed-phase separation	73	136	158

With the next-generation MabPac™ SCX-10 (3 µm) column, both separation efficiency and resolution can be further improved (Figure 5). Separation time can be easily reduced to <10 min by simply increasing the flow rate. Additionally, the peptides have better retention and are better resolved on the MabPac SCX-10 column compared to the ProPac SCX-10 column.

For the synthetic peptide and byproducts separation, the ProPac SCX-10 and MabPac SCX-10 (3 µm) columns provide similar resolution (Figure 6). The latter column performs better for resolving the main product (Peak 1) and a nearby byproduct (Peak 2).

Conclusion

This work shows that the ProPac SCX-10 column delivers high-resolution separations for peptide mapping and provides an alternate or supplementary method for reversed-phase peptide separation. The new MabPac SCX-10 (3 µm) column delivers faster high-resolution peptide mapping. Both columns can be used to separate a synthetic peptide and its byproducts, providing an alternative to reversed-phase separation.

References

1. Dionex (now part of Thermo Scientific) Application Note 521: Automated 2D LC Coupled to ESI-MS/MS for the Analysis of Complex Peptide Samples. Sunnyvale, CA, 2002. [Online] www.dionex.com/en-us/webdocs/5476-AN521_LPN1470.pdf (accessed June 18, 2012).
2. Dionex (now part of Thermo Scientific) Application Note 126: Determination of Hemoglobin Variants by Cation-Exchange Chromatography. Sunnyvale, CA, 2007. [Online] www.dionex.com/en-us/webdocs/4466-AN126_released022707.pdf (accessed June 18, 2012).
3. Dionex (now part of Thermo Scientific) Application Update 183: Separation of Peptides from Enzymatic Digestion of Different Acclaim Columns: A Comparative Study. Sunnyvale, CA, 2011. [Online] www.dionex.com/en-us/webdocs/111297-AU183-LC-Peptides-EnzyDigest-AcclaimCompar-02Nov2011-LPN2973.pdf (accessed June 18, 2012).
4. Dionex (now part of Thermo Scientific) Technical Note 74: High Peak Capacity Nano LC Peptide Separations Using Long Packed Columns. Sunnyvale, CA, 2009. [Online] www.dionex.com/en-us/webdocs/70394-TN74-HPLC-Peptides-LongColumns-29Jan09-LPN2111.pdf (accessed Aug 8, 2012).

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. MabPac SCX-10, 3 µm (4 × 50 mm, P/N 077907)
Mobile Phase:	A: 20 mM TEAP, pH 2.0, 50% acetonitrile B: 100 mM sodium perchlorate in A
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–15 min, 0–100% B; 15.5–25 min, 0% B, at 0.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	Myoglobin (from equine heart) tryptic digest
Sample Preparation:	Reduce, alkylate, dialyze, and digest with trypsin overnight

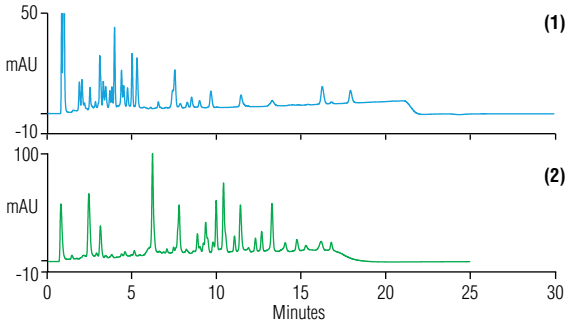

Figure 5. Chromatograms of a myoglobin tryptic digest separated by 1) a ProPac SCX-10 column (10 µm particle size) and 2) a MabPac SCX-10 column (3 µm particle size).

Columns:	**1**. ProPac SCX-10, 10 µm (4 × 250 mm, P/N 075725) **2**. MabPac SCX-10, 3 µm (4 × 50 mm, P/N 077907)
Mobile Phase:	A: 20 mM TEAP, pH 3.9, 50% acetonitrile B: 100 mM sodium perchlorate in A
Gradient:	**1**. 0–20 min, 0–100% B, at 1.4 mL/min **2**. 0–15 min, 0–100% B; 15.5–25 min, 0% B, at 0.4 mL/min
Inj. Volume:	20 µL
Temperature:	30 °C
Detection:	UV, 214 nm
Sample:	A synthetic peptide and its byproducts The sequence of the peptide: (Ac-)YNIQKESTLPLVLRLRGG(–$CONH_2$) Calculated pI of the peptide: 11.4

Peak X is also shown in the blank, so it should not be considered a real peak.

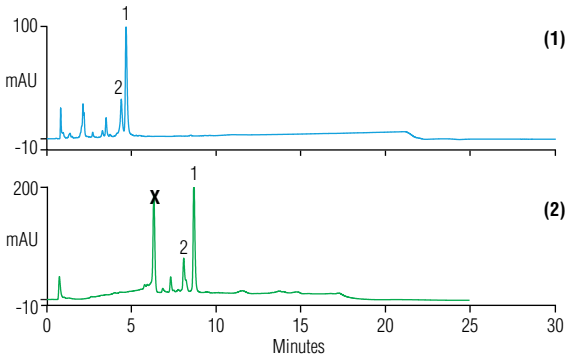

Figure 6. Chromatograms of a synthetic peptide and its byproduct using 1) a ProPac SCX-10 column (10 µm particle size) and 2) a MabPac SCX-10 column (3 µm particle size)

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Austria +43 1 616 51 25
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+32 3 353 42 94
Brazil +55 11 3731 5140
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France +33 1 39 30 01 10
Germany +49 6126 991 0
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Comprehensive Sequence and Post-translational Modifications Analysis of Monoclonal Antibody by Flash Digest and LC-High Resolution MS

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Overview

Purpose: To develop a workflow for fast and comprehensive characterization of peptide sequence, identification and relative quantitation of post-translational modifications (PTMs) of monoclonal antibody (mAb).

Methods: Native and oxidatively stressed IgG mAb were enzymatically digested by Flash Digest™ kit with trypsin. Peptide samples were analyzed by online LC-MS on a Thermo Scientific™ Q Exactive™ mass spectrometer. Peptide sequence mapping, identification and quantification of PTMs were performed by Thermo Scientific™ PepFinder™ software (version 1.0).

Results: Comprehensive and simultaneous sequence and PTM analysis with a particular focus on oxidation of IgG mAb was realized by combining rapid digestion, high resolution, accurate mass (HRAM) data and PepFinder software. This workflow greatly shortens the sample preparation and data analysis time while providing great sensitivity to detect low level PTMs.

Introduction

As well established and fast growing biotherapeutics, mAbs have been approved for the treatment of diseases such as cancer, inflammatory, infectious and autoimmune diseases etc.[1,2] To ensure product efficacy and safety, the quality of biotherapeutics needs to be closely monitored. Various analytical methods have been used to study quality attributes such as structural integrity, aggregation, glycosylation pattern or amino acid degradation. Here, we report a fast and sensitive approach by combing fast enzymatic digestion, high resolution mass spectrometry and user friendly new data processing software for sequence and post translational modifications analysis. This approach provides an effective way to characterize protein therapeutics in bioprocess development.

Methods

Sample Preparation

Differential oxidative stress was induced by 5 mM hydrogen peroxide and quenched by the addition of 1mM sodium thiosulfate at various time points. The native and oxidatively stressed IgG samples were trypsin digested using a Flash Digest kit (Perfinity Biosciences Inc). Digestion time was optimized by incubating native, non-reduced IgG mAb at 70 °C for various durations from 15 to 120 minutes. One portion of the digest was analyzed by online UHPLC-ESI-MS/MS. The other portion was reduced and alkylated before similar analysis. All chemicals were purchased from Sigma Aldrich unless it is specified.

Liquid Chromatography

Native and Oxidatively stressed tryptic peptide samples were analyzed on Thermo Scientific™ Dionex™ UltiMate™ 3000 XRS system and OAS autosampler coupled to the Thermo Scientific™ Q Exactive™ MS. Peptides were separated on an ACQUITY® BEH 130 C18 column (2.1x100mm, 1.7μm, Waters) with column temperature set as 40 °C at a flow rate of 300 μL/min with solvent A (0.05% trifluoroacetic acid in H_2O) and solvent B (0.045% trifluoroacetic acid in acetonitrile).

Injection amount: 8.0 μg digested protein on column

Time [min]	Flow [μL/min]	Mixture [%B]
0	300	0.1
5	300	0.1
94	300	35
94.5	300	95
99.5	300	95
100	300	0.1
110	300	0.1

Mass Spectrometry

The Q Exactive MS interfaced with H-ESI II ion source was employed for MS analysis. Acquisition method was set with full scan (resolution 70,000 at FWHM *m/z* 200) and top 5 data dependent MS/MS (17,500 resolution) in positive mode.

=== HESI Source: ===	
Spray Voltage (+)	3800V
Capillary Temperature (+)	320°C
Sheath Gas (+)	40
Aux Gas(+)	10
Sweep Gas(+)	0
Heater Temperature (+)	300°C
S-lens	50

Full MS Scan in positive mode: Resolution=70,000; AGC=3e6; IT=100ms; Scan range=*m/z* 300-1800; Lock mass=off; Microscans=1

Top 5 data dependent MS/MS: Resolution=17,500; AGC=1e5; IT=250ms; NCE=27; Isolation window=*m/z* 2; Fixed first mass=*m/z* 130

Data Analysis

The mapping of mAb sequence, disulfide linkages and identification of PTMs are performed in PepFinder software. PepFinder software is designed for in-depth characterization of biotherapeutic proteins. It offers automatic workflow for identification of disulfide bonds, glycopeptides and other PTMs, i.e. oxidation, deamidation etc by mono-isotopic mass at MS level and confirmation by MS/MS fragments indicated with a confidence score. The peptide sequence coverage map with color code for signal intensity of each characterized peptide and modification summary report with relative quantitation percentage are generated on the user friendly interface. For unknown /untargeted modifications, the amino acid sites are indicated with accurate mass of the modification for further interpretation.

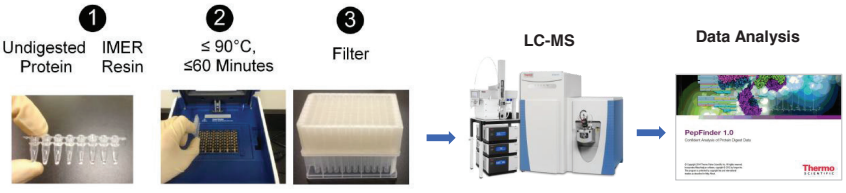
Results

Tryptic Digestion Time Optimization of mAb by Flash Digest Kit

Flash Digest is a very active, highly stable immobilized trypsin reactor that is combined with heating technology for fast reproducible digestions. The trypsin column makes use of a high concentration of trypsin while simultaneously eliminating autolysis in order to push the non-complete digestion due to the decrease of substrate concentration near the completion of digestion reaction.

Using the Flash Digest kit (below workflow 1-3), digestion time was optimized by incubating native, non-reduced IgG mAb at 70 °C at 15, 30, 45, 60, 75, 90, 105 and 120 min. The filtered samples were directly subjected to LC-MS/MS and data analysis.

FIGURE1. Complete Workflow including Flash Digest, LC-MS and Data Analysis



Sequence coverage maps of both light chain and heavy chain of native, non-reduced mAb were generated from PepFinder software. Without reduction of disulfide linkages, a 30-min digestion time is adequate to achieve good sequence coverage of >83% for light chain and >79% for heavy chain, indicating an excellent digestion efficiency. The uncovered sequences on light (Figure 2) and heavy chains (not shown) are due to the non-reduced disulfide bonds on cysteine residue. As shown in Table 1, sequence coverage of light and heavy chains was not increased by extending digestion time to 120 min.

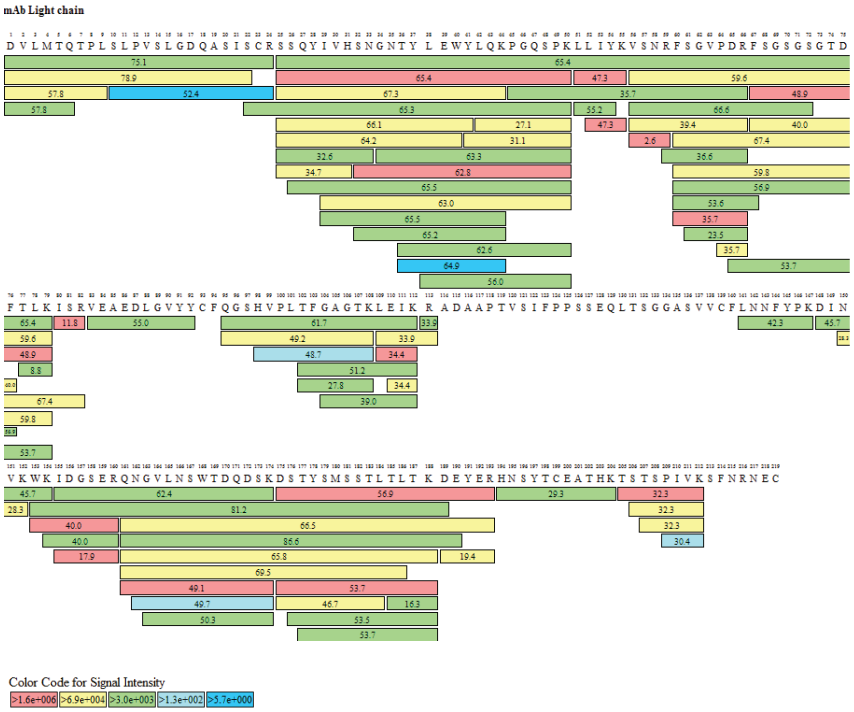
TABLE 1. Sequence Coverage Summary of Native, Non-reduced IgG at Various Digestion Times

Sequence Coverage	15min	30min	45min	60min	75min	90min	105min	120min
Light Chain	78.5%	83.6%	83.6%	83.6%	82.6%	83.6%	83.6%	83.6%
Heavy Chain	79.1%	79.1%	79.1%	79.1%	79.1%	79.1%	79.1%	79.1%

TABLE 2. Selected PTMs of Native, Non-reduced IgG at Various Digestion Times

Relative Abundance	15min	30min	45min	60min	75min	90min	105min	120min
N33+Deamidation (Light Chain)	27.12%	28.12%	28.90%	27.87%	26.96%	29.84%	29.85%	29.85%
N162+Deamidatio n (Light Chain)	15.98%	17.23%	18.38%	19.29%	20.29%	21.39%	21.68%	22.16%
M180+Oxidation (Light Chain)	0.26%	0.35%	0.49%	0.70%	0.97%	0.98%	1.01%	1.31%
N83+Deamidation (Heavy Chain)	1.44%	1.44%	1.62%	1.79%	1.81%	2.13%	2.14%	2.12%

FIGURE 2. Sequence Coverage Map (83.6%) of Native, Non-reduced IgG Light Chain



Sequence Characterization of IgG mAb

When the same IgG is further reduced and alkylated, sequence coverage of both IgG light chain (Figure 3) and heavy chain are 100% and 97.1%, respectively. On the sequence coverage map (Figures 2 and 3), most peptide sequences are mapped multiple times indicating by different color bars below the sequence, which greatly increases the identification confidence. The color bar represents signal intensity of the identified peptide. The number within the bar is the retention time of eluting peptide.

This data is comparable to the result generated by an overnight digestion protocol in which IgG was denatured by guanidine, reduced by DTT and alkylated by IAA, followed by trypsin digestion overnight (data not shown). The sample preparation efficiency is significantly improved with Flash Digest kit.

Simultaneous Identification and Relative Quantitation of PTMs in Oxidatively Stressed IgG Samples

Differential oxidative stress was induced by hydrogen peroxide at 15, 30, 60, 90, 120 mins. After simple trypsin digestion, samples were analyzed by LC-MS/MS. PTMs such as oxidation, deamidation, and glycation at different amino acid sites are identified with high confidence levels as shown in the summary (Table 3) from software.

The relative abundance of each modification in different samples under same LC-MS condition is calculated. The oxidative study of IgG shows that oxidation of methionine (M) 49, 304 and 393 in the heavy chain is dose-dependent as the oxidation reaction time increases from 30 to 120 min, while unquenched sample demonstrated significantly higher percentage of oxidation. M49 oxidation was identified by isotopic mass and confirmed by MS/MS spectrum in PepFinder software. The experimental MS/MS spectrum was annotated automatically in the software. The well matched predicted (top panel) and experimental (bottom panel) spectra are shown in Figure 4, demonstrating the high confident identification and confirmation of peptide with oxidation modification.

Relative percentages of each glycoform, double oxidation of methionine and deamidation of asparagine (N) , H_2O loss of threonine, serine, aspartic acid and glutamic acid, glycation on lysine did not change over the reaction time as expected. Double and triple oxidation of cysteine were monitored but not observed.

FIGURE 3. Sequence Coverage Map (100%) of Digested, Reduced and Alkylated IgG Light Chain

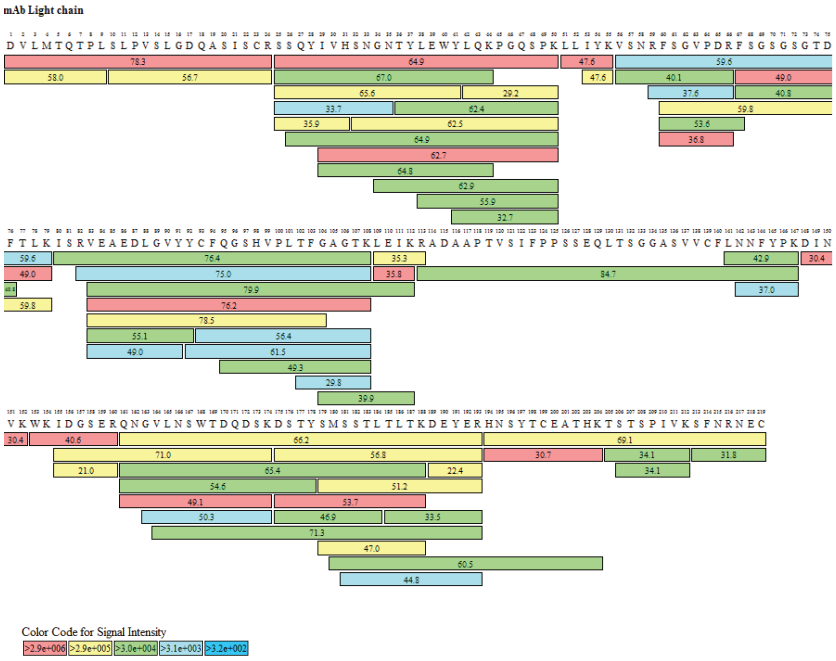
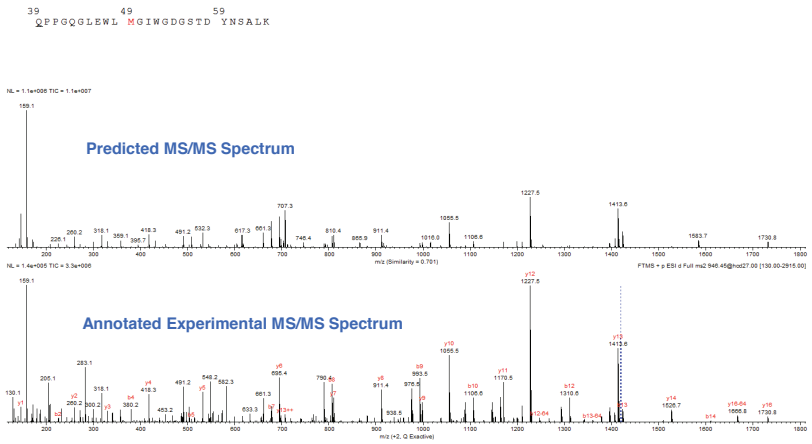


TABLE 3. Summary of Selected Identified PTMs and Major Glycoforms of Oxidatively Stressed IgG Heavy Chain

Relative Abundance	15min	30min	60min	90min	120min	Unquenched
M49+Oxidation	9.67%	9.51%	10.70%	10.84%	11.43%	74.30%
~M49+Double Oxidation	0.12%	0.13%	0.15%	0.13%	0.15%	0.12%
N60+Deamidation	4.34%	5.14%	4.95%	5.55%	5.25%	4.86%
M304+Oxidation	15.35%	18.50%	24.20%	30.35%	36.76%	86.69%
M393+Oxidation	13.99%	14.28%	18.53%	21.67%	25.80%	84.36%
N292+A1G0F	15.48%	16.51%	16.17%	16.86%	16.00%	16.91%
N292+A1G1F	4.04%	4.87%	5.24%	5.17%	4.99%	4.94%
N292+A2G0F	34.82%	34.35%	35.59%	33.71%	35.34%	33.60%
N292+A2G1F	36.86%	36.12%	34.54%	36.53%	34.89%	36.36%
N292+A2G2F	7.57%	7.82%	7.62%	7.11%	7.99%	7.28%

FIGURE 4. MS/MS Spectra of Triply Charged Peptide with Methionine Oxidation



Conclusion

A complete workflow has been developed for the fast and comprehensive sequence and post-translational modifications analysis of monoclonal antibodies.

- A 30-min digestion time demonstrated sufficient digestion efficiency of immobilized trypsin column for IgG mAb. Good sequence coverage of native, non-reduced IgG light and heavy chains were obtained. Further reduction and alkylation increased sequence coverage to 100% and 97% for light and heavy chains, respectively.
- Oxidative study results show that oxidation of methionine 49, 304 and 393 in IgG heavy chain is dose-dependent as the oxidation reaction time. However, major glycoforms did not change as expected.
- This workflow could greatly shorten the sample preparation and data analysis time while providing great sensitivity to detect low level PTMs. Additionally, any unintentionally incurred oxidative stresses during biopharmaceutical production may be rapidly analyzed for impact on production.

Reference

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2. Durocher Y, Butler M (2009) Expression systems for therapeutic glycoprotein production. *Curr Opin Biotechnol* 20: 700–707.

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Degradation Profiling of a Monoclonal Antibody Using Multiple Fragmentation Techniques and a Novel Peptide Mapping Software

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Overview

Purpose: The goal of these analyses is to develop an effective workflow for degradation profiling of a monoclonal antibodies utilizing the sensitive liquid chromatography-mass spectrometry technique in combination with a software tool for automatic identification and relative quantification of protein modifications.

Methods: Enzymatically digested monoclonal antibody was analyzed by liquid chromatography and mass spectrometry with complementary fragmentation techniques. The HCD and ETD data were interpreted by Thermo Scientific™ PepFinder™ Software[1].

Results: Chemical degradation of amino acids was profiled. Both expected and unexpected modifications were identified.

Introduction

Monoclonal antibodies (mAbs) are the fastest growing classes of therapeutic agents. Like other proteins, mAbs are subject to various degradation pathways. MAb degradation can occur during manufacturing process and upon storage in liquid, frozen, or solid state at different pH and temperature conditions. These degradations are heavily characterized in the biopharmaceutical industry throughout the development, manufacturing and storage stages. The characterization of chemical degradation includes amino acid verification, N-terminal and C-terminal processing, deamidation, oxidation, etc. Recent development and optimization of mass spectrometry with multiple fragmentation techniques is expected to significantly improve efficiency and sensitivity of the conventional characterization methods. Furthermore, the use of a novel peptide mapping software yields detailed and ultimate qualitative insight into mAb degradation profiling.

Methods

Sample Preparation

Commercially available monoclonal antibody rituximab was purchased and used in all experiments. Rituximab was supplied at a concentration of 10 mg/mL, formulated in 9.0 mg/mL sodium chloride, 7.35 mg/mL sodium citrate dihydrate, 0.7 mg/mL polysorbate 80,and water. For degradation profiling of the mAb, different conditions for stressing rituximab include pH extremes (pH 10), oxygen (exposure to hydrogen peroxide), light (exposure to natural day light) and temperature (40 °C).

For pH extremes, buffer pH 10 was made by 5% NH_4OH. To stress the mAb protein, 5 µL rituximab was mixed with 45 µL buffer and stored in dark at 40 °C.

For oxidative stress, 5 µL rituximab was mixed with 45 µL 5% H_2O_2 and stored at room temperature for 45 minutes prior to digestion.

For light stress, 20 µL protein was stored in an Eppendorf tube and exposed to natural day light for two days.

For additional temperature stress conditions, 20 µL stock was stored at 40 °C in dark for 48 hours (annotated as “native, 40 °C”).

All mAbs were mixed with 1:1 ratio (v:v) to 2,2,2-Trifluoroethanol (Sigma, St. Louis, Missouri) and reduced in 200 mM dithiothreitol for 20 min at 90 °C in dark. The reduced proteins were then alkylated with 100mM iodoacetamide (Sigma, St. Louis, Missouri) in the dark for 1 hour. The proteins were diluted by 1:10 10 mM ammonium bicarbonate and incubated with 1:20 trypsin to protein (w:w) for 6 hours. Digestion was quenched by acidifying the sample to pH 3 using formic acid.

Liquid Chromatography

An approximate 2.5 µg digest was loaded per injection. Peptides were separated by reverse phase liquid chromatography using an Accucore C18 column (100 x 2.1 mm, 2.6 µm particle size, Thermo Fisher Scientific). For all experiments, the solvents used were water with 0.1 % FA (A) and acetonitrile with 0.1% FA (B). Gradient was performed at 300 µL/min, 5-35 % in 50 minutes.

Mass Spectrometry

Peptides, eluted from reverse phase liquid chromatography, were directly analyzed using a Thermo Scientific™ Orbitrap Fusion™ Tribrid™ Mas Spectrometer. Full MS was acquired at 120,000 resolution with mass range of *m/z* 350-2,000 followed by data dependent MS/MS spectra . Tandem spectra acquired include alternating HCD and ETD spectra. HCD

spectra were collected with precursor ions with charge states from +2 to +8. ETD spectra were only collected with precursor ions with charge states from +3 to+8. Thermo Scientific™ IonMax™ source with the heated electrospray ionization (HESI) was utilized with source parameters as sheath gas at 40, aux gas at 10, sweep gas at 1, vaporizer temperature at 350 ºC, ion transferring tube temperature at 275 ºC and spray voltage at 3,500 v. The digests were analyzed using electron transfer dissociation (ETD) and high energy collision dissociation (HCD) fragmentation.

Data Analysis

The LC-MS data were processed using PepFinder software1.0. The MS spectra were analyzed with this novel peptide mapping software for sequence coverage information and identification of modifications. The software looked for both expected and unexpected modifications. The peptide identification settings include absolute MS signal threshold at 2e4, maximum peptide mass at 10,000, N-glycosylation search within CHO N-glycan, maximum numbers of modification/peptide at 1, mass changes for unspecified modifications at -58 to 162.

Results

Protein Sequence Coverage

Rituximab is an IgG1 class chimeric monoclonal antibody, consisting of two light chains with 213 amino acids and two heavy chains [2]. The antibody is glycosylated at residue Asn301 of each heavy chain. Its attached glycans diverse in composition and length. The variety and relative abundance of the glycoform is essential as part of the antibody characterization. Additionally, mAbs are particularly sensitive to environmental factors such as temperature changes, oxidation, light, ionic content, and shear. Thus, besides complete protein sequence coverage, comprehensive characterizations, which accurately detect these degradation changes during storage, are in great need.

In this study, LC-MS data was acquired and then interpreted by PepFinder software 1.0. As shown in Figure 1, 100 % sequence coverage was achieved for light chain from day light stressed rituximab. Both full MS precursor mass and MS/MS spectra were utilized for matching of the sequence. Color coded blocks beneath amino acid sequences shows the signal intensity of each peptide. The numbers included in the color coded block represent the retention time for each peptide. In the same analysis, 100 % sequence coverage was also obtained for heavy chain from the day light stressed rituximab as shown in Figure 2. A complete protein coverage serves as a foundation for surveying degradation of the whole protein.

Protein Modifications

A list of modification sites of mAb were identified with high confidence MS/MS spectra match and listed in Table 1. To determine the modification sites, each residue on the peptide is theoretically modified, the MS/MS spectrum is predicted, and compared to the experimental spectrum as shown in Figure 3,4,5.

FIGURE 1. Light chain sequence coverage of light stressed rituximab. Color code is for the signal intensity of each peptide. Numbers under the sequences represent retention time of the peptides.

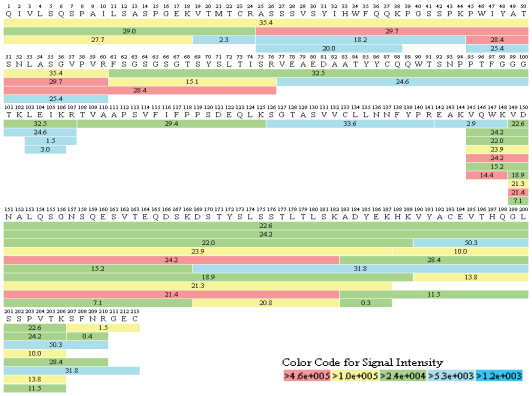


FIGURE 2. Heavy chain sequence coverage of light stressed rituximab.

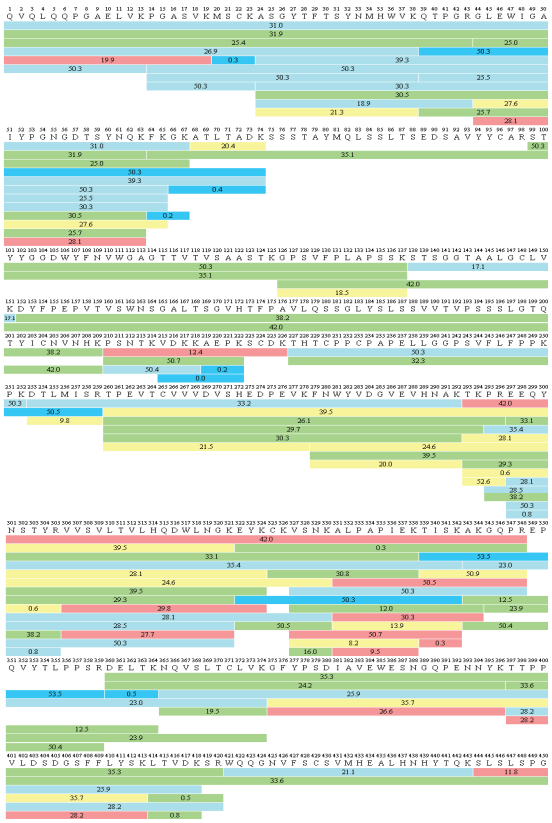


TABLE 1. Modification summary for the residues in heavy chain from light stressed rituximab. All sequence were confirmed by tandem spectra. An ~ sign is labeled in front of the modification site to indicate the approximate location of the modification.

Modification on Heavy Chain	Abundance
~Q1+NH3 loss	100.00%
~N55+Deamidation	9.22%
~N55+NH3 loss	5.05%
M256+Oxidation	6.36%
~C265+57.0220	71.78%
~D284+H2O loss	13.45%
N301+A2G0F	46.54%
N301+A2G1F	54.60%
N301+A2G2F	9.32%
~W317+37.9417	2.66%
N319+Deamidation	5.37%
~N319+NH3 loss	4.33%
~K321+37.9419	4.59%
N329+Deamidation	2.67%
K330+Glycation	1.73%
~A331+70.0422	6.77%
~I340+70.0424	26.36%
~L372+57.0249	96.32%
~S387+37.9458	10.70%
~N388+Deamidation	1.34%
~N388+NH3 loss	5.27%
~S407+H2O loss	2.33%
~H437+57.0342	89.37%

match as shown in Figure 3,4,5. Figure 3 presented both predicted and Fusion ion trap acquired ETD spectrum of T293-R305 (N301+A2G1F) from the heavy chain. The two spectra of the glycopeptide are well aligned for both fragment ion masses and relative intensities. Following are Figure 4 and 5, which show pairs of spectra (predicted versus experimental) from a deamidated peptide (ETD) and a oxidized peptide (HCD), respectively. Correlations between both *m/z* and their relative intensities provide high confidence identification for these modified peptides. Additionally, complementary fragmentation techniques help deliver unambiguous and comprehensive characterization, especially localization of labile modifications such as glycosylation. The relative quantitation of the modifications were based on the peak areas of modified and unmodified forms of the peptide as the equation below. The abundance as shown in Table 1 represents relative area % of the modifications.

$$\text{Relative area \% of modification} = \frac{\text{Area of modified peptide}}{\text{Area sum of all related peptides (native + all modified forms)}} \times 100$$

FIGURE 3. Predicted and experimental ETD spectra of T293-R305 (N301+A2G1F) from heavy chain. Top spectrum is the PepFinder predicted spectrum; bottom spectrum is Fusion acquired ETD spectrum.

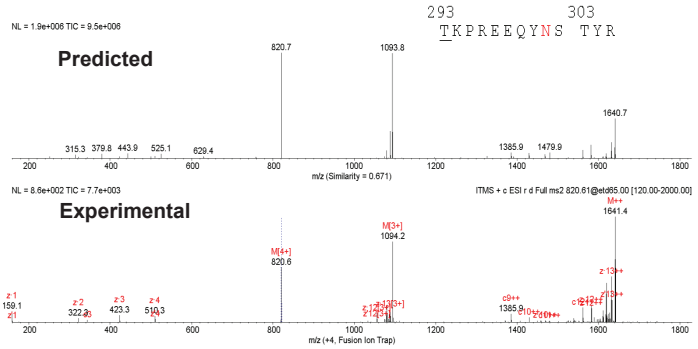


FIGURE 4. Predicted and experimental ETD spectra of V327-K338 (N329+Deamidation) from heavy chain.

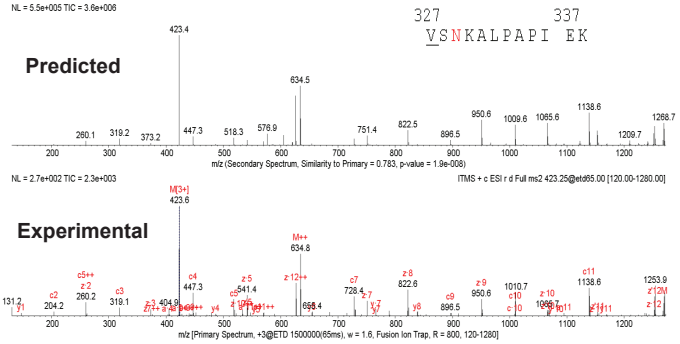
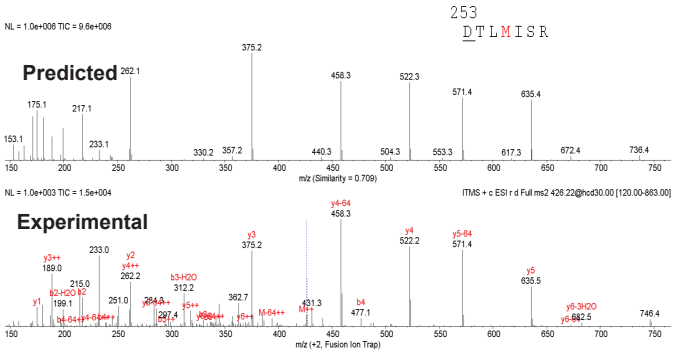


FIGURE 5. Predicted and experimental HCD spectra of D253-R259 (M256+Oxidation) from heavy chain.

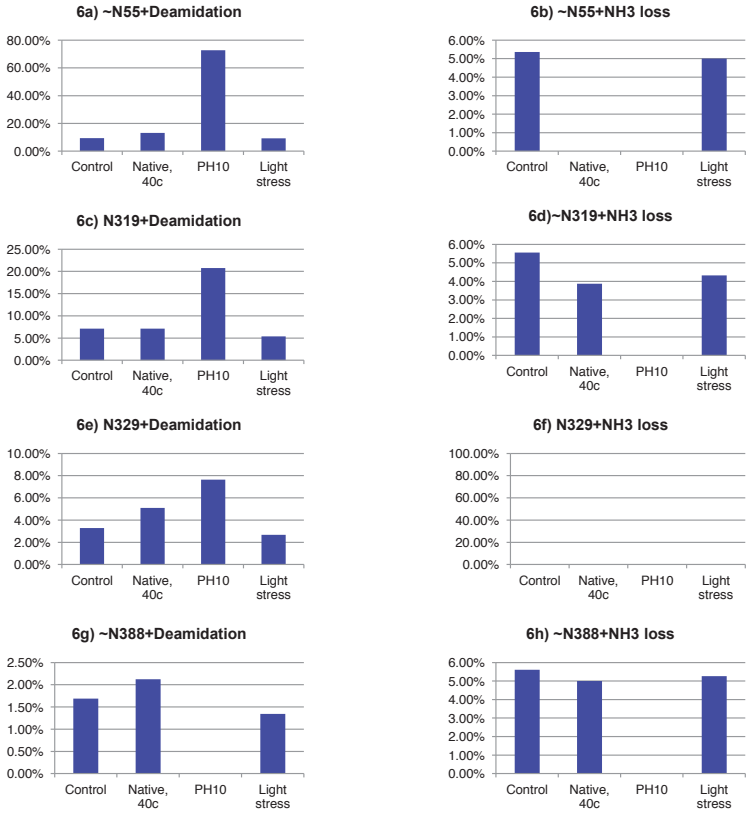


Artificial modifications happen during sample preparation prior to LC-MS analysis. Unstressed sample is thus included as a control. The control is rituximab stored at 4 °C without any stress treatment. It was digested following the same protocol as stressed samples. Modifications levels on the stressed samples are compared to the control. As shown in TABLE 2, oxidation levels were increased from less than 2 % to 72-100 % after treatment with H_2O_2. Figure 6 also shows the variation of deamidation and succinimide intermediate levels among the stressed samples. For example, asparagine (N) 55 was increased to over 70 % abundance at pH 10 compared to other samples at around 10 %. Whereas Succinimide levels of N329 remained below 6 % for all the samples.

TABLE 2. Comparison of oxidation level of methionines from heavy chain between oxidative stressed sample and the control. ND denotes not detected.

Oxidative stress	Abundance	Control	Abundance
~M20+Oxidation	100.00%	~M20+Oxidation	ND
M34+Oxidation	72.82%	M34+Oxidation	ND
M256+Oxidation	100.00%	M256+Oxidation	1.89%

FIGURE 6. Deamidated and succinimide asparagine profiling for native 40 °C, PH10 40 °C, light stress samples versus control. Column charts 6a-6h show abundance levels of deamidated and succimide asparagine of N55, N319, N329 and N388 respectively.



Conclusion

Results from this study show that mAb degradation can be comprehensively characterized using the high resolution/accurate mass spectrometer with complementary fragmentation techniques (HCD, ETD) and PepFinder software as the new generation peptide mapping software.

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Disulfide Bond Analysis on Orbitrap Mass Spectrometry

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Overview

Purpose: To develop a disulfide bond analysis method on Orbitrap Elite.

Methods: The non-reduced peptide samples were analyzed on LC-MS/MS tandem system, using a data dependent top 12 method.

Results: We have found 15 disulfide bonds in our mAb digested samples, all with MS/MS fragment information.

Introduction

Disulfide bonds are one of the most important post-translational modifications of proteins. They are stabilizing the protein's 3D structure and are crucial for their biological function. For biopharmaceutical industry, disulfide bond match and mismatch are highly concerned.

Monoclonal antibodies (mAbs) are one of the fastest growing classes of pharmaceutical products. They play a major role in the treatment of a variety of conditions such as cancer, infectious diseases, allergies, inflammation, and auto-immune diseases. Because mAbs can exhibit significant heterogeneity, extensive analytical characterization is required to obtain approval for a new mAb as a therapeutic product. Mass spectrometry has become an essential tool in the characterization of mAbs.

In this study, the Thermo Scientific™ Orbitrap Elite™ Hybrid Ion Trap-Orbitrap Mass Spectrometer, was evaluated for disulfide bond studies. We analyzed the disulfide bonds in Rituximab, which is known under the trade names Rituxan® (Biogen Idec/Genentech) in the United States and MabThera® (Roche) in Europe, is a recombinantly produced, monoclonal chimeric antibody against the protein CD20. It was one of the first new generation drugs in cancer immune therapy. Rituximab was approved by the U.S. Food and Drug Administration in 1997 and by the European Commission in 1998 for cancer therapy of malignant lymphomas. The variable domain of the antibody targets the cell surface molecule CD20, that can be found in some non-Hodgkin lymphomas. In this study we found 15 of the total 16 disulfide bonds in Rituximab. The Orbitrap Elite MS is well suited for disulfide bond analysis, produces reproducible and high confident mass information for disulfide bond analysis.

Methods

Sample Preparation

The Rituximab sample was aliquoted and digested in two different ways; one half was digested by trypsin without reduction and the other half was digested by chymotrypsin and trypsin together, without reduction.

Liquid Chromatography

The peptide samples were separated on a Thermo Scientific™ Accela HPLC system and the gradient is described in the following table(A: 0.1% formic acid in water,B: 0.1% formic acid in acetonitrile).

TABLE 1. LC gradient used for experiments.

Time	A%	B%	µl/min
0	95	5	300
3	95	5	300
45	78	22	300
53	70	30	300
58	10	90	300
63	10	90	300
64	95	5	300
70	95	5	300

Mass Spectrometry

Source settings: Spray voltage of 3800 V and capillary temperature of 275 °C.

Full MS settings: Resolution=60,000; Maximum IT=200 ms; scan range: 300 to 2000 m/z;

dd-MS² settings: Activation type: CID; Isolation width: 2.00; TopN=12; NCE= 35.0

Data Analysis

The data was analyzed by StavroX™ software (Michael Goetze University of Halle-wittenberg).

Results

Overview

Rituximab is a recombinantly produced, monoclonal chimeric antibody against the protein CD20, which has 16 disulfide bonds totally. FIGURE 1 shows the sequence and disulfide bonds distribution of this mAb.

FIGURE 1. Amino acid sequence of heavy chain (top) and light chain (bottom) Rituximab. The disulfide bonds (blue: intrachain linkage; red: interchain linkage between light chain and heavy chain; green: interchain linkage between different heavy chains) and glycosylation site on heavy chain (Asn residue underlined and bolded) are also labeled.

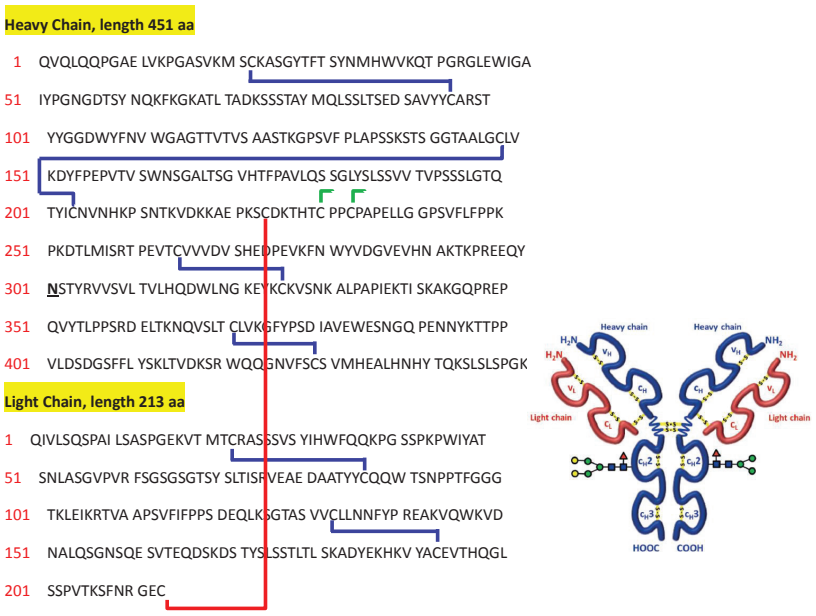


FIGURE 2 shows the chromatogram of the trypsin-only digested and chymotrypsin-trypsin double digested samples, and FIGURE 3 shows the sequence coverage of trypsin-only digested and chymotrypsin-trypsin double digested samples, using the software Proteome Discoverer 1.4. It can be found that both of the coverage rates are nearly 100%, suggested the digestions went to completion.

FIGURE 2. the chromatogram of the two samples.

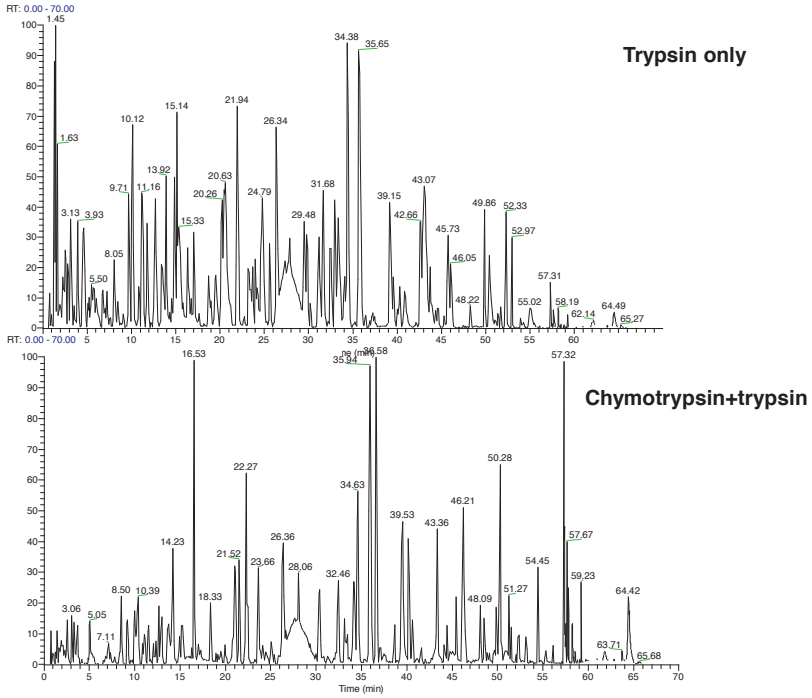
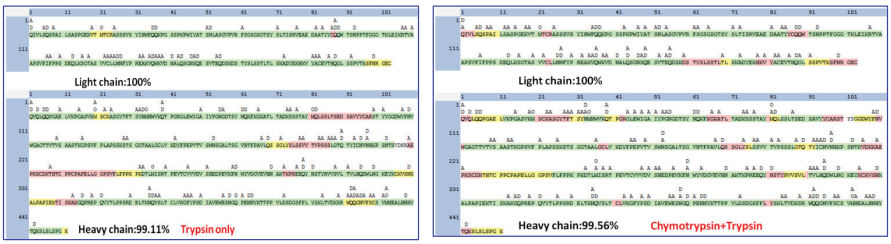


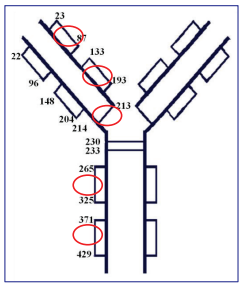
FIGURE 3. the sequence converge map of mAb digested in two different ways.



The result of trypsin-only digested sample

Using StavroX™, we have identified 10 disulfide bonds in the trypsin-only digested Rituximab. FIGURE 4 shows the summary of all identified disulfide bonds.

FIGURE 4. All of the identified disulfide linkage in trypsin-only digested Rituximab.



peptide(1)	protein(1)	from(1)	to(1)	peptide(2)	protein(2)	from(2)	to(2)
[VTMTCR]	>Light	19	24	[VEAEDAATYYCQQWTSNPPTFGGGTK]	>Light	77	102
[SGTASVVCLLNNFYPR]	>Light	126	141	[VYACEVTHQGLSSPVTK]	>Light	190	206
[SCDK]	>Heavy	223	226	[SFNRGEC]	>Light	207	213
[EYKCK]	>Heavy	322	326	[TPEVTCVVVDVSHEDPEVK]	>Heavy	260	278
[NQVSLTCLVK]	>Heavy	365	374	[WQQGNVFSCSVMHEALHNHYTQK]	>Heavy	421	443

FIGURE 5. The annotated MS/MS spectrum of disulfide bond C265-C325 on heavy chain (trypsin digested only).

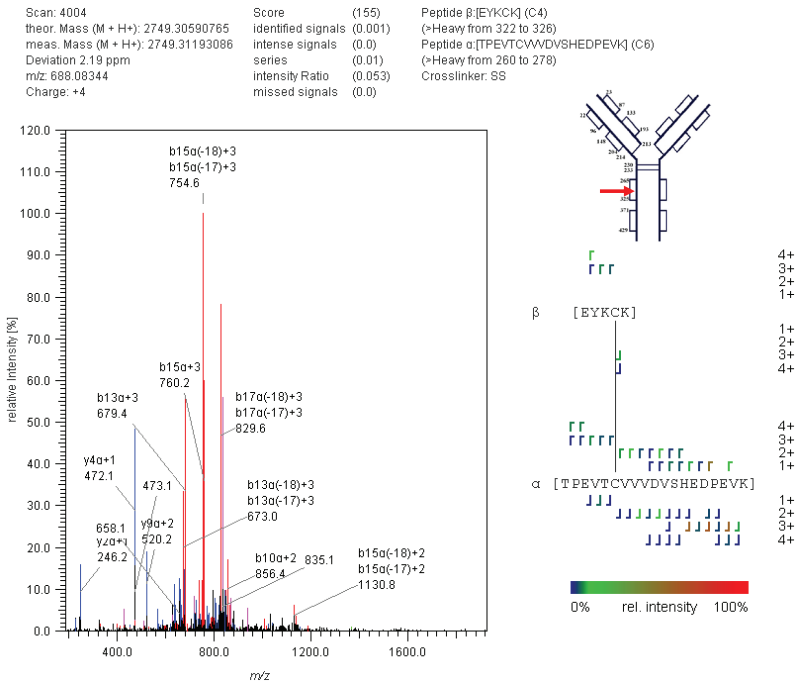


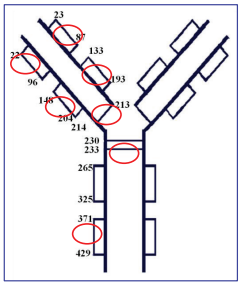
FIGURE 5 is an annotated MS/MS spectrum of disulfide linkage between Cys265 and Cys325 on heavy chain. The accuracy of precursor ion is high (2.19ppm), which indicated that an Orbitrap MS can produce high mass accurate data. It's also easily to find that many continuous, disulfide linkage-included fragment ions were identified. These ions are strong evidence which suggest the existence of the disulfide bond.

The result of double digested sample

In our experiments, we also tried double digestion, to produce more disulfide linkage-included peptides which were suitable for mass spectrometer detection. Chymotrypsin and trypsin were used for double digestion, which can get nearly 100% sequence coverage of the sample (FIGURE 3).

By using the software StavroX™, we have identified 13 disulfide bonds in the double digested Rituximab. FIGURE 6 shows the summary of all identified disulfide bonds in double digested sample.

FIGURE 6. All of the identified disulfide linkage in double digested Rituximab.



peptide(1)	protein(1)	from(1)	to(1)	peptide(2)	protein(2)	from(2)	to(2)
[VTMTCR]	>Light	19	24	[YCQQW]	>Light	86	90
[SGTASVVCL]	>Light	126	134	[ACEVTHQGL]	>Light	192	200
[AEPKSCDK]	>Heavy	219	226	[SSPVTKSFNRGEC]	>Light	201	214
[MSCK]	>Heavy	20	23	[YCAR]	>Heavy	95	98
[GCLVK]	>Heavy	147	151	[ICNVNHKPSNTK]	>Heavy	203	214
[THTCPPCPAPEL]	>Heavy	227	238	[SCDKTHTCPPCPAPELLGGPSVF]	>Heavy	223	245
[TCLVK]	>Heavy	370	374	[SCSVmHEAL]	>Heavy	428	436

FIGURE 7. The annotated MS/MS spectrum of disulfide bond C133-C193 on light chain (double digestion).

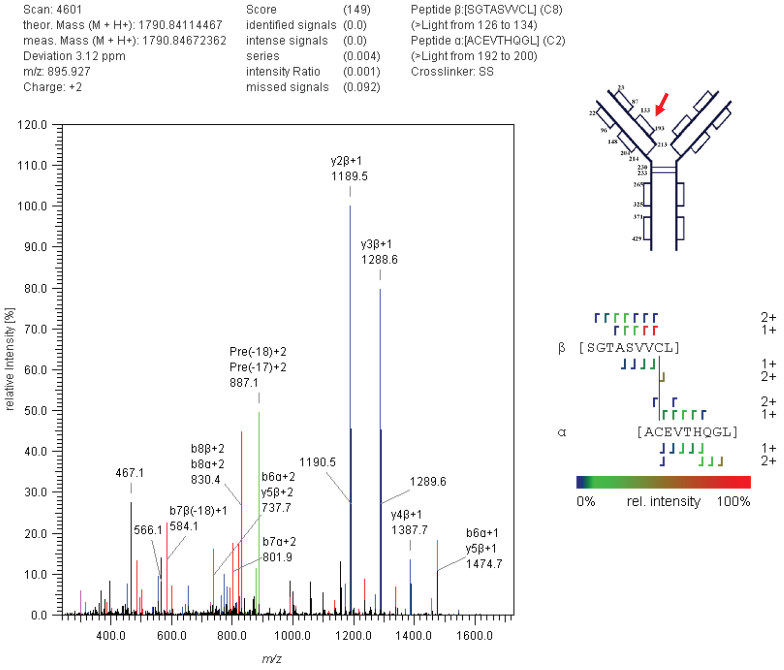
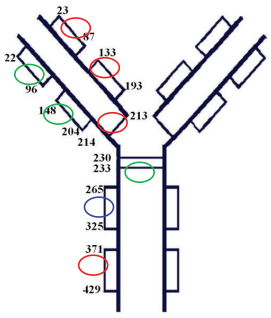


FIGURE 7 shows the disulfide bond between Cys133 and Cys193 on light chain. In this double digestion experiment, which employs chymotrypsin and trypsin at the same time. Because the disulfide bond-included peptides are usually very large, to get more shorter peptides, double digestion is a good choice.

In double digestion result, we can find that the intrachain linkage on the Fab region of heavy chain and one of the interchain between heavy chains are identified, which weren't identified in trypsin only experiment. It is worth noting that the C265-C325 linkage on heavy chain was found in trypsin only experiment, but not identified in double digestion condition; this can be explain that the peptide which contains this linkage was cut into some very short parts——only one or two amino acids, which was beyond the detection line of mass spectrometer.

By combination of these two results, 15 out of 16 disulfide bonds on Rituximab were identified in our experiment successfully.

FIGURE 8. All of the identified disulfide linkage of Rituximab. Red, the disulfide bond can be found in both experiments. Blue , the disulfide bond can just be found in trypsin-only digestion. Green, Blue , the disulfide bond can be found only in double digestion.



Conclusion

- Two different digestion ways were used in this study: one employed trypsin only while the other used the combination of chymotrypsin and trypsin.
- 10 out of 16 disulfide bonds were identified in trypsin-only digestion experiment and 13 out of 16 were found in double digestion way, and the results were complementary.
- Totally, 15 out of 16 disulfide bonds were identified and all of them have confident MS/MS information.
- Biopharmaceutical industry-related disulfide bonds analysis were performed on Orbitrap Elite, with reproducible and high confident mass information.

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Rapid Peptide Mapping via Automated Integration of On-line Digestion, Separation and Mass Spectrometry for the Analysis of Therapeutic Proteins

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Overview

Purpose: An automated system was developed that integrated rapid trypsin digestion without pretreatment, on-line desalting and high resolution LC/MS/MS.

Methods: A rapid sample preparation and separation system equipped trypsin column was used for fast trypsin digestion of reduced as well as native proteins. This sample preparation instrument was coupled to a hybrid quadrupole-Orbitrap mass spectrometer for peptide mapping experiments and the resulting data sets were analyzed.

Results: For the reduced and alkylated antibody sample, a 100% sequence coverage of the light chain and 91% sequence coverage of the heavy chain were observed. For the untreated/native antibody sample, 90% sequence coverage of the light chain and 73% sequence coverage of the heavy chain were observed. Furthermore, a digest of native serum albumin resulted in 51% sequence coverage. The low coverage percentage value associated with the untreated/native protein analytes can be attributed to disulfide bonded peptides that were likely recovered from the analytical system but not recognized by the proteomics software.

Introduction

Biological systems are extraordinarily dynamic. As such, it is often the case that the time associated with protein sample preparation and analysis delays the detection of malformations until it is too late to take action. Furthermore, a lack of hands-free automation puts an enormous strain on analysts to replicate results across multiple labs. Presented here, is the automated integration of rapid sample preparation, separation and MS used for peptide mapping of a reductively alkylated monoclonal antibody, untreated (native) antibody and untreated human serum albumin (1, 2).

Methods

Samples

To reduce the intact mAb, the sample was incubated for 1 hour at 60 C in 6 M guanidine HCl containing 25 mM DTT for complete reduction. Following reduction the samples was reacted with 100 mM iodoacetic acid for 1 hour at room temperature. Native samples were simply diluted to their final concentration in tris-buffered saline pH 7.4 prior to injection. Peptide mapping of all samples was performed using a modified Dionex ultimate 3000 RSLC nano system system equipped with a Perfinity No Reduction or Alkylation (NORA) Trypsin Column, Digest Buffers from Perfinity, a C18 trap column (Halo, 0.3 X 20 mm) and C18 analytical column (Halo 0.3 X 100 mm).

Online Trypsin Digestion

Untreated antibody solutions were directly injected into the system. Digest efficiency was monitored at various times and temperatures. Simultaneous denaturation and enzymatic digestion were performed at 70 C. Reductively alkylated samples were processed at 50 C. 2 minute digestion times were utilized for all samples.

Chromatography

A Dionex Ultimate 3000 RSLC nano system equipped with a microflow flow selector pumping at 20-50 uL/min was used for the desalting and reversed phase operations. Following digestion, the trapping column was brought in-line with the analytical column by valve switching. Both columns were desalted for 3 column volumes using initial gradient conditions. For peptide mapping experiments, peptides were eluted with a 50 min gradient at a flow rate of 20 µL/min. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). LC gradient was 0-50%B in 50 min.

Figure 1 shows the schematics of the LC configuration used for automated peptide mapping. All transfer tubing was 75 µm Thermo Scientific™ Dionex™ nanoViper™ fingertight fitting system. All valves and columns were operated under isothermal condition in a column oven. An isocratic pumping system was utilized for all sample preparation steps while binary pumps were used for the reversed phase gradient. This configuration enables the digestion of a sample while another sample is separated by reversed phase. By parallel processing in this way a sample can be run every 7 minutes.

Mass Spectrometry

Peptides eluted from analytical column were analyzed using a data-dependent top 10 experiment on the Thermo Scientific™ Q Exactive™ hybrid quadrupole-Orbitrap mass spectrometer (Figure 2). Resolution was 70K for the full MS, 17.5K for HCD MS/MS with a dynamic exclusion of 30 seconds. Detailed instrument parameters are listed in Table 1.

Thermo Scientific™ Proteome Discoverer™ Software revision 1.3 was used to search the protein database with the MASCOT™ search engine for all database searches. The disulfide linked peptides were identified using StavroX (1). Raw files generated by the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer were searched directly using a 10 ppm precursor mass tolerance and a 20 amu fragment mass tolerance.

FIGURE 1. Schematics of the LC configuration for automated peptide mapping.

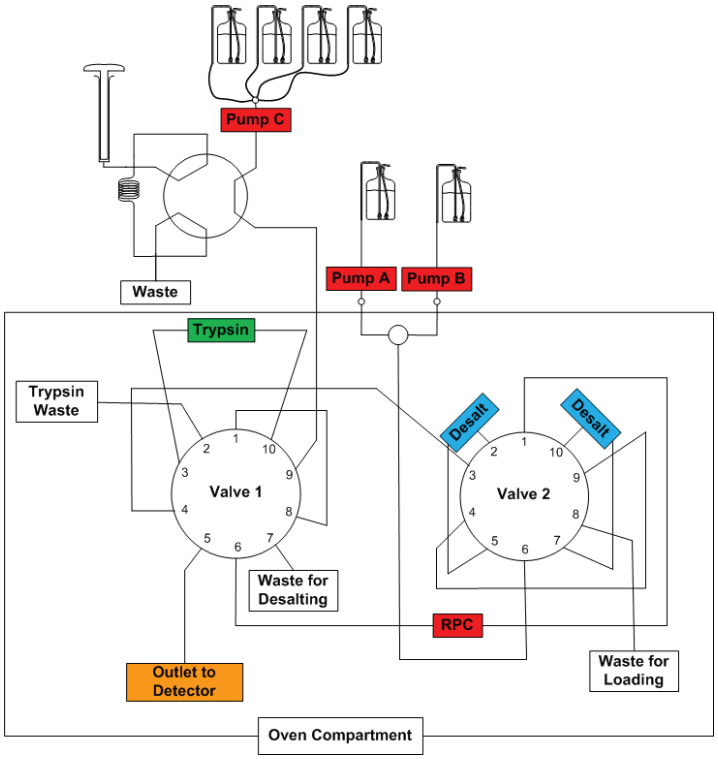


Figure 2. Schematic of the Q Exactive hybrid quadrupole-Orbitrap mass spectrometer.

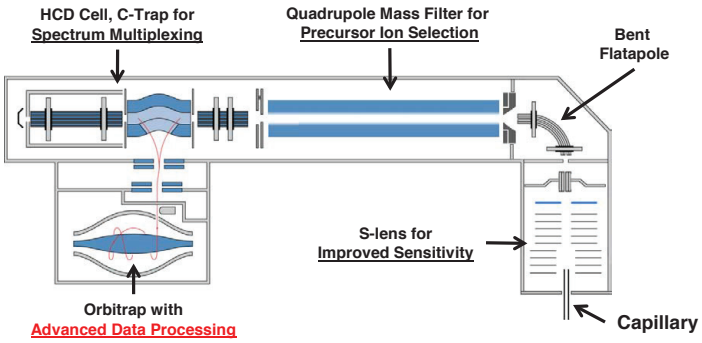


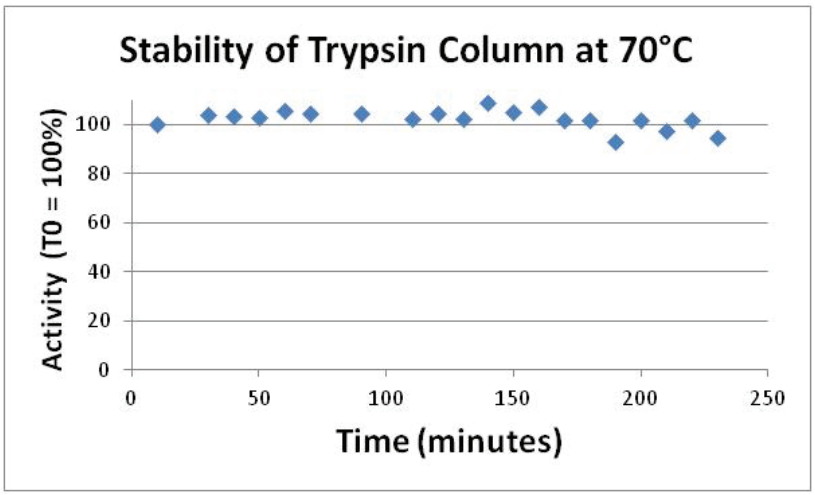
Table 1. The Q Exactive hybrid quadrupole-Orbitrap mass spectrometer instrument method parameters

Parameters	Settings
Full MS scan range	400-2000
MS/MS fixed first mass	100
AGC	1e6, full MS 5e4, MS/MS
Max injection time (ms)	2, full MS 60, MS/MS
Isolation width (m/z)	2.0
NCE	27
Under fill ratio	1%
Dynamic Exclusion	30 sec

Results

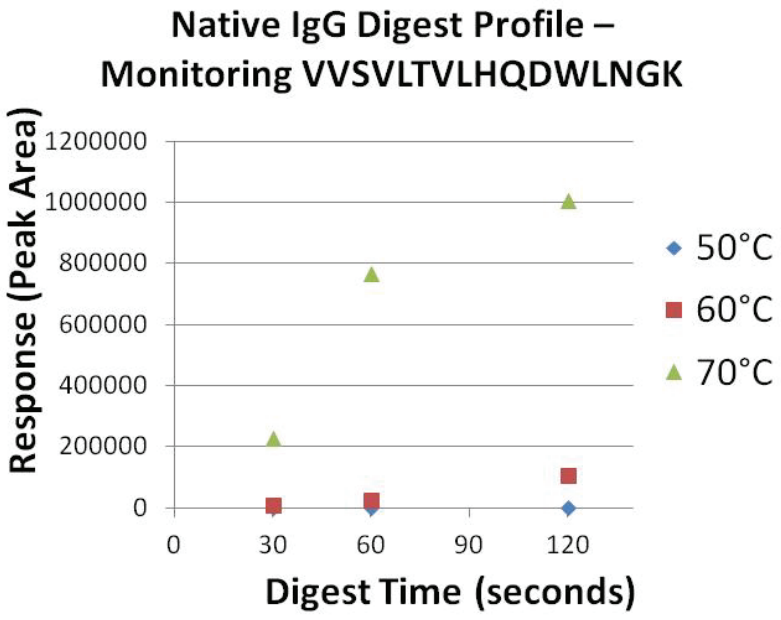
Historically, Kumakura et. Al (2), showed that in the best-case scenario trypsin that was simply immobilized exhibited a 60% reduction of the activity 30 minutes at 70 C. Shown in Figure 3, the proprietary stabilization process of the enzymes three dimensional structure enables prolonged operation under these harsh conditions.

FIGURE 3. Data suggests that a trypsin column was developed capable of operation over prolonged periods at elevated temperatures.



As shown in Figure 4, when the digestions of native antibody samples were performed at various times and temperatures, a dramatic increase in efficiency was observed at 70 C. These results suggest that under these conditions the samples were simultaneously denatured and enzymatically digested. This observation validates results obtained by Vermeer et. al. that showed that the denaturation of antibody variable regions occurs at 60 C and constant region at 70 C (3).

FIGURE 4: Native mAb digested at various times and temperatures.

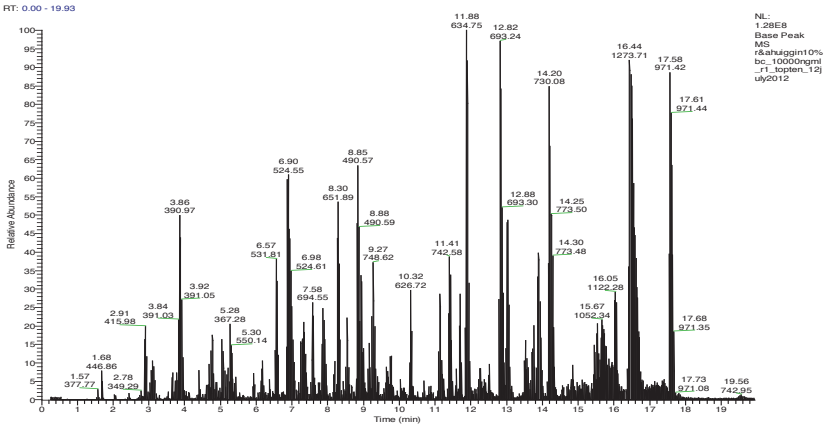


As shown in Table 2, digestion of reduced and alkylated mAb samples yielded sequences coverage exceeding 90%. Even for the samples of digested without pretreatment sequence coverage was 79% for heavy chain and 97% for light chain. The base peak chromatograms obtained for native antibody digestion is shown in Figure 5.

Table 2. Coverage determinations of mAb digests.

Sample	Light Chain	Heavy chain
Reduced and Alkylated	100%	91%
Native	97%	79%

FIGURE 5: Base peak chromatogram of native mAb digestion without reduction and alkylation.



This online digestion experiment was also applied to native human serum albumin. The extensive disulfide linkages in this molecule makes it an especially challenging case for native digestion. Figure 6 shows the coverage determinations of native HSA digests. Figure 7 shows the base peak chromatogram of native HSA digestion without reduction and alkylation. The identification of a large number of canonical peptides suggests native human serum albumin (HSA) was effectively digested. Given the highly bridged nature of HSA this data suggests that many different types of less stable proteins and antibodies can effectively be digested under these conditions.

Since the total digest time for this work was <5 minutes it is possible that this workflow would be extremely useful in situations where close to real time monitoring would be advantageous such as process monitoring. Because reduction and alkylation are not necessary, it is possible that this technology could also be successfully applied to disulfide bond mapping.

TABLE 6. Coverage determinations of native HSA digests.

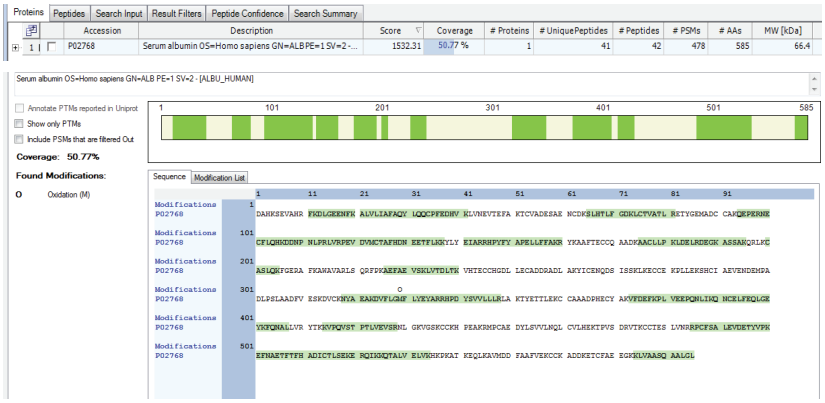
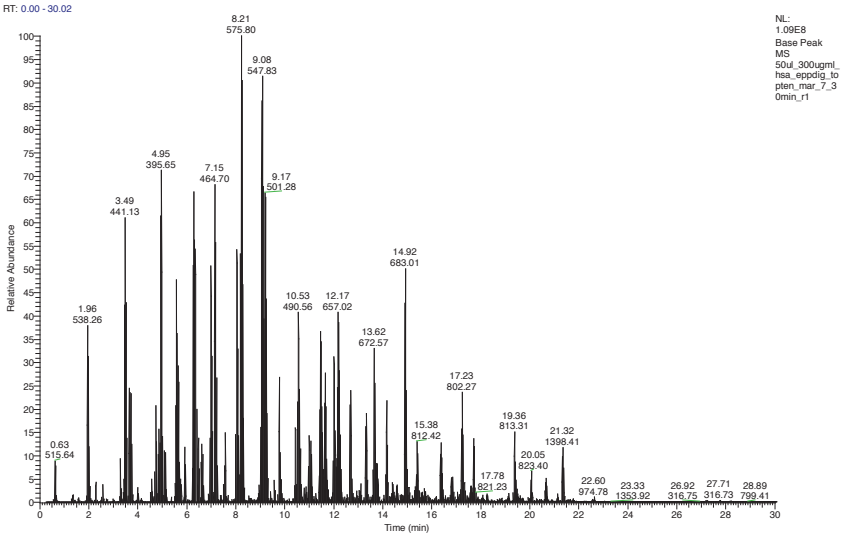


FIGURE 7: Base peak chromatogram of native HSA digestion without reduction and alkylation.



Conclusion

- An automated workflow was developed for protein therapeutics peptide mapping which combined online trypsin digestion and high resolution accurate mass MS.
- A trypsin column was developed capable of operation over prolonged periods at elevated temperatures.
- Operation under denaturing conditions enabled rapid digestion without pretreatment.
- Direct coupling of this system to the Q Exactive Orbitrap hybrid quadrupole-Orbitrap mass spectrometer provided confident amino acid sequence information for the monoclonal antibody.
- A confident peptide mapping experiment including online trypsin digestion and Orbitrap LCMS/MS analysis was achieved within a hour.
- Potential future applications include use of this set-up in fast diagnostics, process monitoring and disulfide bond mapping.

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Differentiate Minor Difference of Protein Structure in Biosimilar and Reference Products Using High-Resolution Orbitrap LC-MS/MS

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Overview

Purpose: To develop a high-resolution LC/MS workflow for the analysis of the protein structure differences between biosimilar and reference products using a Thermo Scientific™ Orbitrap™ bench-top hybrid quadrupole-Orbitrap mass spectrometer.

Methods: A top-ten data-dependent high-energy collision dissociation (HCD) method was performed to analyze the samples using a bench-top mass spectrometer. Data was analyzed using a new software that is under development.

Results: An LC-MS/MS workflow was developed for differentiating minor differences of protein structure between biosimilar and reference products using a benchtop Orbitrap LC-MS/MS and a new software that is under development. This workflow provides qualitative and quantitative biosimilar to reference product comparison.

Introduction

Biosimilar products are required by regulatory authorities to have appropriate and comparable quality, safety and efficacy with a reference biologic product. Mass spectrometry can offer in-depth characterization to explore the similarity and difference between a candidate biosimilar and a reference biologic. In this study, we developed a robust approach for comparability study of biosimilar and reference products. Any minor difference in sequence modification and glycosylation can be well characterized and compared by using combination of high resolution Orbitrap LC-MS/MS with a powerful software to systematically interpret the results.

In this study, tissue plasminogen activator (TPA) and a generic variant of TPA (TNK) are well characterized by the robust approach. In addition, two TNK forms (G-TNK as a reference product and I-TNK as a biosimilar form) are also compared to explore the similarity and difference.

Methods

Samples

Three samples, TPA, I-TNK, G-TNK, were digested using trypsin after reduction and alkylation. Tenectelplase (TNK) is a recombinant TPA with the following minor sequence changes:
T103 -> N (Becomes N-glycosylation site)
N117 -> Q (Removes N-glycosylation site)
KHRR (296-299) -> AAAA

Liquid chromatography

Peptides were separated using Thermo Scientific™ EASY-Spray™ technology containing a 50-cm C_{18} column (2 µ particle size) and a Thermo Scientific™ EASY-nLC™ LC. LC solvents are 0.1% formic acid in H_2O (Solvent A) and 0.1% formic acid in acetonitrile (Solvent B). Flow rate was 250 µL/min. A 60 min gradient was used to elute peptides from the column.

Mass spectrometry

A top-ten data-dependent high-energy collision dissociation (HCD) method was performed using a Thermo Scientific™ Q Exactive™ MS system to analyze the samples. The following MS and MS/MS settings were used: MS scan range 100-2000 *m/z*. FT-MS was acquired at 70,000 resolution at *m/z* 200 with AGC target of $1x10^6$. MS2 was acquired at 17,500 resolution at *m/z* 200 with AGC target of $2x10^5$. The spray voltage was 1.8kV. Capillary temperature was 275 °C. S-lens level was set at 55.

Data analysis

Data was analyzed using a new software that is under development. This software provides automated analyses of liquid chromatography/tandem mass spectrometry (LC-MS/MS) data for large-scale identification and quantification of known and unknown modifications. Peptide identification is achieved by comparing the experimental fragmentation spectrum to the predicted spectrum of each native or modified peptide. Peak areas of related peptide ions under their selected-ion chromatograms (SIC) are used for relative quantification of modified peptides.

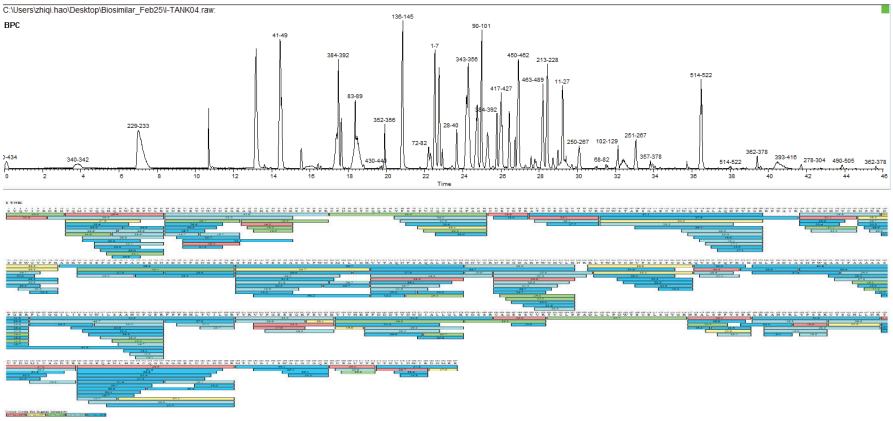
Results

Nine LC-MS/MS data files, three repeat runs for each of the samples: TPA, I-TANK and G-TANK, were analyzed and the results were compared.

1. Peptide identification and protein sequence coverage

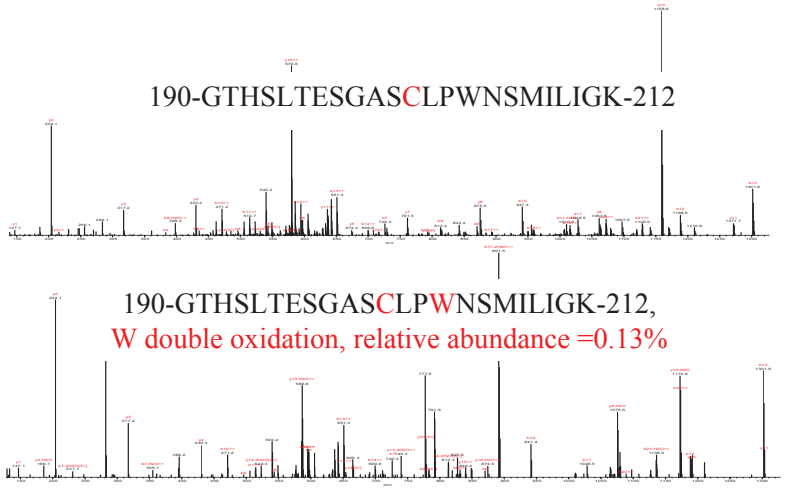
The top ten data-dependent acquisition using the Q Exactive MS produced high quality, high-resolution and accurate mass MS/MS spectra which yielded high rates of identification. For each raw file, 40% to 50% of the MS/MS spectra resulted in high confidence peptide identification (data not shown). 100% protein sequence coverage was achieved for each of the nine data files. Figure 1 shows an example of the peptide map and sequence coverage view for one of the data file.

Figure 1. Peptide map (top) and sequence coverage (bottom) of I-TNK.



The Q Exactive MS provides very high throughput and sensitivity. More than 5 orders of magnitude of abundances of identified peptides was routinely achieved (data not shown), which ensures confident identification of low abundance modifications, non specific cleavage versions as well as sequence variants. Figure 2 shows the high quality MS/MS spectra of a peptide (top) and its double oxidized (on W) version (bottom) which is of 0.1% in abundance.

Figure 2. MS/MS spectra of a peptide (top) and its double oxidized version (bottom) which is of 0.13% in abundance.



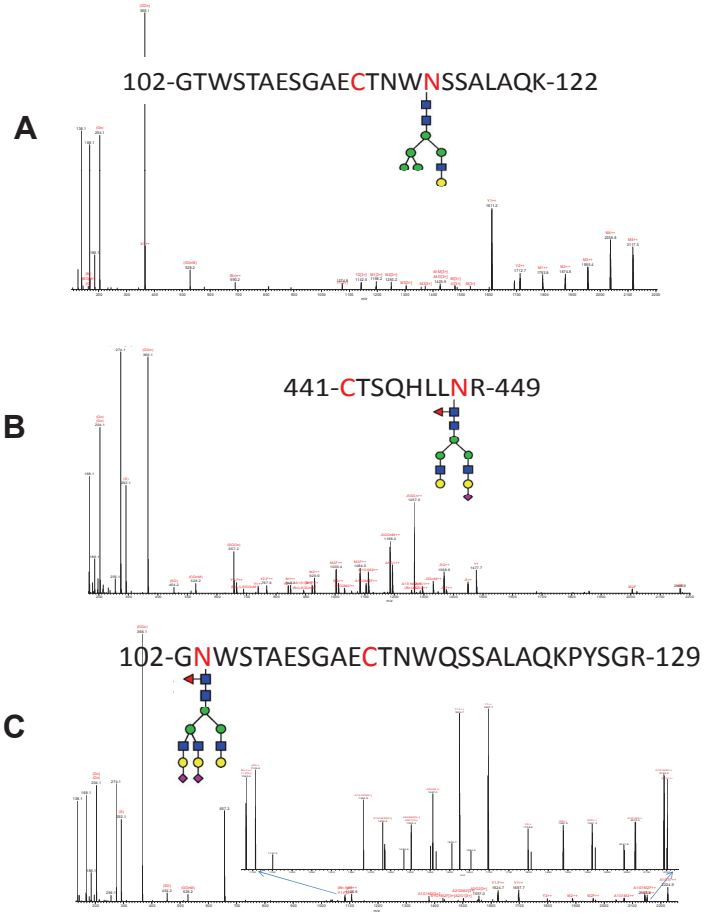
2. Glycosylation of TPA, I-TNK and G-TNK

A total of four glycosylation sites were identified, among which three of them are over 99% glycosylated. They are N 448 in all of the three samples, N103 in I-TNK and G-TNK, and N117 in TPA. The forth glycosylation site, N184, was identified only in I-TNK and only 19% of this site is glycosylated (Table 1). I-TNK has an additional glycosylation site (N184) compared to G-TNK even though these two proteins share the same amino acid sequence, suggesting differences in the manufacturing procedure. Examples of MS/MS spectra of three identified glycopeptides are shown in Figure 3.

Table 1. Identified glycosylation sites, percentage of glycosylation and the number of glycoforms identified with high confidence.

Site of glycosylation	Sample	# glycoforms	% glycosylation
N 103	I-TNK	18	>99
N 103	G-TNK	11	>99
N117	TPA	14	>99
N 184	I-TNK	12	19
N 448	TPA	44	>99
N 448	I-TNK	36	>99
N 448	G-TNK	47	>99

Figure 3. Examples of HCD spectra of identified glycopeptides. A: glycosylation on N117. B: glycosylation on N448. C: glycosylation on N103.



The type of glycosylation forms and their relative abundance in the three samples were compared and the following were observed :

1. Glycosylation forms on N448 and their relative abundance are consistent among all the three samples (Table 2A). Most of glycans on this site contain sialic acid.
2. Glycoforms on N103 are similar between I-TNK and G-TNK, while the relative abundance profile is quite different. Although the most abundant form, A2S1G1F, is the same in the two samples, the second and the third most abundant forms are different. For the top five most abundant forms, only two of them were shared in the two samples(Table 2B).
3. Glycans on N117 are of the type of high mannose, which is completely different from the glycans identified on other sites (Table 2C).
4. Glycosylation on N184 was only identified in I-TNK (Table 2D) and all of the glycans contain sialic acid.

Table 2. Comparison of glycoforms in the three samples. Only those with relative abundance higher than 1% in at least one of the samples are included. Abbreviations for glycan structure (1) : Antenna A, core fucose (Fuc) F, mannose (Man) M, galactose (Gal) G, *N-acetyl neuraminic acid (NANA) S, N-glycolyl neuraminc acid (NGNA) Sg.*

A

N448 Glycoform	TPA	I-TNK	G-TNK
N448+A2G2F	6.41%	5.40%	3.23%
N448+A2S1G0	5.18%	2.57%	ND
N448+A2S1G0F	0.52%	0.21%	1.79%
N448+A2S1G1F	23.11%	16.86%	14.43%
N448+A2S2F	37.96%	35.34%	37.59%
N448+A3G3F	0.59%	1.29%	0.80%
N448+A2Sg1S1F	1.32%	0.70%	0.56%
N448+A3S1G2F	1.59%	2.48%	0.91%
N448+A3S2G0	1.43%	0.86%	0.57%
N448+A3S2G1F	5.19%	7.00%	5.04%
N448+A4S2G2F	0.98%	ND	2.20%
N448+A4S1G3F	0.39%	1.16%	0.56%
N448+A3S3F	9.33%	11.61%	16.50%
N448+A4S3G1F	1.17%	6.55%	2.62%
N448+A4S4F	1.67%	7.20%	6.51%

B

N103 Glycoform	I-TNK	G-TNK
N103+A2G0F	ND	1.61%
N103+A2G1F	0.27%	4.49%
N103+A2G1M4F	ND	27.99%
N103+A2S1G0F	ND	1.72%
N103+A2G2	2.36%	ND
N103+A2G2F	14.89%	ND
N103+A2S1G1	5.82%	1.91%
N103+A2S1G1F	41.74%	51.76%
N103+A2S2	3.15%	ND
N103+A2S2F	26.09%	9.94%
N103+A3S1G2F	2.19%	1.89%
N103+A3S2G1F	2.16%	1.08%

C

N117 Glycoform	TPA
N117+A1G1M5	3.57%
N117+A1S1M4	2.63%
N117+A1S1M5	6.74%
N117+M5	52.41%
N117+M6	28.46%
N117+M7	6.00%

D

N 184 Glycoform	I-TNK
N184+A2S1G1F	3.22%
N184+A2S2F	4.74%
N184+A3S2G1F	2.01%
N184+A3S3F	2.99%
N184+A4S3G1F	1.50%
N184+A4S4F	1.67%

3. Other covalent modifications identified and quantified

Besides glycosylation, other covalent modifications that were indentified in these three samples included cysteine alkylation, deamidation, overalkyation, Cys+DTT, oxidation, formylation, glycation, etc. Also identified are low abundance semi-tryptic and non tryptic peptides (data not shown).

Figure 4 shows an example of a peptide that was identified in 3 different forms: native and deamidated on two different Asp residues, respectively. A total of 12 deamidation sites were indentified with high confidence in the three samples. Deamidation on N140 was only identified in I-TNK and G-TNK, not in TPA. Other sites and relative abundance of N-deamidation were consistent across all three samples (Table 3).

Figure 4. Identification and localization of two deamidation sites, N-140 and N-142 , on peptide 136-LGLGNHNYCR-145. Base peak chromatogram (A) and high resolution HCD spectra (B) of this peptide in native form or with deamidation either on N-140 or on N-142.

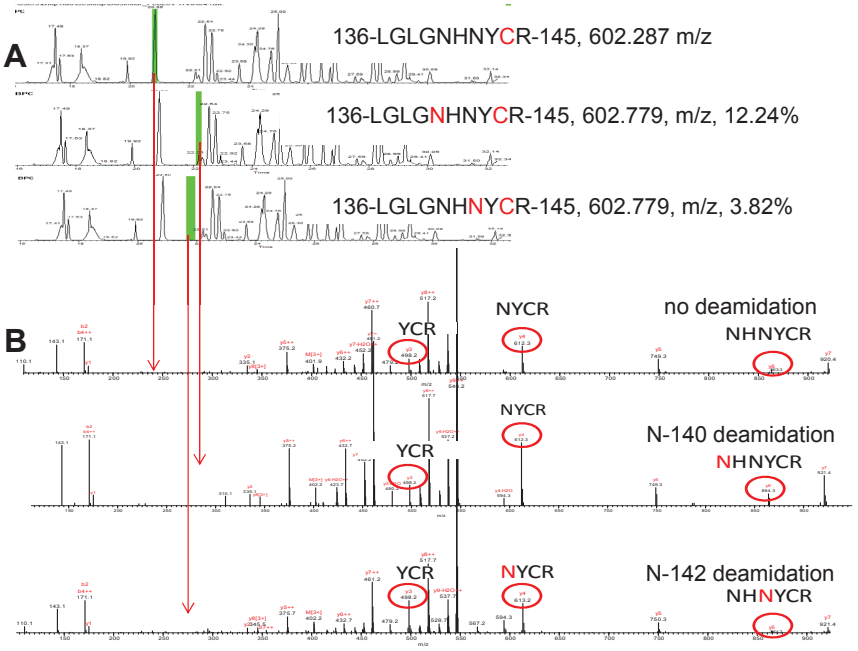


Table 3. Identified deamidation sites and relative abundance of deamidation.

Location of N-deamidation	TPA	I-TNK	G-TNK
N140	ND	12.24%	10.21%
N142	3.68%	3.82%	2.70%
N205	2.08%	1.61%	0.15%
N218	0.63%	0.11%	0.31%
N234	0.15%	ND	ND
N37	29.83%	22.83%	19.64%
N370	8.24%	13.56%	0.50%
N454	3.62%	2.71%	2.27%
N469	3.71%	2.05%	1.24%
N486	11.20%	10.80%	7.64%
N516	3.68%	2.87%	2.20%
N524	1.32%	0.51%	1.80%

Conclusion

An LC-MS/MS workflow was developed for differentiating minor difference of protein structure in biosimilar and reference products. This workflow provides qualitative and quantitative comparison of a biosimilar to a reference product.

1. 100% sequence coverage was obtained for all the nine data files analyzed. A five order magnitude dynamic range for identified peptide abundance was achieved.
2. The identified covalent modifications, both expected and unexpected, include cysteine alkylation, deamidation, overalkyation, Cys+DTT, oxidation, formylation, glycation. Relative abundance of the modified forms was calculated and a comparison between files was generated.
3. The site and type of glycosylation were identified and relative abundance of glycoforms was calculated. Comparison of glycosylation sites, type and relative abundance of glycoforms indicates the differences in glycosylation among the three samples.

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Sheathless Capillary Electrophoresis Mass Spectrometry (CESI-MS) as a Versatile and Powerful Tool for the Characterization of Monoclonal Antibodies

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Overview

Purpose: To assess the performance of sheathless capillary electrophoresis electrospray ionization (CESI) coupled to a hybrid quadrupole-Orbitrap mass spectrometer for the characterization of tryptic digests of monoclonal antibodies

Methods: CESI-MS - sheathless hyphenation of capillary electrophoresis (CE) with electrospray ionization mass spectrometry (ESI-MS) through the use of a separation capillary with a porous tip

Results: 100% sequence coverage & in-depth characterization of glycosylations in a single CESI-MS run

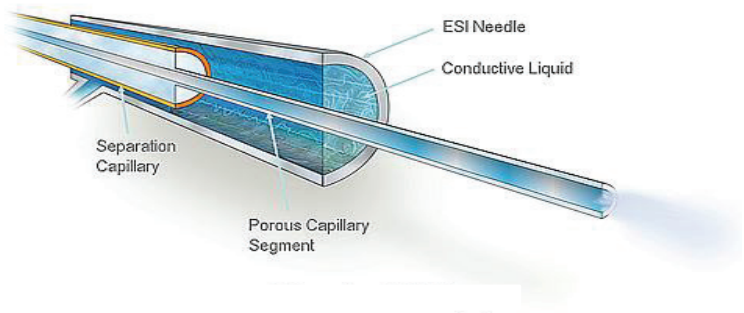
Introduction

In the last decade, the percentage of therapeutics based on monoclonal antibodies (mAbs) have been growing significantly. The number of molecules currently in clinical trials (innovators and/or biosimilars) indicate that the slope is becoming even steeper. Characterization of mAbs is important as unpredicted impurity or heterogeneity can impact therapeutic safety and/or efficacy. mAbs are large glycosylated molecules (≈ 150 kDa), containing several other post-translational modifications (PTMs). As a result, the analytical characterization of mAbs is usually complex and cumbersome. Aiming at improving the capabilities of the analytical toolbox available for mAb characterization, a novel platform combining sheathless capillary electrophoresis with fast, highly resolving and accurate mass (HRAM) mass spectrometry, has been assessed for primary sequence and glycosylation characterization of mAbs. Using two different molecules, Trastuzumab and Bevacizumab, the capabilities of the sheathless CESI-MS platform have been evaluated. Initially focusing on peptide mapping, the versatility of the platform has been demonstrated. It has been shown that the platform was capable of separating and unambiguously identifying, within a single run, very different molecules ranging from small dipeptides to very large peptides (Mw> 8000 Da). Taking advantage of the good compatibility between the speed of CESI and the duty cycle capabilities of the mass spectrometer in MS/MS mode, 100% sequence coverage has been obtained for the two tested molecules. As glycosylation represents such an important attribute of mAbs, the capabilities of the platform for the study of glycoforms was further assessed. In this context, it was found that the sensitivity of the platform was an enabling parameter.

Methods

Samples were prepared following a classical but short (2-hour digestion) protocol involving DTT, iodoacetamide, RapiGest and trypsin. CESI experiments were carried out with a Beckman Coulter™ CESI prototype system equipped with a temperature controlled autosampler and a power supply with the ability to deliver up to 30 kV. Prototype fused-silica capillaries with porous tip were used. Solutions of 10% acetic acid and ammonium acetate (pH 4 and various ionic strengths) were employed as background electrolyte (BGE) and leading electrolyte, respectively. CESI separations were performed at 20 kV, and about 100 fmol (45 nL of a 2 mM solution) of each tryptic digest were injected per analysis. Eluted peptides were analyzed using a Thermo Scientific™ Q Exactive™ bench-top quadrupole Orbitrap mass spectrometer with a data-dependent top ten HCD method. High resolution HCD spectra were analyzed using Thermo Scientific™ Proteome Discoverer™ software version 1.3.

Schematic of CESI Sprayer



100% Sequence Coverage with CESI-MS

Bevacizumab and Trastuzumab were initially considered to assess the capabilities of CESI-MS for achieving high sequence coverage of mAbs. Each molecule was digested for 2 hours with trypsin and the obtained peptide mixture was analyzed directly after by CESI-MS.

FIGURE 1. Triplicate analysis of a tryptic digest of Bevacizumab

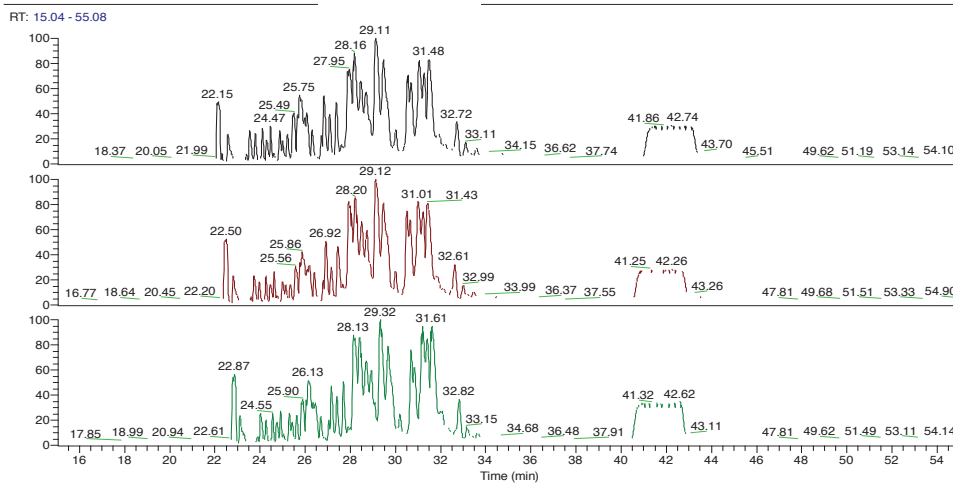


FIGURE 2. Triplicate analysis of a tryptic digest of Trastuzumab

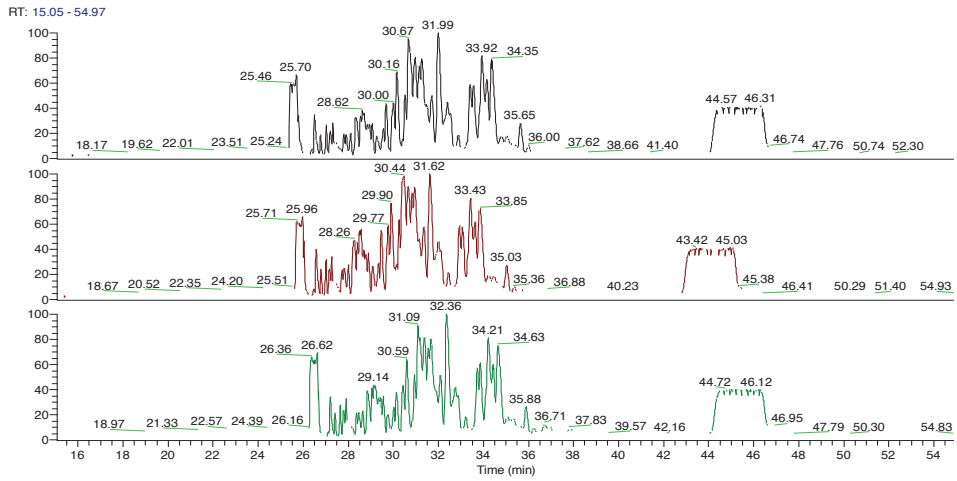
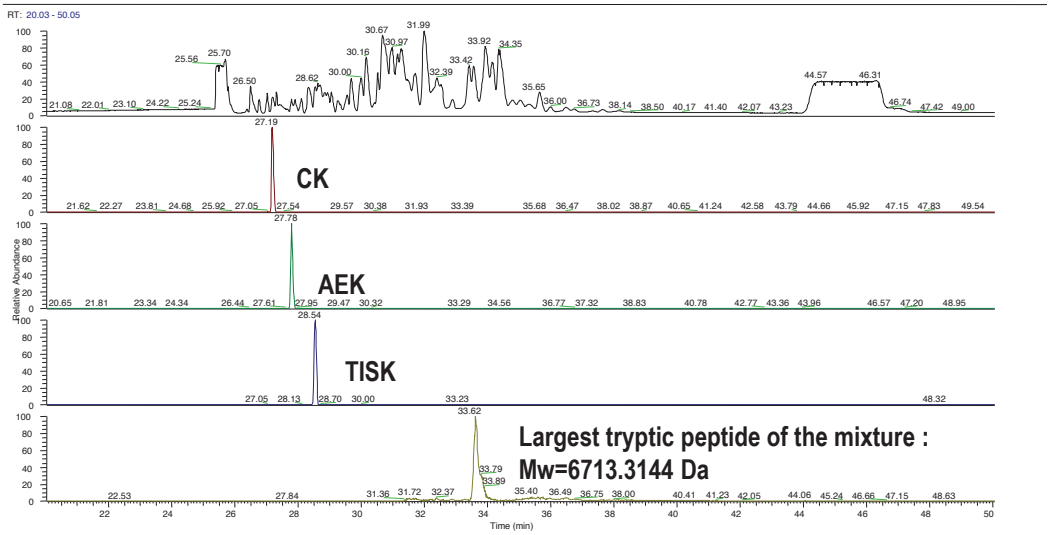


FIGURE 3. Extracted ion electropherograms of various peptides (tryptic digest of trastuzumab)



- ❑ CESI capable of analyzing very small (< 300 Da) and very large peptides (>8000 Da) under same experimental conditions
- ➢ CESI-MS provided 100% sequence coverage for each of the tested molecules with only 1 single digest analyzed in 1 single run

In-depth Glycosylation Study

In addition to achieving high sequence coverage, the following glycosylation study demonstrates further advantages of CESI-MS for the characterization of mAbs

FIGURE 4. Impact of glycan structure on the migration time of a glycopeptide (EEQYNSTYR)

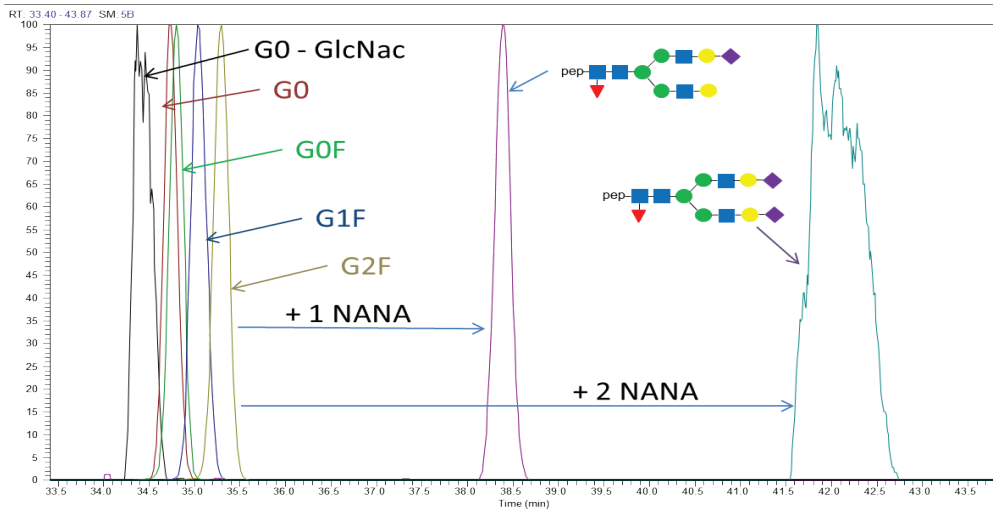


FIGURE 5. Extracted ion electropherograms of NANA and NGNA containing glycopeptides

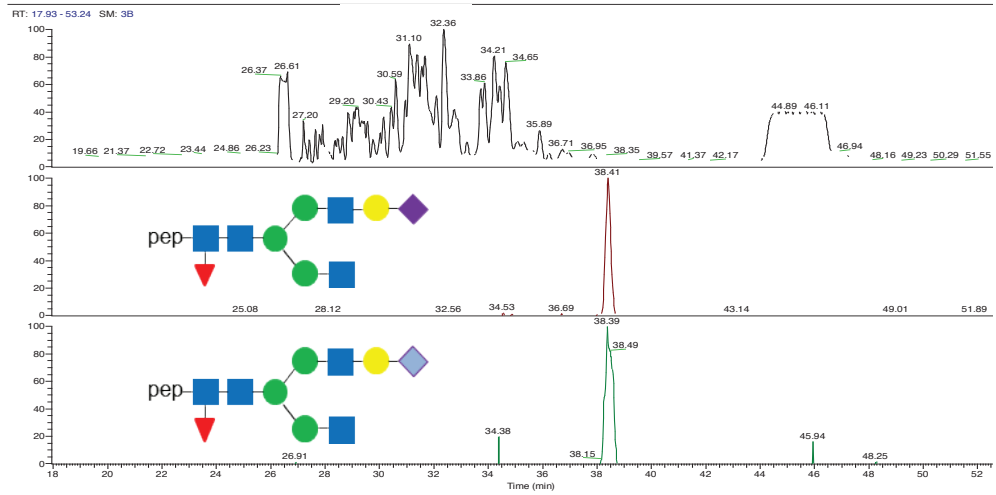
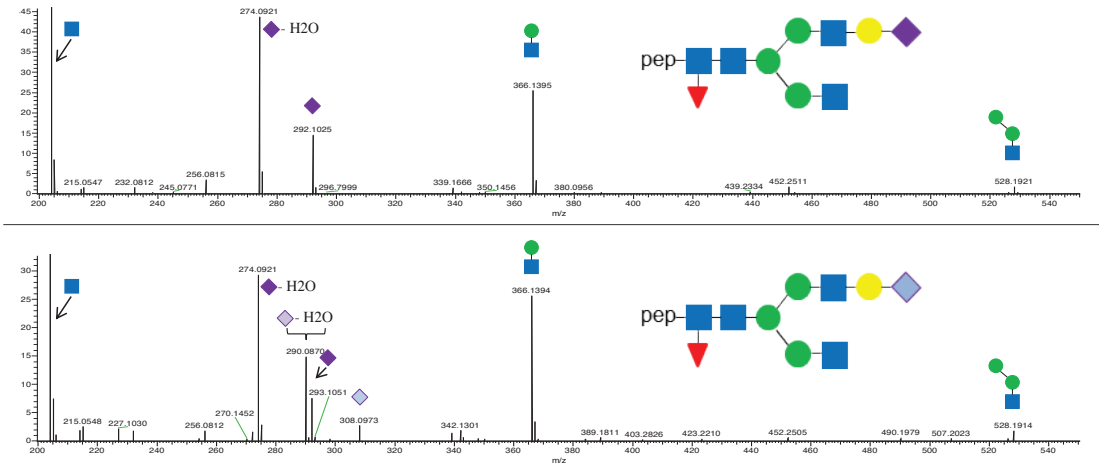
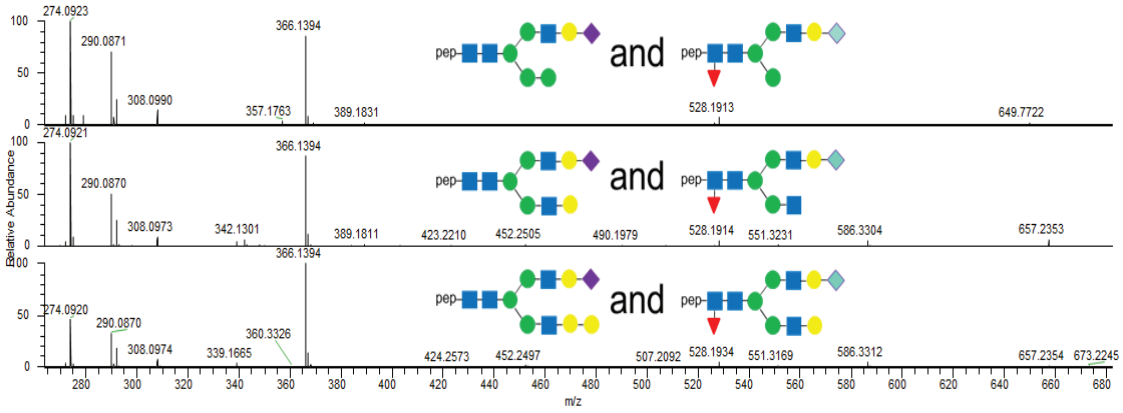


FIGURE 6. MS/MS spectra of NANA and NGNA containing peptides



- Besides MS¹ mass accuracy and the presence of diagnostic ions in MS/MS spectra, migration shifts can also be used to increase confidence in glycopeptide identification
- NGNA containing glycopeptides also show traces of NANA in MS/MS spectra
  - ➢ Presence of isobaric species

FIGURE 7. Potential Isobaric Species



Summary of Detected Glycopeptides

Glycopeptide (EEQYNSTYR)	z	m/z theo	m/z exp	Mass Accuracy (ppm)	Relative Abundance (%)	Presence of diagnostic ions in MS2 (204, 274, 290, 292, 308, 366, 526, etc.)	Electrophoretic Migration Pattern
	3	932.6996	932.7084	9.5	39.98		
	3	878.6820	878.6897	8.8	37.23		
	3	986.7172	986.7230	5.9	7.51		
	3	829.9960	830.0021	7.3	4.91		
	3	884.0136	884.0188	5.9	3.21		
	3	865.0064	865.0125	7.0	1.06		
	3	1083.7490	1083.7549	5.5	0.93		
	3	810.9888	810.9944	6.9	0.89		
	3	802.6449	802.6499	6.2	0.83		
	3	1029.7314	1029.7366	5.1	0.68		
	3	816.3205	816.3255	6.2	0.47		
	3	962.0382	962.0433	5.3	0.39		
	3	938.0312	938.0362	5.3	0.39		
	3	762.3029	762.3060	4.1	0.33		
	3	1180.7808	1180.7868	5.1	0.29		
	3	913.3523	913.3571	5.3	0.28		
	3	1000.3927	1000.3946	1.9	0.17		
and	3	967.3699	967.3748	5.1	0.12		
	3	870.3381	870.3444	7.3	0.10		
and	3	1035.0630	1035.0680	4.8	0.06		
and	3	1089.0806	1089.0863	5.2	0.05		
	3	1040.7348	1040.7415	6.5	0.04		
	3	1054.4103	1054.4168	6.2	0.04		
	3	1186.1124	1186.1183	5.0	0.02		

Conclusions

A CESI prototype was coupled to a Q Exactive mass spectrometer for the characterization of two monoclonal antibodies at the peptide level.

- CESI-MS was capable of analyzing very small (< 300 Da) and very large peptides (>8000 Da) under same experimental conditions
  - ➢ 100% sequence coverage achieved for each molecule with 1 single run & 1 enzymatic digest
- High ionization efficiencies provided by CESI and highly sensitive MS
  - ➢ Allowed for in-depth analysis of glycosylations with relative abundance varying more than 3 orders of magnitude

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