Rapid Online Desalting Using a Supermacroporous Reversed Phase Cartridge for Mass Spectrometry Analysis of a Monoclonal Antibody

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ABSTRACT

Here, we present mass spectrometry (MS) analysis of the monoclonal antibody (mAb) rituximab following sample cleanup using a reversed phase desalting cartridge in two approaches. First, we evaluate the high throughput analysis of an intact mAb over 100 runs using an optimized 4 minute gradient for loading/desalting, elution/MS analysis, and cartridge regeneration. MS detection enabled accurate mass characterization and relative quantification of the different mAb glycoforms. Using high mass loading, we were able to detect 8 different glycans, which combined to give 13 glycoforms. Sample carryover was reduced to 0.8% by area following one blank run. Second, we evaluated the analysis of the same mAb following IdeS digestion and TCEP reduction to separate the mAb into Fc/2, Fd', and LC fragments. Using an extended gradient, the different components were separated on the desalting cartridge into separate peaks. Deconvolution of the resulting mass spectra provided exact determination of the molecular weight of the separated fragments.

INTRODUCTION

Monoclonal antibodies (mAbs) have rapidly grown to become one of the top classes of biotherapeutics due to their ability for targeted drug delivery to specific cells and tissues. Variability in cellular protein production results in structural heterogeneity primarily derived from differences in post-translational modifications that can adversely affect mAb performance. For this reason, development typically involves the screening of hundreds of monoclonal cell populations to select a cell line that produces a mAb with the desired properties including structural fidelity, antigen specificity and activity, and stability. Mass spectrometry (MS) is an essential technique for characterizing structural heterogeneity due to its ability to determine the type and relative quantities of these post-translation modifications affecting mAb properties. To economically evaluate large numbers of mAbs from different monoclonal cell lines, high throughput methods for MS analysis must be developed.

Sample preparation prior to MS analysis is essential to ensure accurate mass and structural characterization. A variety of salts, stabilizers, detergents, and other adduct forming components that interfere with MS detection are commonly present in the matrix of recovered and purified mAbs. These artifacts preclude the direct injection of mAb in the sample matrix to the MS for high throughput analysis. Reversed phase (RP) chromatography is a common approach for the removal of these components as the protein binds to the column under aqueous loading conditions; whereas, water soluble (MS interfering) components are eluted. A gradient from low to high organic content (generally acetonitrile) then elutes the protein from the column to the MS instrument for analysis. Additional benefits to RP desalting include (1) denaturing conditions which result in increased m/z values for improved MS characterization and (2) increased mobile phase volatility at the source for improved ionization process. This work demonstrates the use of a small, RP cartridge packed with a supermacroporous resin for the rapid removal of matrix components prior to MS analysis of a mAb. First, we demonstrate a high throughput method for analysis of an intact mAb. Second, we show the analysis of a mAb following IdeS digestion and reduction to characterize the Fc/2, Fd', and LC fragments.

MATERIALS AND METHODS

Chemicals and Reagents

Rituximab in formulation buffer was obtained from Hoffmann-La Roche Ltd. FabRICATOR® (IdeS) protease was purchased from Genovis. Tris-(2-carboxyethyl)phosphine, hydrochloride (TCEP) was obtained from Fisher Scientific.

Columns

- Thermo Scientific™ MSPac DS-10 desalting cartridge, 2.1x10mm, P/N 089170 with
- Thermo Scientific™ Acclaim™ cartridge holder, P/N 069580

Figure 1. 2.1x10mm MSPac DS-10 Desalting Cartridge with Acclaim Cartridge Holder

LC Instruments

Thermo Scientific™ Vanquish™ UHPLC system consisting of
System Base (P/N VH-S01-A)
Binary Pump H (PN VH-P10-A)
Split Sampler HT (PN VH-A10-A)
Column Compartment H (PN VH-C10-A)
Diode Array Detector (PN VH-D10-A) equipped with a LightPipe flow cell, 10 mm (PN 6083.0100)
Active Pre-Heater (P/N 6732.0110)
Biocompatible 2-position/6-Port Switching Valve (150 MPa) (PN 6036.1560)
MS Connection Kit Vanquish (PN 6720.0405)

Mobile Phases

Mobile Phase A: Water + 0.1% formic acid
Mobile Phase B: 80/20 (v/v) Acetonitrile/Water + 0.1% formic acid

Sample Preparation and LC-MS Conditions for High Throughput Intact mAb Analysis

10mg/mL rituximab was diluted 1:1000 with mobile phase A to give a 10 µg/mL solution in 0.7 µg/mL polysorbate 80, 7.35 µg/mL sodium citrate dihydrate, and 9 µg/mL NaCl

Data processing was handled using Thermo Scientific™ Dionex™ Chromeleon™ data system, V. 7.2 SR3, and Thermo Scientific™ Protein Deconvolution™ software, V. 4.0.

Table 1. Intact mAb LC Gradient Conditions

Time (min)	A	B	Flow Rate (mL/min)	°C
0.0	70	30	0.5*	70
1.0	70	30	0.5*	
1.01	70	30	0.2	
3.0	0	100	0.2	
3.2	0	100	0.2	
3.3	0	100	1.0	
3.5	0	100	1.0	
3.6	70	30	1.0	
4.0	70	30	0.5*	

Table 2. Intact mAb MS Divert Valve Configuration

Time (min)	Valve Configuration	Analysis Stage
0	To Waste	Sample loading and desalting
1.5	To MS	Elution to MS for analysis
3.1	To Waste	Cleaning and equilibration for loading next sample

Table 3. Intact mAb MS Instrument and Conditions

MS Instrument			
Thermo Scientific™ Q Exactive™ Plus hybrid quadrupole-Orbitrap™ mass spectrometer			
MS Source Parameters	**Setting**	**MS Method Parameters**	**Setting**
Source	HESI-II	Method type	Full MS only
Sheath gas pressure (psi)	35	Full MS mass range (*m/z*)	1800 - 5000
Auxiliary gas flow (arbitrary units)	10	Resolution settings (FWHM at *m/z* 200)	17.500 K
Vaporizer temperature (°C)	260	Target value	3e6
Capillary temperature (°C)	260	Max injection time (ms)	150
S-lens RF voltage	80	Microscans	10
Source voltage (kV)	3.5	SID (eV)	20

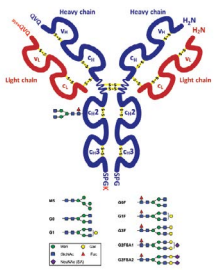

Figure 2. Rituximab general structure and associated glycans

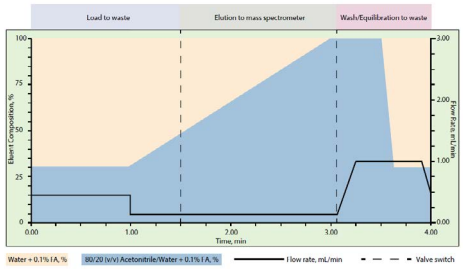

Figure 3. Schematic depiction of high throughput LC-MS method with dashed lines indicating divert valve switching.

Sample Preparation and LC-MS Conditions for Ides Digested and Reduced mAb Analysis

IdeS protease was used to digest Rituximab according to the manufacturer's protocol. After sample digestion, the resulting protein fragments were reduced in 5 mM TCEP for 30 minutes at 60°C. The final sample concentration was 1 mg/mL. Figure 4 at right shows a schematic of the resulting mAb following IdeS digestion and reduction.

Data processing was handled using Chromeleon data system, V. 7.2 SR2, and Protein Deconvolution Software, V. 3.0.

Table 4. IdeS Digested and Reduced mAb LC Gradient Conditions

Time (min)	A	B	Flow Rate (mL/min)	°C
0.0	75	25	0.2	50
1.0	75	25		
11	30	70		
11.1	0	100		
13	0	100		
14	75	25		
18	75	25		

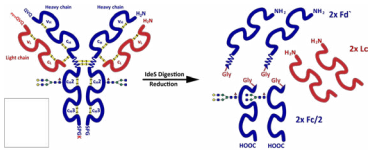

Figure 4. IdeS digestion and reduction of a mAb to produce Fd, Lc, and Fc/2 fragments.

Table 5. IdeS Digested and Reduced mAb MS Instrument and Conditions

MS Instrument			
Thermo Scientific™ Q Exactive™ HF hybrid quadrupole Orbitrap mass spectrometer			
MS Source Parameters	**Setting**	**MS Method Parameters**	**Setting**
Source	HESI-II	Method type	Full MS only
Sheath gas pressure (psi)	40	Full MS mass range (*m/z*)	550 – 3000
Auxiliary gas flow (arbitrary units)	10	Resolution settings (FWHM at *m/z* 240)	15 K
Vaporizer temperature (°C)	260	Target value	3e6
Capillary temperature (°C)	260	Max injection time (ms)	200
S-lens RF voltage	50	Microscans	3/10
Source voltage (kV)	3.5	SID (eV)	10

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RESULTS

High Throughput Desalting and MS Analysis

Method development

For this study, Rituximab was used as a model mAb. The general structure for rituximab and all associated glycans detected in this study are shown in Figure 2. Prior to the high throughput method application, experiments were conducted to determine an ideal loading flow rate of 0.5 mL/min and SID (in-source collision induced dissociation) voltage of 20 eV (data not shown). At this flow rate, no decrease in peak area or signal intensity was observed relative to lower flow rates. Above 20 eV for SID voltage (range 0-100 eV), protein fragmentation was observed while below 20 eV incomplete removal of adducts was observed resulting in poor spectrum quality. The final high throughput gradient used is depicted graphically in figure 3 with loading, elution/MS detection, and cartridge regeneration phases illustrated with dashed lines to indicate switching of the MS divert valve (see Tables 1 and 2 for exact details). During loading, a high flow rate was used to maximize the desalting efficiency of the column. For gradient elution, the flow rate was decreased to 0.2 mL/min to be consistent with the optimized flow rate for the electrospray ionization (ESI) source. During wash and equilibration, the flow rate was increased to maximize cartridge cleaning.

Efficiency of Rapid mAb Desalting and MS Analysis

To evaluate the performance of the desalter using the fast 4 minute method, 100 ng of rituximab was loaded and the resulting mass spectrum evaluated. Figure 5 shows the total ion chromatogram (TIC, figure 5A) and resulting mass spectrum (figure 5B) for analysis of the peak as shown by the grey region. Inspection of the enlarged region in figure 5C shows a clean mass spectrum that is absent of any interference from salts or sample artifacts. In contrast to these results, figure 5D shows an affected mass spectrum with significant interference from formulation buffer components. In this analysis, 2.5 ng of a comparable mAb were separated on a 100 µm x 250 mm ProSwift C4 RP-5H column using a water/acetonitrile gradient at 70ºC.

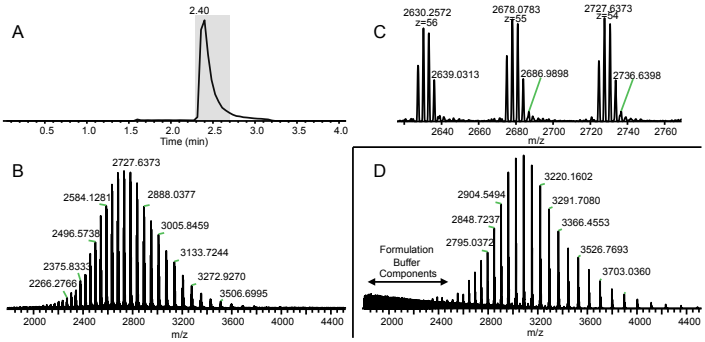

Figure 5. Loading, desalting and MS analysis of 100 ng rituximab: (A) Total Ion Chromatogram, (B) average full mass spectrum of gray region in (A), (C) enlarged region of mass spectrum showing 3 most abundant charge states, and (D) an example of poor desalting of a mAb performed on a 100µm x 25cm C4 reversed phase column.

High Throughput Analysis and Cartridge Ruggedness

To evaluate the desalting efficiency and robustness of the solid phase in a high throughput application, 100 consecutive 10 ng mAb injections were analyzed using the optimized high throughput LC-MS method. Figure 6 shows (A) the spectrum quality of the four most abundant states for runs 20, 40, 60, 80 and 100 and (B) the associated deconvoluted mass spectra. The ruggedness of the desalting cartridge is exemplified by the absence of salt interference in the mass spectra and excellent reproducibility of the glycoform pattern over 100 runs. These results demonstrate the utility of the desalting cartridge for rapid analysis of a large sample set when paired with a fast, straightforward method.

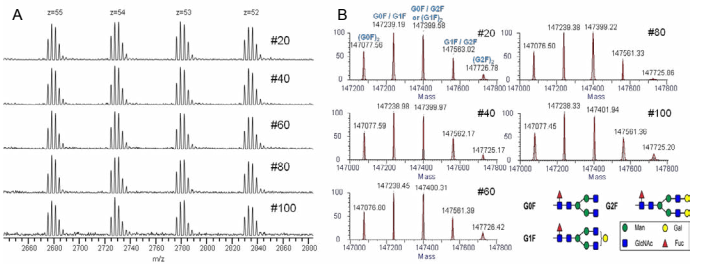

Figure 6. (A) Enlarged view of charges states showing mass spectrum quality over 100 runs (runs 20, 40, 60, 80, and 100) for 10 ng mAb injections and (B) the associated deconvoluted mass spectra showing glycoform detection.

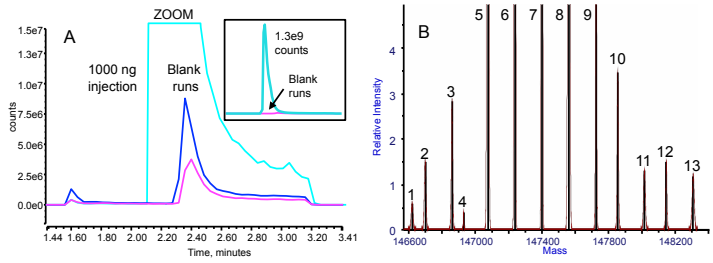

Figure 7. (A) TIC for 1000 ng injection and following 2 blank runs and (B) enlarged region of the deconvoluted spectrum showing low abundance glycoforms, see table 6 for masses.

High Mass Loading and Carryover Analysis

High mass loading levels are commonly used for detailed analysis of low abundance glycoforms and other mAb variants. Figure 7 shows (A) the TICs for a 1000 ng injection and subsequent 2 blank runs and (B) the deconvoluted mass spectrum of the 1000 ng injection detailing the detection of low abundance glycoforms with masses and structures summarized in table 6. Using a high mass loading enabled the detection of eight different glycans on the mAb heavy chains (see Figure 1 for details), resulting in 13 different glycoforms.

Since sample carryover from run to run can interfere with variant characterization, it is important minimize this interference for high throughput methods. Inspection of the blank runs shows a total carryover of 1.48% by area for the first blank run and carryover of 0.79% by area in the second blank run. These results indicate one blank run is required to reduce carryover for MS analysis of structurally distinct mAbs when doing high mass loading analyses.

Table 6. Intact mAb Glycoforms

Peak	MW (Da)	Modifications
1	146623.00	(Man5)2
2	146701.11	Man5/G0
3	146861.92	Man5/G1
4	146931.52	G0/G0F
5	147076.45	G0F/G0F
6	147237.98	G0F/G1F
7	147400.22	G0F/G2F or (G1F)2
8	147560.95	G1F/G2F
9	147724.64	G2F/G2F
10	147854.70	(G1F/G2F)SA1
11	148014.19	(G2F/G2F)SA1
12	148145.02	(G1F/G2F)SA2
13	148305.57	(G2F/G2F)SA2

Analysis of IdeS Digested and Reduced mAb

mAb IdeS Digestion and Reduction and LC-MS Analysis

Reduction and/or digestion of mAbs to generate smaller protein fragments is a common approach for improving MS analysis for two reasons: (1) the smaller protein fragments may enable acquisition of isotopically resolved charge states with monoisotopic determination of mass by deconvolution and (2) structural mAb modifications can be isolated to a specific region (e.g., Fc, Lc, and Fd') of the antibody. IdeS is a common mAb digestion agent, which cleaves mAbs below the hinge region. A reduction agent such as TCEP (or DTT) is then generally used to reduce the interchain disulfide bonds resulting in the generation of Fd', Lc, and Fc/2 fragments (See figure 4 for schematic depiction) with molecular weights in the range of 23 – 26 kDa. These steps introduce additional salts and sample artifacts that can suppress or otherwise interfere with MS detection and thus should be removed for optimal mass characterization. Figure 8 shows the LC-MS analysis of Rituximab following IdeS digestion and TCEP reduction using an un-optimized gradient. Since the MS divert valve was not used, the peak at ~0.5 minutes corresponding to the cartridge void volume shows the elution of salts and other sample matrix components. MS analysis (discussed further below) shows that the Fc/2, Lc, and Fd' fragments (peaks 1, 2, and 3, respectively) can be separated from each other using the small desalting cartridge. Intact mAb (peak 4) and partially digested/reduced species (broadly eluted as peak 5) are also separated using this cartridge.

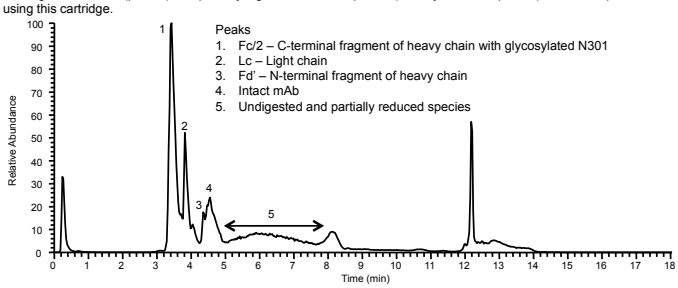

Figure 8. TIC of Fd', Lc, and Fc/2 mAb fragments for 0.8 µg total protein loading.

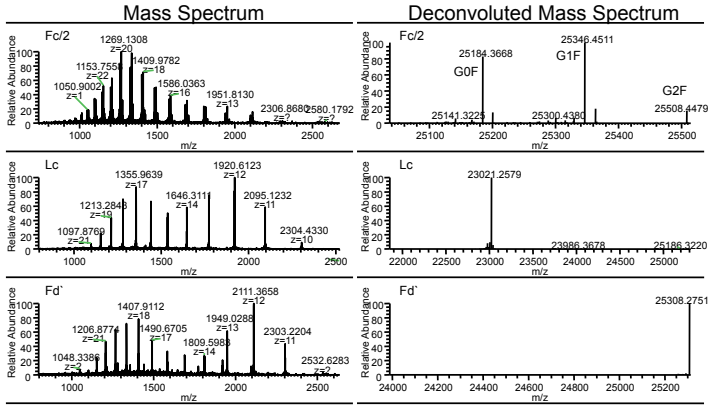

Figure 9. (Left) Mass spectra and (right) the deconvoluted mass spectra with molecular weights for Fc/2, Lc, and Fd' rituximab fragments for the corresponding peaks shown in figure 8.

Table 7. mAb Fragments with Monoisotopic and Measured Molecular Weights

Fragment	Modifications	# C	# H	# N	# O	# S	MW (monoisotopic) [Da]	MW measured [Da]	Mass deviation [ppm]
Lc	N-terminal pyroglutamic acid, 2 internal S-S bonds	1016	1570	272	328	6	23021.28593	23021.2579	-1.22
Fd`	N-terminal pyroglutamic acid, 2 internal S-S bonds	1125	1724	292	354	10	25308.30854	25308.2751	-1.32
Fc/2	G0F glycan 2 internal S-S bonds	1122	1736	286	361	6	25184.46011	25184.3668	-3.71
Fc/2	G1F glycan 3 internal S-S bonds	1128	1746	286	366	6	25346.51294	25346.4511	-2.44
Fc/2	G2F glycan 4 internal S-S bonds	1134	1756	286	371	6	25508.56576	25508.4479	-4.62

Mass Spectra and Structural Identification

The desalting efficiency and separation power of the desalting cartridge result in protein peaks that provide a clean mass spectrum for each individual fragment as shown on the left of Figure 9. Deconvolution (right, figure 9) of the mass spectra allows determination of the monoisotopic molecular weight with mass deviations ≤ -4.62 ppm as shown in Table 7. Furthermore, the location of glycan modifications can confidently be located on the Fc region of the mAb. These two results highlight the power of using digestion/reduction as a practical tool for improving the mass characterization of mAbs with regards to both mass accuracy and variant location. Additionally, these results further highlight the desalting and sample cleanup capabilities of the desalting cartridge based on the high quality of the mass spectra to give monoisotopic determinations of molecular weight.

Table 8. Measured and Theoretical MW of Unresolved Fragments (Figure 6, Peak 5)

Fragment	MW measured [Da]	Theoretical MW [Da]
Lc	23036.352	23035.353
Fc/2 + G1F (2 internal S-S bonds)	25361.822	25362.019
Hc + G1F (4 internal S-S bonds)	50602.141	50668.124
Hc+ Lc (6 internal S-S bonds)	73680.188	73699.445
(2 x Lc + 2 x Fd`)[a] (7 internal S-S bonds)	96713.414	96712.9

Table 6 summarizes the MS analysis of the unresolved mAb fragments labeled as peak 5 in figure 6. This analysis is only capable of providing approximate molecular weights of the analyzed species with a maximum molecular weight deviation of 66 Da from the calculated theoretical molecular weight. In general, the observed MW deviations are attributable to the poorer spectrum quality for the fragments eluted in this complex region of the chromatogram. These results illustrate the importance of developing a robust digestion and reduction method for analysis of mAb fragments; however, despite these drawbacks for the sample used in this analysis, the desalting cartridge is capable of separating all the species present to elute a clean sample that can be readily analyzed by mass spectrometry.

CONCLUSIONS

A small, 2.1 x 10 mm reversed phase cartridge can be used with water/acetonitrile eluents to desalt and remove hydrophilic matrix components from mAbs and elute the protein in a sharp peak providing strong MS signal and obtaining a clean mass spectrum free of interference from matrix components.

The use of a fast, 4 minute method including sample loading/desalting, elution to MS, and cartridge regeneration steps can be used to analyze are large sample set of at least 100 injections per cartridge to provide information on the glycoforms of mAbs.

Large sample loading amounts of 1 µg mAb can be analyzed to provide in-depth information on low abundance glycoforms with only one blank run required to reduce carryover below 1%.

The properties of the supermacroporous resin enable the separation of mAb fragments following IdeS digestion and reduction resulting in minimal peak overlay to produce a clean protein spectrum for each fragment and determination of monoisotopic masses.

Download corresponding application notes for complete method and experimental details:

- AN 21465 - Fast Online Desalting of mAbs Using a Reversed-Phase Desalting Cartridge for LC-MS Analysis
- AN 21239 - LC-MS Analysis of Digested mAbs Using a Reversed-Phase Desalting Cartridge and High-Resolution, Accurate Mass Spectrometry (HRAMS)

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