Profiling the Distribution of N-Glycosylation in Therapeutic Antibodies using the QTRAP® 6500 System

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INTRODUCTION

Immunoglobulin G molecules have become attractive as targeted therapeutic proteins, due to their high specificity and long circulation time. Glycosylation patterns determine the stability and bio-disposition of these recombinant protein drugs in vivo, as well as the efficacy, folding, binding affinity, specificity and pharmacokinetic properties. Therefore, a complete characterization of the biotherapeutic IgG glycosylation is desirable.

In this study, we demonstrated how a comprehensive MS/MS analysis of the glycopeptides can be achieved by targeting the known nature of the glycosylation structures. We used Trastuzumab, a humanized mAb, and SilumAb in this proof of concept study. Monoclonal IgGs have a known glycosylation site and set of glycan isotypes (complex, high mannose and hybrid). We applied a robust MRM triggered MS/MS workflow to profile and confirm glycopeptides and their glycan isoforms. Overlaying several specific MRMs for each glycopeptide group and searching each full scan MS/MS spectrum with SimGlycan® provides added confidence to the identification of each glycopeptide isoform (Figure 1).

MATERIALS AND METHODS

Sample Preparation: Monoclonal antibodies (mAb human) were supplied by Bristol-Myers Squibb (Sunnyvale, USA). The mAb solution was first denatured 8M Guanidine HCl, reduced with DTT (37C for 1 hour), then alkylated with iodoacetamide (RT for 45 min). A Zeba column (VWR) was used to remove guanidine and any impurities before digestion (used according to manufacturers' guidelines). Recovery of 65% was observed. The first aliquot of trypsin was added (37C for 2 hours) followed by a second aliquot (37C for 2 more hours). ***LC Analysis:*** Microflow LC was performed using the NanoLC™ 425 system (SCIEX) using a using a C18 RP column (CXP CL18-120 0.5x150mm, SCIEX) running at 15 µL/min. Samples were directly loaded onto a heated column at 30˚ C using a 30 min gradient to 2-35% acetonitrile, 0.1% formic acid). Injections of 3.3 to 6 pmol of protein digest on column per run were performed.
Mass Spectrometry: The eluent was analyzed using QTRAP® 6500 system equipped with an IonDrive™ Turbo V Source and a 25 µm I.D. electrospray probe (SCIEX). Analyst® Software 1.6.2 with the *Scheduled* MRM™ Pro Algorithm was used for data acquisition. A variety of scan modes was explored to quantify and confirm the glycopeptides from the digested mAb sample. Excel spreadsheet was created to automatically to compute targeted MRM transitions for known fragment ions (Table 1).

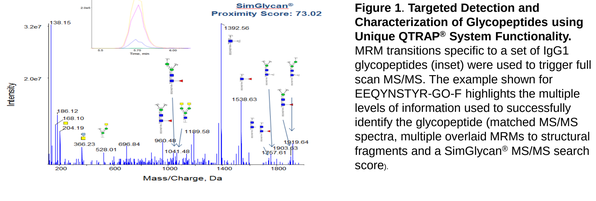


Figure 1. Targeted Detection and Characterization of Glycopeptides using Unique QTRAP® System Functionality. MRM transitions specific to a set of IgG1 glycopeptides (inset) were used to trigger full scan MS/MS. The example shown for EEQYNSTYR-GO-F highlights the multiple levels of information used to successfully identify the glycopeptide (matched MS/MS spectra, multiple overlaid MRMs to structural fragments and a SimGlycan® MS/MS search score).

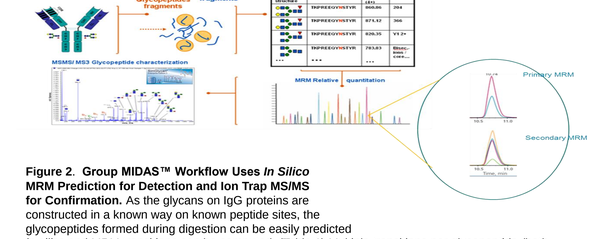
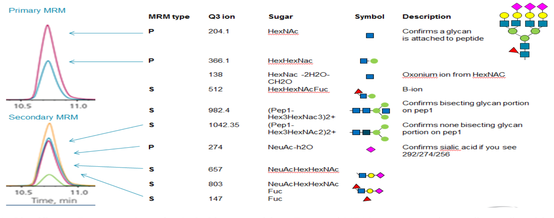
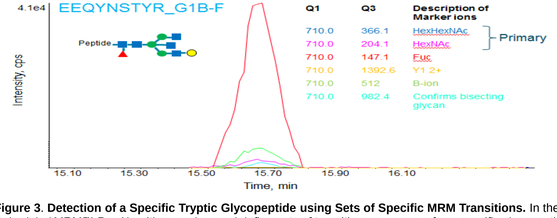


Figure 2. Group MIDAS™ Workflow Uses *In Silico* MRM Prediction for Detection and Ion Trap MS/MS for Confirmation. As the glycans on IgG proteins are constructed in a known way on known peptide sites, the glycopeptides formed during digestion can be easily predicted *in silico* and MRM transitions can be computed. (Table 1) Multiple transitions per glycopeptide (both primary and secondary MRM transition) are used to increase the specificity of detection with group triggered MRM workflow in the *Scheduled* MRM™ Pro Algorithm. Both additional MRMs and full scan MS/MS spectra can be collected in a targeted manner to characterize and quantitatively profile glycopeptides from IgG.



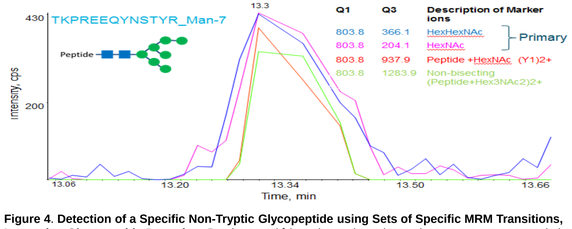
MRM type	Q3 ion	Sugar	Symbol	Description
P	204.1	HexNAc		Confirms a glycan is attached to peptide
P	366.1	HexHexNac		
S	138	HexNac -2H2O-CH2O		Oxonium ion from HexNAC
S	512	HexHexNAcFuc		B-ion
S	982.4	(Pep1+ Hex3HexNac3)2+		Confirms bisecting glycan portion on pep1
S	1042.35	(Pep1+ Hex3HexNAc2)2+		Confirms none bisecting glycan portion on pep1
P	274	NeuAc-H2O		Confirms sialic acid if you see 292/274/256
S	657	NeuAcHexHexNAc		
S	803	NeuAcHexHexNAc Fuc		
S	147	Fuc		

Table 1. Significant Fragment Ions for IgG Glycopeptides. Targeted MRM assays can be easily predicted for all possible IgG glycan structures, using the known structures and peptide sequence for parent ion computation and the common known fragment ions that are produced during MS/MS, including diagnostic marker ions . An Excel spreadsheet was constructed that enabled the computation of specific MRM transitions for all possible IgG glycopeptides. Here, glycoform specific fragment ions for a tetra-antennary glycopeptide with peptide sequence EEQYNSTYR are computed as an example.



Q1	Q3	Description of Marker ions	
710.0	366.1	HexHexNAc	Primary
710.0	204.1	HexNAc	
710.0	147.1	Fuc	
710.0	1392.6	Y1 2+	
710.0	512	B-ion	
710.0	982.4	Confirms bisecting glycan	

Figure 3. Detection of a Specific Tryptic Glycopeptide using Sets of Specific MRM Transitions. In the *Scheduled* MRM™ Pro Algorithm can be used define sets of transitions as a group for a specific glycopeptides. Within the group, MRMs expected to be most intense can be set as primary transitions (m/z 204 (GlucNAc) or 366 (HexHexNAc)), and additional confirmatory MRMs can be defined as secondary transitions. Overlay of primary and secondary MRM transitions for EEQYNSTYR-G1FB is shown and the monitored fragment ions are listed in the inset. Detection of a bisecting ion confirms a G1B-F glycoform on peptide 1, as demonstrated in Figure 3 by the presence of signal for the bisecting core ion (m/z 982.4^{2+}).
Using group-triggered MIDAS™ Workflow, improved dwell and cycle times are achieved by only acquiring secondary transitions when a glycopeptide is eluting as detected by primary transitions.



Q1	Q3	Description of Marker ions	
803.8	366.1	HexHexNAc	Primary
803.8	204.1	HexNAc	
803.8	937.9	Peptide +HexNAc (Y1)2+	
803.8	1283.9	Non-bisecting (Peptide+Hex3NAc2)2+	

Figure 4. Detection of a Specific Non-Tryptic Glycopeptide using Sets of Specific MRM Transitions, Increasing Glycopeptide Detection. Previous work[1] has shown that a large glycan structure can mask the tryptic cleavage site and therefore neglecting this longer peptide would result in an incomplete glycan profile. Therefore, Peptide 2 was also included in the study (TKPREEQYNSTYR contains 1 missed tryptic cleavage). The same diagnostic marker ions were monitored for peptide 2 (Table 1). Here, the overlay of the additional secondary MRMs highlights a high mannose type glycoform, mannose-7. The specific core ion (m/z 1283.9^{2+}) confirms the high mannose type structure. It was found that by monitoring peptide 2, additional high mannose glycoforms could be detected (such as mannose-7 and 9) and a tri-antennary A3 isoform as shown in Table 3.

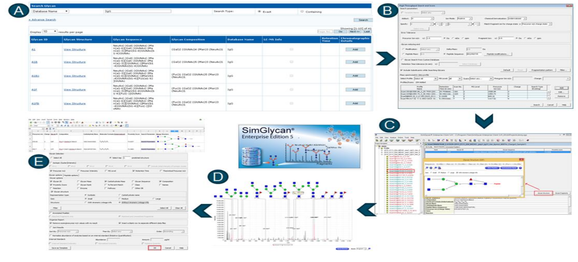


Figure 5. Glycopeptide Identification Workflow in SimGlycan® Enterprise Edition. A) A specific database is created containing the known glycoforms for IgGs. B) MIDAS™ workflow files are then submitted for batch searching. C) Search results are then returned and all matched glycoforms are shown in green. Selecting a result will show the glycan structure. D) Each full scan MS/MS can be annotated using the search results and the known glycopeptide structure. E) A report is then generated using the report creation window.
Both the MIDAS Workflow data using MRMs to m/z 204 and the Group triggered MIDAS data targeted the additional structural ions were submitted for search. Results from the searches are summarized in Table 2 and 3, including the SimGlycan® proximity scores to highlight successfully confirmed MS/MS spectra. Mostly higher scores (proximity score >70) are assigned to candidate glycopeptides with the carbohydrate fragments matched to high intensity peaks in the MS/MS data.

Table 2. Summary of Glycopeptides Found on EEQYNSTYR Peptide for Trastuzumab and SiluMab. A glycopeptide was considered detected when both the MRM area of the m/z 204 peak was ~200 cps or greater, multiple MRMs were overlaid, and when the SimGlycan score on the triggered MS/MS spectrum exceeded 70. MRM signal was detected for 23 different glycopeptides on peptide 1 for Trastuzumab, however only 17 of these were further confirmed by MS/MS. For SiluMab, 16 glycoforms on peptide 1 were confirmed by MRM and MS/MS.

		Trastuzumab 6 pmol			SiluMab 6pmol		
Symbolic glycan structure	Glycopeptide & glycan name	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)
	EEQYNSTYR_Man-7	-	-	-	63.65*	14.46	3150
	EEQYNSTYR_Man-9	-	-	256	73.56	18.93	3814
	EEQYNSTYR_G2-F	73.80	4.5	14877	71.92	5.63	38668
	EEQYNSTYR_A1-G1-F	-	4.59	15412	72.65	4.51	9692
	EEQYNSTYR_GO_F	73.02	4.60	58687	72.52	5.7	743131
	EEQYNSTYR_A1-GO-F	76.09	4.63	113938	68.57*	4.54	18426
	EEQYNSTYR_A1-G1	-	4.82	3002	-	4.55	23346
	EEQYNSTYR_G1-F	72.65	5.12	343124	72.72	5.62	25609
	EEQYNSTYR_G1	73.55	5.51	656	-	-	-
	EEQYNSTYR_G1B-F	74.36	7.01	362	-	-	-
	EEQYNSTYR_G1B	77.59	7.02	88578	78.71	14.63*	1125
	EEQYNSTYR_GOB	77.47	10.57	2190	71.42	10.57	1011
	EEQYNSTYR_A2	72.76	12.17	564	-	-	433
	EEQYNSTYR_A1-GO	72.25	12.19	12795	-	10.52	23346
	EEQYNSTYR_A2B-G2	36.42*	12.23	1098	-	-	-
	EEQYNSTYR_GO	74.23	12.28	164989	74.96	4.64*	242
	EEQYNSTYR_Man-6	76.98	12.30	3757	73.08	14.42	2521
	EEQYNSTYR_Man-5	76.04	12.57	5562	74.52	13.6	1497
	EEQYNSTYR_G2	-	14.16	2182	71.43	18.60*	645
	EEQYNSTYR_G2B	75.09	14.8	7192	-	-	-
	EEQYNSTYR_A1	-	15.85	1229	75.27	28.54	980
	EEQYNSTYR_A1FB	65.90*	15.86	2176	-	-	1358
	EEQYNSTYR_GOB-F	55.78*	15.88	22379	73.33	15.8	25609
	EEQYNSTYR_A1F	-	15.89	573	-	-	340
	EEQYNSTYR_A2F	75.04	15.90	705	-	-	996
	EEQYNSTYR_A2FB	-	15.92	550	-	-	-
	EEQYNSTYR_G2B-F	72.71	22.8	211	71.03	9.41*	282

Table 3: Summary of Glycopeptides Found on TKPREEQYNSTYR Peptide for Trastuzumab and SiluMab. MRM signal detected 12 glycoforms on peptide 2, and 10 of these were further confirmed by MS/MS.

		Trastuzumab 6 pmol			SiluMab 6pmol		
Symbolic glycan structure	Glycopeptide & glycan name	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)	SimGlycan MS/MS Score	RT (min)	MRM Area of 204 (cps)
	TKPREEQYNSTYR_GO-F	65.62*	4.73	4750	-	4.6	7774
	TKPREEQYNSTYR_G1S1	76.93	12.99	1405	-	-	-
	TKPREEQYNSTYR_Man-8	-	13.34	3018	-	12.17	2148
	TKPREEQYNSTYR_G2-F	76.91	13.35	2053	-	13.32	836
	TKPREEQYNSTYR_Man-5	76.47	13.35	2027	-	12.12	1034
	TKPREEQYNSTYR_Man-6	74.52	13.35	1307	-	12.4	303
	TKPREEQYNSTYR_Man-7	76.55	13.35	3626	-	12.43	308
	TKPREEQYNSTYR_G2	76.86	13.7	1540	-	13.7	199
	TKPREEQYNSTYR_A1	76.90	15.8	802	-	16.91	895
	TKPREEQYNSTYR_A2FB	-	16.9	1126	-	17.95	1373
	TKPREEQYNSTYR_A3	74.18	19.46	1366	-	-	-
	TKPREEQYNSTYR_Man-9	74.29	25	3110	-	24.4	414

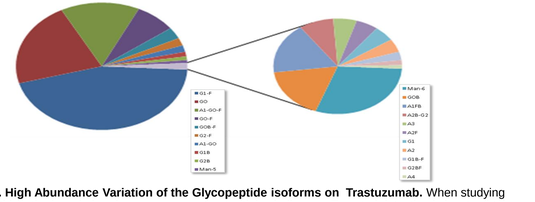


Figure 6. High Abundance Variation of the Glycopeptide isoforms on Trastuzumab. When studying glycopeptides, the broad abundance range creates multiple challenges. Targeted detection is key to obtain good quality MS/MS on the many glycopeptide isoforms. Here, MRM peak areas for m/z 204 for each of the glycopeptide 1 isoforms are compared to highlight the dynamic range, the inset shows isoforms that make up the lowest 1.5% of signal.

CONCLUSIONS

In this application note, the feasibility of the Group triggered MIDAS™ workflow was demonstrated for the characterization and profiling of glycopeptides derived from therapeutically relevant antibodies (Trastuzumab and SiluMab) using the QTRAP® 6500 System.

- HexNAc (m/z 204) and HexHexNAc (m/z 366) were used as primary MRMs to trigger further confirmatory secondary MRMs such as Fucose, bisecting-ions, core-ions, Y1^{2+} ion, B-ion (HexHexNAcFuc+H+)$^{+}$ and sialic acid-ions
- Overlaid primary and secondary MRMs, as well as good proximity scores from SimGlycan on the triggered MS/MS spectra provided confident detection
- Total 23 glycopeptide isoforms for Trastuzumab and 16 for SiluMab were identified and quantified by monitoring both peptide 1 and peptide 2 (missed cleavage)
- Wide abundance variation in the detected glycopeptides highlights the benefit of using targeted detection on the QTRAP® 6500 system.
- Future work includes separation optimization, such as different column types or CESI

REFERENCES

1. Novel LC-MS/MS Workflows for Quantitative Analysis of Glycoform Distribution in Human Immunoglobulin Proteins, Christof Lenz and Jenny Albanese (2008), Technical Note, SCIEX.
2. Rapid Characterization of Biologics using a CESI 8000 – AB SCIEX TripleTOF® 5600+ System, Mark Lies, Clarence Lew, Jose-Luis Gallegos-Perez, Bryan Fonslow, Rajeswari Lakshmanan and Andras Guttman, Technical Note, SCIEX Separations.
3. Rapid Level 3 Characterization of Biologics using a CESI 8000, Bryan Fonslow and Andras Guttman, Technical Note, SCIEX Separations

TRADEMARKS/LICENSING