Journal of Chromatographic Science 2015;53:443–449
doi:10.1093/chromsci/bmu229

Article

Rapid Level-3 Characterization of Therapeutic Antibodies by Capillary Electrophoresis Electrospray Ionization Mass Spectrometry

Clarence Lew[1], Jose-Luis Gallegos-Perez[1], Bryan Fonslow[1], Mark Lies[1] and Andras Guttman[1,2*]

[1]SCIEX Brea, and Framingham, MA, USA, and [2]MTA-PE Translational Glycomics Group, University of Pannonia, Veszprem, Hungary

*Author to whom correspondence should be addressed. Email: a.guttman@neu.edu

Received 5 October 2014; revised 9 December 2014

With the increase of the number of approved protein therapeutics in the market, comprehensive and reproducible characterization of these new generation drugs is crucial for the biopharmaceutical industry and regulatory agencies. One of the largest groups of biotherapeutics is monoclonal antibodies (mABs) possessing various posttranslational modifications and potential degradation hotspots during the manufacturing process that may affect efficacy and immunogenicity. The exceptionally high separation power of capillary electrophoresis (CE) in conjunction with mass spectrometry fulfills Level-3 characterization requirements necessary to reveal such modifications and degradations. In this paper, a comprehensive characterization example will be given for a representative mAB Trastuzumab (Herceptin), illustrating the benefits of the integration of CE and electrospray ionization in a unified bioanalytical process coupled with high-resolution mass spectrometry. Peptides separated in a wide size range (3–65 amino acids) were identified with 100% sequence coverage and quantified, including degradative hotspots such as glutamic acid cyclization, methionine oxidation, aspargine deamidation and C-terminal lysine heterogeneity using only 100 fmol of a single protease digest sample. The low flow rate of the system (>20 nL/min) ensured maximized ionization efficiency and dramatically reduced ion suppression.

Introduction

Monoclonal antibodies (mAbs) make up an important class of biotherapeutics undergoing significant growth in the pharmaceutical industry. Currently, >30 mAbs have been approved for use in treatment of a number of indications ranging from various forms of cancer to autoimmune and infectious diseases. Therapeutic pipelines for mAbs and mAb-like molecules like bi-specific antibodies, single chain variable fragments and antibody drug conjugates are expanding. Due to approaching innovator patent expirations, a growing number of mAb biosimilar and biobetter products are also in development. Consequently, there has been a shift towards more comprehensive characterization of both innovator mAbs and their biosimilar alternatives. Changes in primary amino acid sequences, quality attribute modifications and post-translational modifications (PTMs; such as glycosylation) may impact therapeutic efficacy, bioavailability and biosafety.

Capillary electrophoresis (CE) technology using absorbance- or fluorescence-based detection methods has long been applied in the biopharmaceutical industry for characterization of mAb purity and heterogeneity in the forms of capillary zone electrophoresis, capillary isoelectric focusing and capillary SDS gel electrophoresis (1). Studies exploring the transferability of this technique illustrate excellent robustness when performed in different laboratories in various geographical locations by different instrument operators (2, 3). The exceptionally high separation power of CE in conjunction with the sample identification capability of mass spectrometry fulfills Level-3 characterization requirements necessary to reveal mAB modifications and degradations. Some important attributes including primary sequence, presence of degradation hotspots and PTMs like glycosylation are only detected, localized and quantified by peptide analysis. Integration of CE and electrospray ionization (ESI) using a sheathless etched capillary interface (4) into a single dynamic process (CESI) readily facilitated this work (5). CESI provides a separation mechanism orthogonal to LC–MS-based approaches for separation, identification, quantification and validation of sequence variants as well as PTMs. The extremely low flow rate of CESI (~20 nL/min) is highly beneficial in maximizing ionization efficiency and minimizing ion suppression (6). An example of this is the strong ionization of such hydrophilic species as glycopeptides. The inherent separation efficiency of CE provides sharp peptide peaks for sensitive and reproducible identification, as well as relative quantitation. Using the open tubular format of CESI without any solid stationary phase, all peptides (both hydrophobic and hydrophilic) migrate through the capillary, supporting high mAb sequence coverage and essentially eliminating sample losses and carry over.

This paper provides a comprehensive characterization example of a representative mAB Trastuzumab (Herceptin) as a continuation of our previous collaborative work (7), illustrating the benefits of CESI coupled with high-resolution mass spectrometry. Small and large peptides in the range of 3–65 amino acids have been separated, identified with 100% sequence coverage and quantified, including degradative hotspots such as glutamic acid cyclization, methionine oxidation, asparagine deamidation and C-terminal lysine heterogeneity using a single separation of only 100 fmol of trypsin protease-digested sample. Importantly, qualitative and quantitative analysis of mAb purity, stability and glycoform heterogeneity is possible from a single CESI-MS run with the use of a single protease in the digestion reaction (trypsin).

Experimental

Chemicals

The Trastuzumab sample was a kind gift of Dr Alain Beck of Pierre Fabre (France). Gold trypsin was purchased from Promega (Madison, WI, USA), Rapigest from Waters, (Milford, MA, USA). All other chemicals were from Sigma-Aldrich (St Louis, MO, USA).

© The Author 2015. Published by Oxford University Press. All rights reserved. For Permissions, please email: journals.permissions@oup.com

Sample preparation

A hundred micrograms of Trastuzumab were solubilized using Rapigest (Waters) followed by reduction with dithiothreitol and alkylation by iodoacetamide. The resulting sample was digested overnight with Gold trypsin (Promega) at 37°C, dried and resuspended in 300 μL of 133 mM ammonium acetate (pH 4), yielding a final concentration of 0.33 μg/μL of digested antibody.

CESI-MS and CES-MS/MS

CESI-MS and CESI-MS/MS analysis was performed on a CESI 8000 High Performance Separation – ESI Module (SCIEX, Brea, CA, USA) using a 30 μm ID × 90 cm bare-fused silica capillary housed in an OptiMS CESI cartridge (Sciex), with recirculating liquid coolant set to 25°C, and coupled to a TripleTOF 5600+ mass spectrometer (AB SCIEX, Redwood Shores, CA). Fifty nanoliters of sample (equivalent to 100 fmol of digested antibody) in 100 mM leading electrolyte were introduced into the bare-fused silica separation capillary and transient-isotachophoresis (t-ITP) was applied to focus the sample components for the electrophoretic separation. The background electrolyte was 1.67 M acetic acid and 20 kV constant voltage was applied for the separation. Under the influence of the electric field, the analytes migrated within the separation capillary according to their charge to hydrodynamic volume ratio and upon reaching the porous sprayer tip of the column they were introduced into the mass spectrometer by ESI.

Data acquisition and analysis

Information-dependent acquisition (IDA) mode with high-resolution TOF MS survey scan followed by several MS/MS scans was utilized to acquire the data. The IDA parameters were as follows: 100 ms TOF MS survey scan, 50 ms IDA on the top 30 ions which exceed 200 cps, rolling collision energy to induce fragmentation. The dynamic exclusion time was set to 10 s. The total cycle time was equal to 1.8 s and the IDA parameters were optimized so that the duty cycle of the MS readily supported the high-speed CE separation. By enabling auto calibration during CE-MS batches, the instrument was automatically calibrated once every three runs, limiting deviation of the mass measurement accuracy. Data analysis was performed using AB SCIEX BioPharmaView™, ProteinPilot™ and PeakView® software packages (AB Sciex). If necessary, some confirmation was manually performed (gray sections in Figure 2A).

Results

Although a number of mABs have been approved by regulators for commercial use, only just a few of these have been as comprehensively characterized as Trastuzumab. The work reported in this paper utilized a Level 3 (bottom-up) analysis approach in which the therapeutic antibody was digested using only a single enzyme (trypsin). Please note that development of a universal enzyme approach for all biologics would greatly simplify sample preparation and also help in overcoming matrix effects. From the viewpoint of workflow efficiency, the single enzyme approach can be beneficial under circumstances in which numerous molecules are analyzed in parallel and where it is unclear which choice of enzyme would provide the best results.

Figure 1 depicts the Level 3 CESI-MS characterization of the tryptic digest of Trastuzumab. The upper panel shows the base peak electropherogram of the experiment (8). In the lower panel of Figure 1, the selected precursor ions from the IDA are observed. The abundance of these ions over all the m/z value range increased the probability of obtaining more peptide identifications. These ions were analyzed by MS/MS, making possible to identify tryptic peptides with high confidence, including very small and very large species (from 3 up to 63 amino acids). The open tubular arrangement of the CE capillary allowed the detection of hydrophobic and hydrophilic peptides avoiding discrimination due of lack of interactions with the capillary surface. The migration time variation of the separating peptides was <30 s ($\leq$0.83% RSD) over the 60 min separation. The relative abundance of modified and unmodified peptides varied <2%.

Achieving 100% sequence coverage

Figure 2 depicts the protein sequence coverage using a combined software and manual approach. The left panel shows the automated sequence information of the heavy and light chains (HC and LC). Manual determination was used for short peptide sequences (lower panel) that are commonly excluded from protein database search parameters (gray sequence sections). Using this combined approach, 100% amino acid sequence coverage was obtained from this single set of data for both the HC and LC of Trastuzumab. The left panel also shows the two protein groups and representative peptide identifications that have been assigned to the HC and LC. Short peptide matches were further validated manually (right panel) based on their MS/MS spectra. The right panel of Figure 2 further highlights the excellent separation capability of the CESI-MS setup for small peptides. Table I lists the CESI-MS identified degradation hotspots in Trastuzumab.

Glycopeptide mapping

Trastuzumab contains a conserved consensus glycosylation site at Asn300 of the HC possessing various glycoforms. Because mAbs are made up of two HCs, various combinations of glycoforms may be present, resulting in significant complexity. With CESI, very high ionization efficiency of the glycopeptides was achieved, generating strong signals and allowing for MS/MS identification of even low abundance species. As the migration time of these glycopeptides differs depending upon glycosylation structure, the mobility of the glycopeptides becomes a valuable aid in structural assignment. Since tryptic peptides separated by CESI-MS retain all associated glycosylation, specific amino acid linkage can be identified allowing confirmation that linkage occurs at Asn300. As Table II depicts, in this Trastuzumab sample, 14 different glycoforms were identified on the EEQYNSTYR peptide (Figure 3). The relative abundances of the glycans ranged from 47% to as low as 0.29% (Table II). Approximately half of these glycan species were present in low relative abundance but were still ionized and identified by CESI-MS.

Discussion

Detailed analysis of degradation hotspots

The characteristic highly resolved CE peaks shown in Figure 1 were used for comparative peptide mass mapping, i.e., extracting peptide peak areas with the combined high resolution separation of CESI in the time dimension and high resolving power of MS in

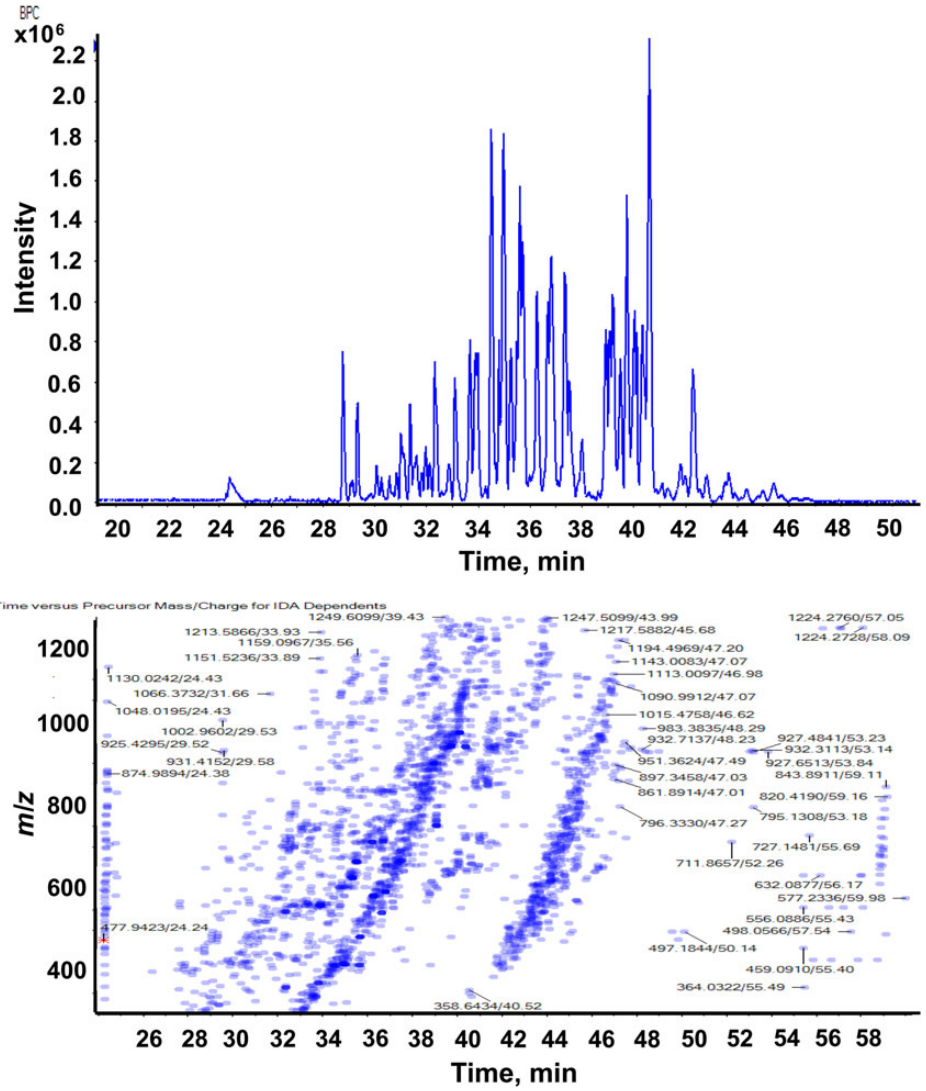


Figure 1. CESI-MS analysis of the tryptic digest of Trastuzumab. Upper panel: base peak electropherogram; lower panel: distribution of precursor m/z values over time. Conditions: OptiMS capillary (90 cm × 30 μm) with etched sprayer. Injection: 50 nL; background electrolyte: 1.67 M acetic acid; Separation voltage: 20 kV; Separation temperature: 25°C.

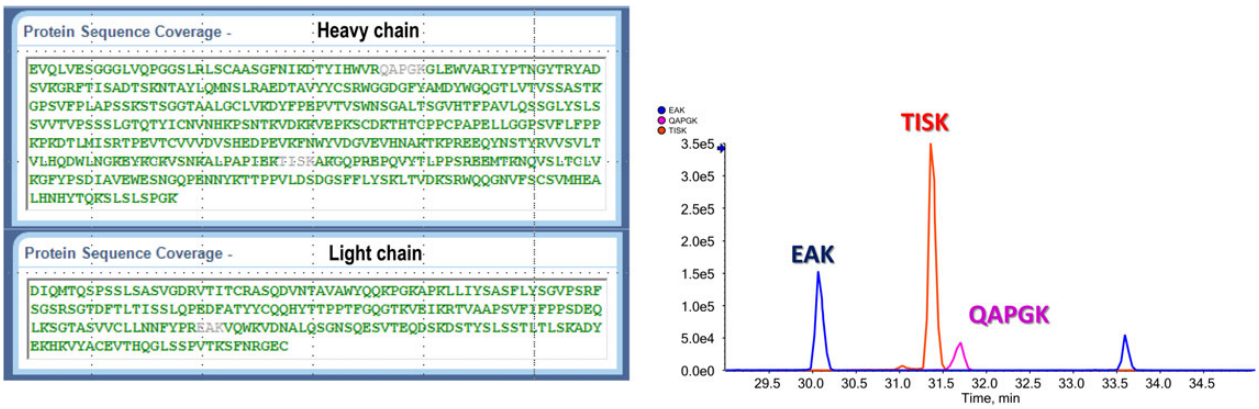


Figure 2. Database Search results of CESI-MS data (left panel) and additional manual processing (right panel) resulted in 100% sequence coverage of Trastazumab HC and LC.

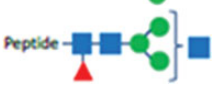
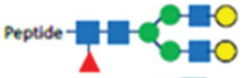
the mass dimension. A hundred percent sequence coverages were achieved in this way for each of the CESI-MS analyses performed, indicating the advantages of CE separations for comprehensive characterization of an mAb from a single digestion step using only trypsin and a single 60 min separation. Extracted ion electropherograms (EIEs) were analyzed to

Table I
List and Positions of Degradation Hotspots in Trastuzumab by CESI-MS

Modification	Protein localization	Sequence and associated mass shift	Monoisotopic mass $[M+H]^+$	Migration time (min)	Relative abundance (%)
Pyroglutamate formation	N-terminal	E(−18.010565)VQLVESGGGLVQPGGSLR	1,863.9923	46.22	3.26
Methionine oxidation	Met255	DTLM(15.994915)ISR	851.4291	35.79	1.56
Methionine oxidation	Met83	NTAYLQM(15.994915)N	970.4299	14.62	1.14
Asparagine deamidation	Asn55	R.IYPTN(0.984016)GYTR.Y	1,085.5262	36.65	92.47
	Asn387	K.GFYPSDIAVEWESN(0.984016)GQPENNY.K	2,545.1154	40.82	
	Asn387 & Asn392	K.GFYPSDIAVEWESN(0.984016)GQPEN(0.984016)NY.K	2,546.0994	4169	
	Asn387 & Asn393	K.GFYPSDIAVEWESN(0.984016)GQPENN(0.984016)Y.K	2,546.0994	41.86	79.00*
	Asn30	R.ASQDVN(0.984016)TAVAWYQQ.P	1,708.8289	39.07	48.06
	Asn152	K.VDNALQSGN(0.984016)SQESVTEQDSK.D	2,136.9527	41.14	13.00
C-terminal lysine loss	C-terminal	K.SLSLSPGK(128.094963)	660.3563	40.5	>99

*Overall value.

Table II
Structural Identification of the Glycosylation of the EEQYNSTYR Glycopeptide on the Trastuzumab Heavy Chain (Asn300)

Glycan abbreviation (structural name)	Glycopeptide identified as R.EEQYN(Glycan)STYR.V Asn300	Glycan mass (Da)	Monoisotopic mass $[M+H]^+$	Migration time (min)	Relative abundance (%)[a]
A1 (G0-GlcNAc)		1,095.3966	2,284.9036	40.42	1.39
MS (Man5)		1,216.4228	2,405.9349	40.5	3.52
FA1 (G0F-GlcNAc)		1,241.4545	2,430.9665	41.02	2.41
A1G1 (G1-GlcNAc)		1,257.4494	2,446.9614	41.02	0.48
A2 (G0)		1,298.4760	2,487.9880	41.02	5.50
FA1G1 (G1F-GlcNAc)		1,403.5073	2,593.01931	41.08	0.33
FA2 (G0F)		1,444.5339	2,634.0459	41.00	39.06
A2G1 (G1)		1,460.5288	2,650.0408	41.35	1.86
FA2G1 (G1F)		1,606.5867	2,796.0987	41.36	36.68
FA1G1S1 (Hex4NAc3FS)		1,694.6027	2,884.1147	46.06	0.30
FA2G2 (G2F)		1,768.6395	2,958.1515	41.79	6.80
FA2BG1 (Hex4NAc5F)		1,809.6661	2,999.1781	41.62	0.29
FA2G1S1 (Hex4NAc4FS)		1,897.6821	3,037.1785	46.37	0.63
FA2G2S1 (A1F)		2,059.7349	3,249.2469	46.63	0.74

[a]As a quantitative approximation, relative abundances were calculated using peak areas of glycosylated peptides of the same sequence at the charge state detected (+2 or +3).
Glycan structure interpretation followed the Consortium of Functional Glycomics (CFG) protocol.

determine the presence of degradation hotspots, which can commonly occur during processing and storage of biopharmaceuticals. To illustrate the capabilities of CESI-MS, identification and quantification of degradative hot spots on peptides within Trastuzumab are delineated in Table I as pyroglutamate formation (N-terminal glutamate cyclization), methionine oxidation,

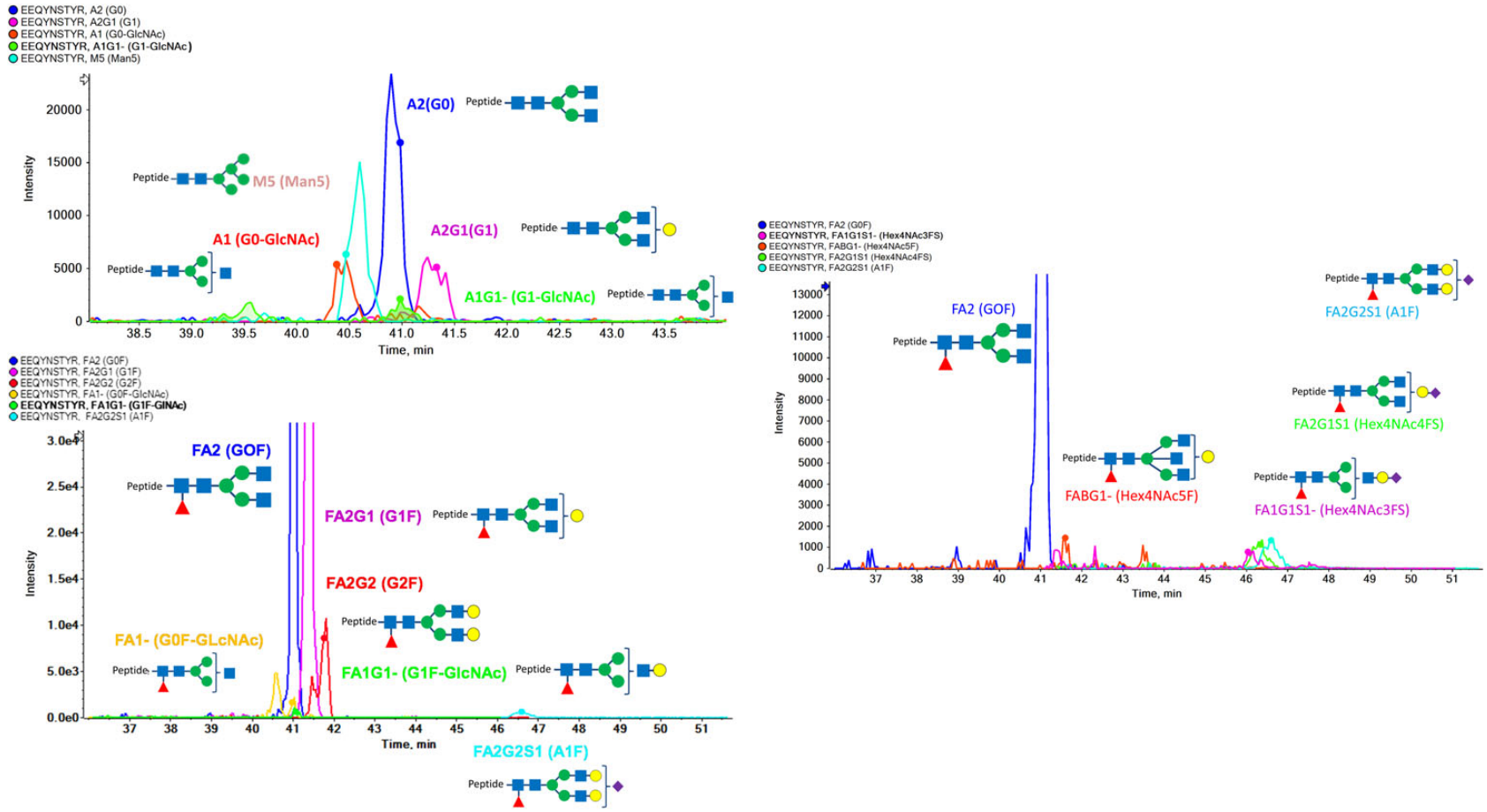


Figure 3. Glycosylation structures on the EEQYNSTYR peptide identified by CESI-MS. Upper left panel: non-fucosylated structures; lower left panel: fucosylated structures, right panel: special structures with bisected *N*-acetylglucosamine and sialic acids. Further glycopeptide characterization is shown in Table II. Glycan structure interpretation followed the CFG protocol.

asparagine deamidation and C-terminal lysine loss. Additional less abundant sites of the same degradative hotspots, among a few others, such as lysine glycation and/or tryptophan dioxidation were also identified but not described here due to their very low levels.

N-Terminal modification can result in cyclization of glutamine to form pyroglutamate. Although not deemed critical, pyroglutamate formation has been proposed to increase the *in vivo* half-life of antibodies and is an indicator of manufacturing process control (9). MS/MS spectra revealed both cyclized and unmodified forms of the N-terminal peptide, which differed by 18 amu. Extracted ion data suggested only a small pyroglutamate modification of 3.26%. Pyroglutamate cyclization leads to loss of a positive charge resulting in lower electrophoretic mobility of the modified peptide relative to the unmodified one. This is advantageous since the modified and unmodified forms can be well separated by CESI in addition to structural confirmation using MS/MS.

Methionine (Met) oxidation is another common modification that is also associated with mAb manufacturing and storage but linked to a decrease in mAb stability and biological activity (10). Analyzing the extracted ion data revealed oxidation at Met255 and Met83 on the HC (Table I) and indicated the presence of both oxidized and unmodified methionine at both sites in the Trastuzumab sample. MS/MS diagnostic ions confirmed the MetOx modification by a +16 amu molecular mass addition relative to unmodified Met in both sites. From EIEs, the relative abundances of MetOx255 and MetOx83 were determined to be 1.56 and 1.14%, respectively.

Extracted ion analysis of the data indicated quite a few incidences of asparagine deamidation, specifically at Asn55, Asn387, Asn392 and Asn393 in the HC and Asn30 and Asn152 in the light chain. MS/MS diagnostic ions for each of these identified +1 amu difference confirming each instance of deamidation (Table I). With CESI, these two forms can be readily separated from each other, making their assignment and differentiation from ionization artifacts much easier. This is particularly important to emphasize since deamidated peptides are not always resolved by LC–MS analyses and may co-elute with unmodified peptides. Additionally, the isotopic envelopes of the peptides, only differing by 1 amu, can also overlap and therefore misassigned by automated identification softwares, even with high-resolution MS and MS/MS data. Separation of these peptides by CESI-MS can address both of these challenges with LC–MS.

The analysis of C-terminal lysine heterogeneity was also shown in Table I. The loss of a positively charged lysine on the HC can be confirmed by a large migration shift. From the extracted ion electropherograms (EIEs), C-terminal lysine loss on the HC was >99%.

Glycopeptide analysis

One of the most important effector function-related PTMs common to mAbs is glycosylation. The presence or absence of specific glycan residues can significantly alter the efficacy, stability and immunogenicity of mAbs. Since CE has long been reported as a robust technique for oligosaccharide analysis, application of this fundamental capability of CE to separate glycosylated species based on charge and hydrodynamic radius (i.e., differential electromigration) offering another dimension of analysis by which glycoform identity can be readily determined. Similarly to degradative PTM analysis, there are notable benefits from CESI-MS analysis of glycopeptides. First, with the low nL/min flow rates, glycopeptides are readily ionized among other peptides, illustrated by the comprehensiveness and dynamic range of glycopeptides identified (Table II). Additionally, since glycopeptides regularly co-elute in LC–MS analysis, CESI-MS separation of glycopeptide is advantageous. The electromigration-based separation of glycopeptides helped to confirm glycan structures. Unwanted fragmentation of glycans prior to MS and MS/MS (i.e., in-source fragmentation) were identified as electrophoretic peak shoulders and non-Gaussian shape peaks. Thus, the separation of glycopeptides by CESI-MS added both sensitivity and confidence to glycosylation characterization.

Conclusions

In this paper, we illustrated the use of CE electrospray ionization mass spectrometry (CESI-MS) technique for rapid sequencing and degradation hotspot analysis (Level 3 characterization) of the therapeutic antibody Trastuzumab. Starting with efficient sample preparation using a single enzyme, single digestion protocol followed by a single separation step, 100% primary sequence coverage for both the HC and LC of this important mAb therapeutic was rapidly obtained. In addition, identification of key amino acid modifications was accomplished, resulting in the elucidation of glutamine cyclization, methionine oxidation, asparagine deamidation and C-terminal lysine heterogeneity. From the same CESI-MS separation, glycopeptide analysis was performed, resulting structural identification for as many as 14 glycoforms, many in low abundance. Strong ionization signals for accurate mass measurement and subsequent MS/MS coupled with structure-based mobility alterations greatly simplified the determination and assignment of the bound glycan structures. Because of its ultra-low flow capability, CESI-MS provided remarkable sensitivity enhancements for poorly ionizing molecular species like glycopeptides, and when coupled to a fast accurate MS platform, efficiently delivered broad mAb characterization from a single protease digestion and single CESI-MS run.

Acknowledgments

The authors thank Dr Alain Beck (Pierre Fabre, France) for providing the Trastazumab sample. The generous support of Jeff Chapman is also greatly appreciated. AG recognizes the support of the MTA-PE Translational Glycomics project (#97101).

References

1. Guttman, A.; Bioanalytical tools for the characterization of biologics and biosimilars; *LC.GC Magazine*, (2012); 30: 412–421.
2. Nunnally, B., Park, S.S., Patel, K., Hong, M., Zhang, X., Wang, S.X., *et al.* A series of collaborations between various pharmaceutical companies and regulatory authorities concerning the analysis of biomolecules using capillary electrophoresis; *Chromatographia*, (2006); 64: 359–368.
3. Salas-Solano, O., Babu, K., Park, S.S., Zhang, X., Zhang, L., Sosic, Z., *et al.* Intercompany study to evaluate the robustness of capillary isoelectric focusing technology for the analysis of monoclonal antibodies; *Chromatographia*, (2011); 73: 1137–1144.
4. Moini, M.; Simplifying CE-MS operation. 2. Interfacing low-flow separation techniques to mass spectrometry using a porous tip; *Analytical Chemistry*, (2007); 79(11): 4241–4246.

5. Busnel, J.M., Schoenmaker, B., Ramautar, R., Carrasco-Pancorbo, A., Ratnayake, C., Feitelson, J.S., *et al.* High capacity capillary electrophoresis-electrospray ionization mass spectrometry: coupling a porous sheathless interface with transient-isotachophoresis; *Analytical Chemistry*, (2010); 82(22): 9476–9483.
6. Schmidt, A., Karas, M., Dulcks, T.; Effect of different solution flow rates on analyte ion signals in nano-ESI MS, or: when does ESI turn into nano-ESI?; *Journal of the American Society for Mass Spectrometry*, (2003); 14(5): 492–500.
7. Gahoual, R., Burr, A., Busnel, J.M., Kuhn, L., Hammann, P., Beck, A., *et al.* Rapid and multi-level characterization of trastuzumab using sheathless capillary electrophoresis-tandem mass spectrometry; *MAbs*, (2013); 5(3): 479–490.
8. Williams, B.J., Russell, W.K., Russell, D.H.; Utility of CE-MS data in protein identification; *Analytical Chemistry*, (2007); 79(10): 3850–3855.
9. Liu, Y.D., Goetze, A.M., Bass, R.B., Flynn, G.C.; N-terminal glutamate to pyroglutamate conversion in vivo for human IgG2 antibodies; *The Journal of Biological Chemistry*, (2011); 286(13): 11211–7.
10. Pan, H., Chen, K., Chu, L., Kinderman, F., Apostol, I., Huang, G.; Methionine oxidation in human IgG2 Fc decreases binding affinities to protein A and FcRn; *Protein Science*, (2009); 18(2): 424–433.