Comprehensive characterization of trastuzumab using chip-based integrated icIEF-MS technology and correlation of intact and bottom-up peptide mapping analysis

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ABSTRACT

Presented here are results of comprehensive charge variant analysis of trastuzumab. In addition to intact PTM analysis by microfluidic chip-based integrated icIEF-MS technology, glycopeptide analysis was done using HRMS with fragmentation by electron activated disassociation (EAD). Intact analysis using the Intabio icIEF-MS system provides a rapid means to identify both charge-based and neutral modifications while preserving important intact immune-modulating structures such as the pairing of glycans within the Fc binding domain. Bottom-up analysis by EAD was highly effective in confirming the glycosylated asparagine residue (N300) along with the N-linked glycan structures attached to the mAb.

Six charge variant peaks with isoelectric points (pI) ranging between 8.83 and 9.13 were separated for trastuzumab by imaged capillary isoelectric focusing (icIEF). Quantification of focused charge variants by UV absorbance was used to calculate percent composition values which ranged between 1.7 and 61.0%. Following characterization by UV absorbance imaging, the focused charge variant peaks were mobilized to an on-chip ESI emitter tip for introduction into a TOF HRMS. The resulting deconvoluted mass spectrum was utilized to identify c-terminal lysine process variants, succinimide intermediates, glycation, deamidation and sialylated glycans to their corresponding charge variant in the trastuzumab charge profile. Glycopeptide analysis of trastuzumab by EAD was then used to refine the assignment of glycan pairs and support the presence of glycated lysine residues.

INTRODUCTION

Monoclonal antibodies (mAb) are an important class of therapeutic proteins utilized in a wide range of treatments. During manufacturing, post translational and chemical alterations may occur to the mAb structure, resulting in a drug product comprised of a heterogenous mixture of proteoforms. Separation, characterization and identification of these alterations is essential to establishing critical quality attributes (CQA's) and ensure consistent safety, efficacy and potency performance of a therapeutic mAb. Recent improvements in intact protein analysis such as icIEF-MS have enabled the rapid measurement of multiple CQAs. Analytical workflows that incorporate these new intact protein analyses with traditional bottom-up characterization techniques offer a much more comprehensive assessment of mAb variants in a therapeutic preparation.

MATERIALS AND METHODS

Sample preparation:
For sample analysis by the research breadboard Intabio icIEF-MS system, trastuzumab was reconstituted with 7.4 mL of 18 MΩ water to 21 mg/mL. Formulation excipients were removed by a spin desalting column, 7k Da MWCO (Thermo Fisher Scientific) and 500 µL desalted aliquots were stored at -80ºC. To prepare the 200-µL icIEF-MS samples, 80 µg of desalted trastuzumab was combined with 3% Pharmalyte 8-10.5 pH (Cytiva), 3% Pharmalyte 5-8 pH (Cytiva), 7.5 mM L-arginine (Fisher Bioreagents) and 6.25 µg pI 5.52 and 9.99 markers. The sample was vortexed, loaded into a sample vial and placed into the Intabio icIEF-MS system.
For peptide mapping, trastuzumab samples were denaturated in 7.2M guanidine hydrochloride, 100mM Tris pH 7.2, followed by reduction and alkylation with 10mM DL-dithiothreitol and 30mM iodoacetamide. Digestion was performed with trypsin/Lys-C enzyme at 37ºC for 16 hours.

Separation conditions:
icIEF separation, imaging and electrospray ionization was performed on a research breadboard Intabio icIEF-MS system. The sample was focused using 1% formic acid (anolyte) and 1% diethylamine (catholyte) for 1 min at 1500 V and then 1 min at 3000 V followed by 4.5 min at 4500 V. Mobilization and ESI were performed with 25% acetic acid and 25% acetonitrile flowing at 2.5 µL/min for 6.0 min while 3000 V was applied between the anolyte and mobilizer and 5500 V was applied at the tip.
For peptide separation, 4 µg of the trypsin/Lys-C digest were separated with CSH C-18 column (1.7 particle size, 130 Å, 21x100 mm, Waters) using a ExionLC system. The mobile phase A consisted of 0.1% formic acid in water and the organic phase B was 0.1% formic acid in acetonitrile. A gradient prolife at a flow rate of 300 µL/min was used to elute the peptides while the column temperature was maintained at 50ºC. Peptides were introduced into the MS using ESI at a 5500 V potential and a nebulizer gas pressure of 50 psi.

MS conditions and analysis:
For intact trastuzumab analysis, a ZenoTOF 7600 system from SCIEX was used to analyze the mobilized peaks. During the mobilization step, the MS was set to scan 2,000-6,000 *m/z* at 2 Hz. The interface was set at 100ºC with a curtain gas setting of 25 psi. The declustering potential was set at 285 eV and the collision energy was set at 60 eV.
Peptide analysis was acquired using an information dependent acquisition (IDA) method using the ZenoTOF 7600 system. The interface was set at 450ºC with curtain gas set at 35 psi. The MS was set to scan from 100-3000 *m/z*. EAD parameters were 12 V for collision energy, 7 eV for electron KE, 5500 nA for electron beam current, an ETC of 100 and an accumulation time of 0.09 sec. The resulting intact spectrum was analyzed in the research breadboard Intabio icIEF-MS workflow in BYOS (Protein Metrics Inc.). After deconvolution, mass isoforms were identified by a delta mass from a reference of 145173.5 Da for the deglycosylated trastuzumab. Peptide MS/MS spectra was also analyzed by BYOS with mass tolerances for peptides and fragments set at 6 and 20 ppm respectively. MS/MS scores lower than 100 were filtered.

Figure 1. Streamlined workflow using the Intabio icIEF-MS system.

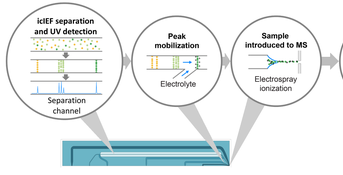
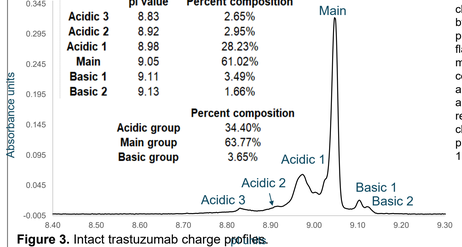


Figure 1. Illustrates the streamlined process of icIEF-MS analysis using the Intabio icIEF-MS system. After loading the separation channel with sample, an electrical potential is applied between the anolyte (anode) and catholyte (ground) channels to start the focusing process. During focusing, absorbance images at 280 nm are taken of the forming gradient and stacking sample components. At the completion of focusing, the ground is switched from the catholyte to the mobilizer channel. The anion in the mobilizer solution disrupts the pH gradient and transports the focused bands via electrophoresis to the incorporated ESI tip for electrospray and downstream MS analysis.

Figure 2. Trastuzumab charge variant characterization and quantitation

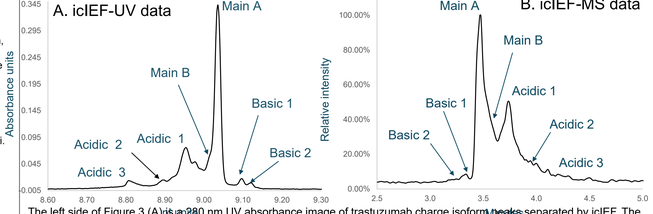


	pI value	Percent composition
Acidic 3	8.83	2.65%
Acidic 2	8.92	2.95%
Acidic 1	8.98	28.23%
Main	9.05	61.02%
Basic 1	9.11	3.49%
Basic 2	9.13	1.66%

Percent composition	
Acidic group	34.40%
Main group	63.77%
Basic group	3.65%

Figure 2. Shows the icIEF focused charge profile of trastuzumab imaged by 280 nm absorbance. The charge profile has 2 basic and 3 acidic peaks flanking the main charge isoform. The main charge isoform has a percent composition of 63.8% with the basic and acidic groups constituting 3.65 and 34.4 percent composition, respectively. Resolution between the charge isoforms can resolve the 0.02 pH unit difference in pI between Basic 1 and Basic 2 charge variants.

Figure 3. Intact trastuzumab charge profiles



The left side of Figure 3 (A) is a 280 nm UV absorbance image of trastuzumab charge isoform peaks separated by icIEF. The right side of Figure 3 (B) is the base peak electropherogram (BPE) of the same corresponding peaks after mobilization, electrospray and detection by MS. The UV and BPE profiles are mirror images of each other as charge variants with higher pI are the first to be analyzed in the MS system.

Figure 4. Confirmation of icIEF-MS glycan pairs by peptide mapping with EAD.

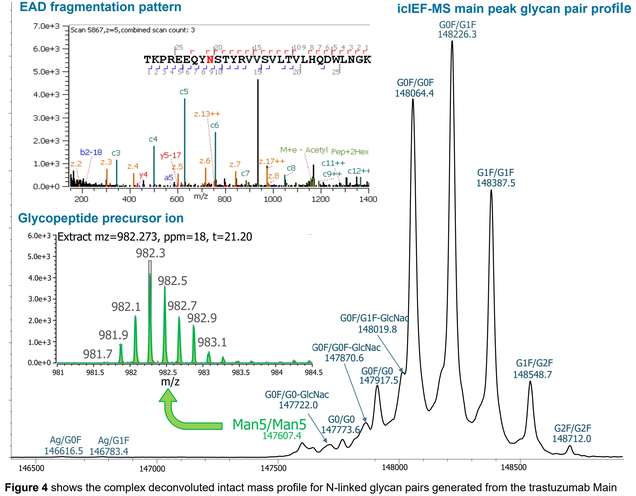


Figure 4 shows the complex deconvoluted intact mass profile for N-linked glycan pairs generated from the trastuzumab Main Peak A mass spectrum. Complimentary analysis of trastuzumab glycopeptides using EAD fragmentation allows for the confirmation of the glycation site in the amino acid sequence and detailed glycan structure. In this example the glycation site is identified as N 300 and the attached glycan structure is Mannose 5.

Table 1. N-linked glycans observed with icIEF-MS confirmed by peptide analysis with EAD fragmentation.

Composition	Glycan	icIEF-MS	EAD confirmation
HexNac(2) Hex(5)	Man 5	Yes	Yes
HexNac(2) Hex(6)	Man 6	Yes	Yes
HexNac(3) Hex(3)	G0-GlcNac	Yes	Yes
HexNac(3) Hex(3) Fuc(1)	G0F-GlcNac	Yes	Yes
HexNac(3) Hex(4) Fuc(1)	G1F-GlcNac	Yes	Yes
HexNac(4) Hex(3)	G0	Yes	Yes
HexNac(4) Hex(3) Fuc(1)	G0F	Yes	Yes
HexNac(4) Hex(4)	G1	Yes	Yes
HexNac(4) Hex(4) Fuc(1)	G1F	Yes	Yes
HexNac(4) Hex(5) Fuc(1)	G2F	Yes	Yes
HexNac(4) Hex(5) Fuc(1) NeuAc(1)	G2F1S	Yes	
HexNac(4) Hex(5) Fuc(1) NeuAc(2)	G2F2S	Yes	Yes

Table 1. Shows the comparisons between the putative glycan pair assignments by icIEF-MS and their confirmation with EAD. Glycopeptide mapping was essential in identifying G2F rather than G1F as the sialyated species. EAD also eliminated potential G3F pairs from the trastuzumab structure further supporting the G2F/G2F+Hex intact mass in multiple acidic peaks of the charge profile as the result of lysine glycation.

Table 2. Trastuzumab mass list and modifications measured by the Intabio icIEF-MS system.

Peak	Mass	Name
Basic 2		
1	147733.02	Clip
2	148052.02	G0F/G0F+Succ
3	148198.99	G0F/G0F+Lys
4	148352.18	G0F/G1F+Lys
5	148515.97	G1F/G1F+Lys
Basic 1		
6	147900.03	G0/G0F+Succ
7	148049.5	G0F/G0F+Succ
8	148210.21	G0F/G1F+Succ
9	148380.72	G1F/G1F+Succ
10	148541.46	G1F/G2F+Succ
Main A		
11	146616.47	Ag/G0F
12	146783.43	Ag/G1F
13	147607.43	M5/M5
14	147721.98	G0F/G0-GlcNac
15	147773.55	G0/G0
16	147870.58	G0F/G0F-GlcNac
17	147917.51	G0F/G0
18	148019.79	G0F/G1F-GlcNac
19	148064.41	G0F/G0F
20	148226.33	G0F/G1F
21	148387.48	G1F/G1F
22	148548.7	G1F/G2F
23	148712.02	G2F/G2F

Peak	Mass	Name
Main B		
25	146620.06	Ag/G0F
26	146774.15	Ag/G1F
27	147575.8	G0/G0-GlcNac
28	147608.98	M5/M5
29	147716.51	G0F/G0-GlcNac
30	147767.18	M5/M6
31	147854.31	G0F/G0F-GlcNac
32	147917.92	G0F/G0
33	148064.47	G0F/G0F
34	148225.95	G0F/G1F
35	148387.45	G1F/G1F
36	148549.01	G1F/G2F
37	148711.48	G2F/G2F
38	148870.01	G2F/G2F +Hex
Acidic 1		
39	146616.43	Ag/G0F
40	146781.93	Ag/G1F
41	147575.55	G0/G0-GlcNac
42	147606.77	M5/M5
43	147721.51	G0F/G0-GlcNac
44	147772.39	G0/G0
45	147857.8	G0F/G0F-GlcNac
46	147918.58	G0F/G1
47	148066.06	G0F/G0F
48	148226.97	G0F/G1F
49	148387.45	G1F/G1F
50	148548.57	G1F/G2F
51	148683.78	G0F/G2F+NeuAc
52	148841.52	G1F/G2F+NeuAc
53	149002.48	G2F/G2F+NeuAc

Peak	Mass	Name
Acidic 2		
54	147780.71	G0/G0
55	147916.92	G0F/G0
56	147944.68	M6/M6
57	148024.87	G0F/G1F-GlcNac
58	148067.47	G0F/G0F
59	148227.54	G0F/G1F
60	148390.04	G1F/G1F
61	148549.98	G1F/G2F
62	148717.25	G2F/G2F
63	148843.46	G1F/G2F+NeuAc
64	148875.37	G2F/G2F +Hex
65	148990.98	G2F/G2F+NeuAc
66	149132.5	G1F/G2F+2NeuAc
Acidic 3		
67	147847.06	G0F/G0F-GlcNac
68	147931.36	M6/M6
69	148065.53	G0F/G0F
70	148226.5	G0F/G1F
71	148390.54	G1F/G1F
72	148547.9	G1F/G2F
73	148688.09	G0F/G2F+NeuAc
74	148712.96	G2F/G2F
75	148835.5	G1F/G2F+NeuAc
76	148860.5	G2F/G2F +Hex

Table 2. Multiple proteoforms were identified from the 6 charge variant peaks separated and analyzed by the research breadboard Intabio icIEF-MS system. Basic Peak 2 primarily contains c-terminal lysine variants, while Basic Peak 1 is comprised solely of succinimide intermediate isoforms. Completely processed C-terminal lysine are present in both the Main and Acidic Peaks. Glycation is first detected in Main Peak B and there is a +1.5 Da shift in the G0F/G0F mass indicating a potential deamidation event. As predicted by the glycopeptide EAD results sialic acid terminated glycans are observed in Acidic Peaks 1, 2 and 3.

CONCLUSIONS

- icIEF-MS is a sensitive and comprehensive approach, requiring only 120 ng of trastuzumab to characterize 7 charge variant peaks and identify multiple molecular features (CQAs)
- Addition of glycopeptide analysis by EAD fragmentation was highly useful in confirming possible paired glycan structures and supports the observation of glycation at the intact level by icIEF-MS

REFERENCES

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