Online IEX-MS Characterization and Monitoring of mAb Charge Heterogeneity Using an Optimized Cation Exchange Resin and Compact TOF Mass Spectrometer

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INTRODUCTION

Ion exchange chromatography (IEX) is a method of choice for the analysis of charge heterogeneity encountered with biotherapeutic drug candidates. Traditionally, IEX separations require high concentrations of salts that are not compatible with mass spectrometry (MS) analysis, which has left a gap in the characterization of charge variants. It has been shown[1,2,3] that direct, MS-based characterization of these charge variants is possible if volatile salts are employed. In this study, MS-compatible IEX separations are combined with a new small footprint benchtop time-of-flight (TOF) MS instrument and applied to a case study on identifying the charge variants formed upon forced degradation.

METHODS

Forced Degradation of Trastuzumab

A sample of Trastuzumab (50 µ L @ 20 mg/mL) was buffer exchanged into 100mM sodium phosphate, pH 8.0 using BioRad Micro Bio-Spin® chromatography columns (#732-6221), according to manufacturer protocol. The buffer exchanged Trastuzumab sample was further diluted to 2 mg/mL in 100mM sodium phosphate, pH 8.0 and was equally split. One half was frozen at –80 °C until analysis and the other was incubated at 25 °C for 1 week.

IdeS Digestion of Trastuzumab and Other mAb samples

50 µ g each Trastuzumab (T0 & 1 week stressed) sample was digested by incubating at 37 °C for 30 min with 50 units of FabRICATOR® enzyme (Genovis, A0-FR1-008) in 25mM NaCl, 25mM Tris, 1mM EDTA, pH 8.0 (with a final sample concentration of 1 mg/mL). 1 mg/mL samples of nonreduced Trastuzumab (T0 & 1 week), NIST mAb, and Infliximab were also prepared for analysis. 10 µ g of each sample was injected for IEX-MS analysis.

IEX-MS

Analyses were performed on the new BioAccord compact TOF MS instrument, which consists of an ACQUITY I-class UPLC with TUV detector and RDa detector.

Figure 1. BioResolve SCX column and BioAccord system (ACQUITY I-class UPLC with TUV and RDa detector, controlled by UNIFI software for acquisition and data processing).

LC Conditions:

Column:	ACQUITY BioResolve SCX (3 µ m, 2.1 x 50 mm)
Column Temp:	30 °C
Flow Rate:	0.1 mL/min
Mobile Phases:	MS-compatible volatile salt buffers (recipes are proprietary)
Gradient (NR):	Hold at 40% B for 1 minute, then 40%-98% B over 20 min (linear), wash at 98%B for 1 min, and re-equilibrate at 40% B for 7 min
Gradient (IdeS):	Hold at 2% B for 1 minute, then 2%-98% B over 20 min (linear), wash at 98% B for 1 min, and re-equilibrate at 2% B for 7 min
Total Run Time:	30 min
Injection Volume:	10 µ L of 1 mg/mL sample

MS Conditions (RDa detector):

Capillary Voltage:	1.5kV
Cone Voltage:	150V
Desolvation Temp:	350 °C
Intelligent Data Capture (IDC):	Enabled.

RESULTS

Separation and Identification of NIST mAb Charge Variants

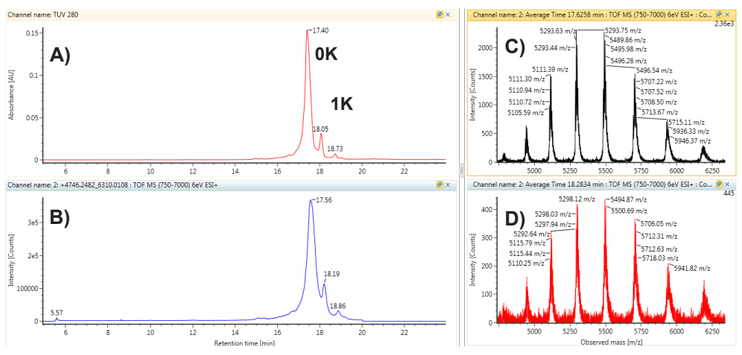


Figure 2. Nonreduced NIST mAb IEX separation and raw MS spectra for main and basic variant peak. A) UV (280nm) trace; B) TIC trace (m/z 4000-6300). The right panel shows combined raw spectra for the C) main peak (NIST mAb + 0K) and D) basic species peak (NIST mAb + 1K)).

Separation and Identification of Infliximab Charge Variants

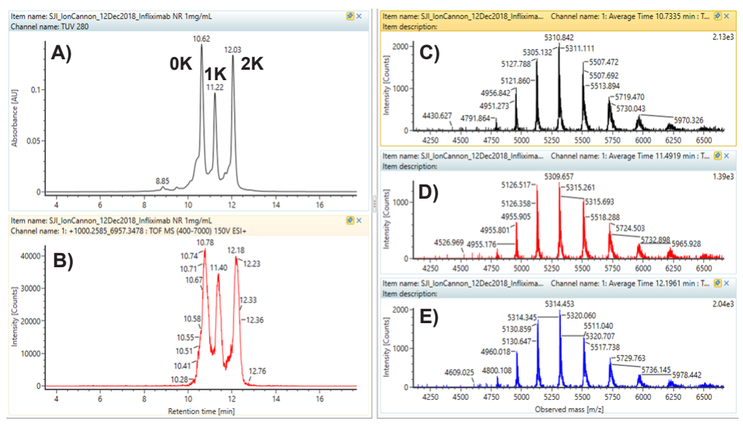
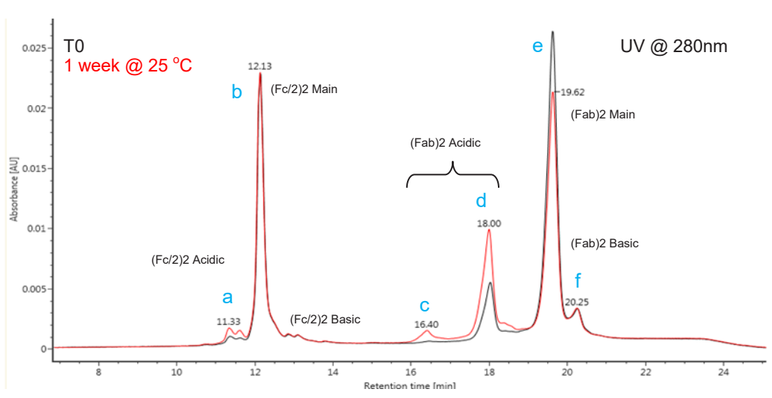


Figure 3. Nonreduced Infliximab IEX separation and raw MS spectra for 3 prominent C-terminal lysine variants. A) UV (280nm) trace; B) TIC trace (m/z 1000-7000). The right panel shows combined raw spectra for the C) Infliximab + 0K, D) Infliximab + 1 K, and E) Infliximab + 2K.

RESULTS

IdeS-Digested Trastuzumab T0 & 1 week

Trastuzumab is a monoclonal antibody with well known susceptibility for deamidation at N30T in the light chain and, to a lesser extent N55G in the heavy chain, which have been well characterized[4]. This antibody is easily deamidated under elevated pH conditions, and is therefore an ideal case study for charge variant monitoring via IEX-MS.



(Fc/2)2	Acidic	Main	Basic
T0	7.1%	84.4%	8.5%
1wk25C	11.6%	81.2%	7.2%
Δ	+4.5%	-3.2%	-1.3%

(Fab)2	Acidic	Main	Basic
T0	20.9%	70.3%	8.9%
1wk25C	34.9%	56.4%	8.7%
Δ	+14.1%	-13.8%	-0.2%

Figure 4. UV (280nm) chromatogram overlay of T0 and 1 week stressed Trastuzumab, IdeS-digested, and corresponding peak integrations.

Figure 4 shows the 280nm UV overlays for injections of IdeS digests of unstressed vs stressed Trastuzumab samples. (Fc/2)2 and (Fab)2 acidic and basic species are well resolved with the BioResolve SCX column. After just 1 week of pH 8.0 stress, there is a significant increase in the (Fab)2 acidic species, as well as a slight increase in Fc region acidic species.

With this online IEX-MS setup, the various species can be directly investigated by mass spectrometry. Figure 4 shows both the combined raw spectra (left) and resulting MaxEnt1 deconvolutions (right) for each peak.

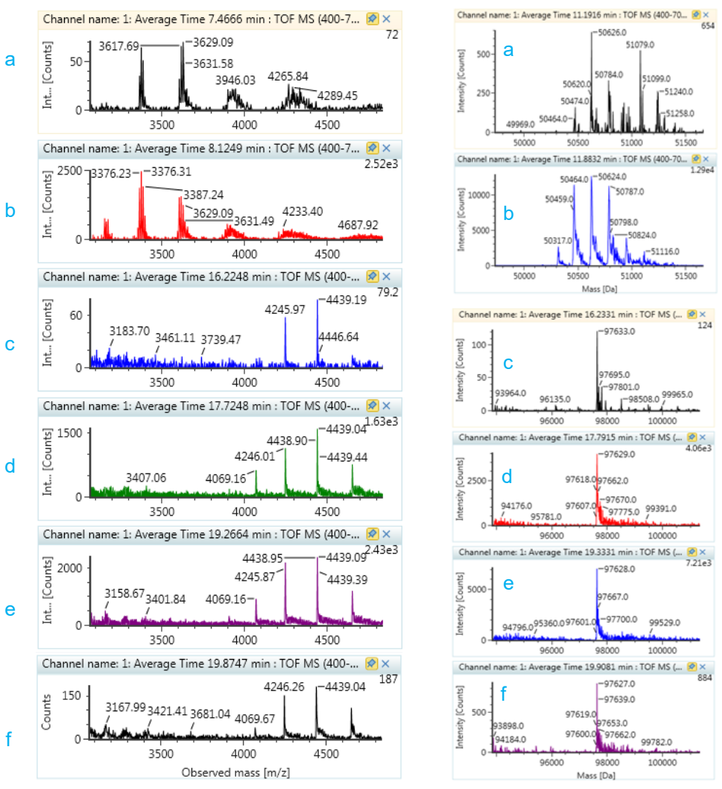


Figure 5. Left panel shows combined raw spectra for peaks a-f in Figure 4, right panel shows the corresponding MaxEnt1 deconvolutions of each peak.

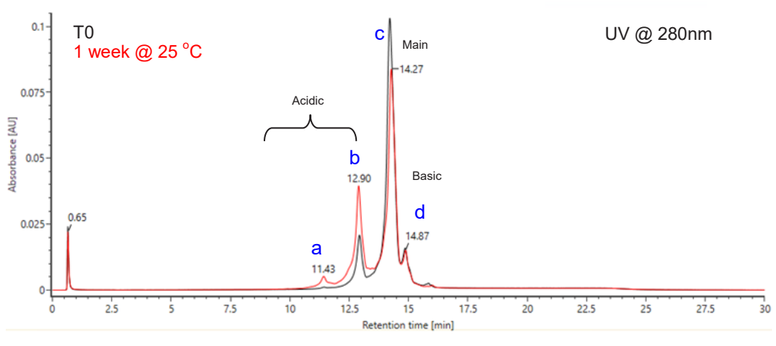
Peak	Retention Time (min)	Peak ID	Δ Mass (Da)[a]	Possible Assignment[b]
a	11.33	(Fc/2)2 Acidic 1	+ 2.0 +292.0	Deamidation + Sialic Acid
b	12.13	(Fc/2)2 Main	-	Unmodified (Fc/2)2
c	16.40	(Fab)2 Acidic 2	+ 5.0	Deamidation
d	18.00	(Fab)2 Acidic 1	+ 1.0	Deamidation
e	19.62	(Fab)2 Main	-	Unmodified (Fab)2
f	20.25	(Fab)2 Basic 1	- 1.0	Possible Disulfide or Conformational Variants

[a] For (Fc/2)2, a representative glycoform is used to calculate Δ mass
[b] RDa detector mass accuracy spec is < 20 ppm for intact mAb based on NIST mAb standard

Table 1. IdeS digestion IEX-MS results and possible assignments for each peak

Nonreduced Trastuzumab T0 & 1 week

Figure 6 shows UV overlays for nonreduced unstressed vs 1 week stressed Trastuzumab, with charged variant species well resolved. An increase of acidic species and decrease of main peak purity is observed for the stressed sample. Figure 7 displays the combined MS spectra and deconvoluted masses for these species.



	Acidic	Main	Basic
T0	14.0%	74.7%	11.3%
1wk25C	32.7%	58.3%	9.0%
Δ	+18.7%	-16.4%	-2.3%

Figure 6. UV (280nm) chromatogram overlay of T0 and 1 week stressed Trastuzumab, nonreduced, and corresponding peak integrations.

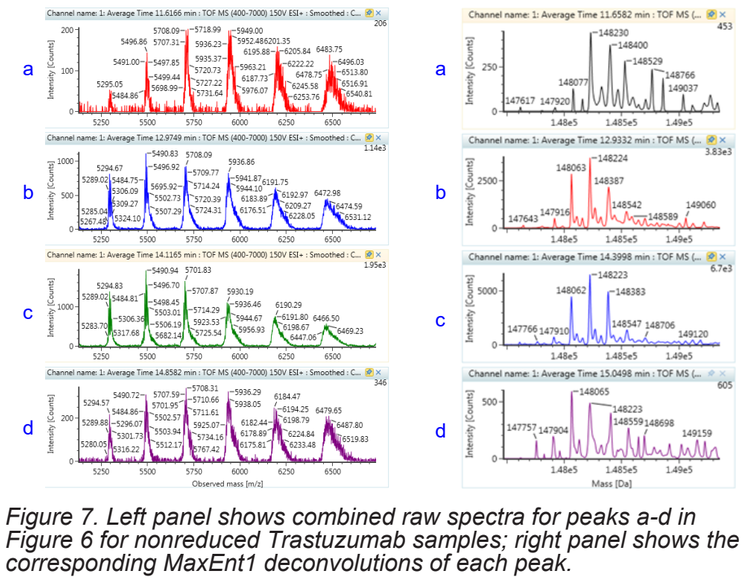


Figure 7. Left panel shows combined raw spectra for peaks a-d in Figure 6 for nonreduced Trastuzumab samples; right panel shows the corresponding MaxEnt1 deconvolutions of each peak.

Peak	Retention Time (min)	Peak ID	Δ Mass (Da)[a]	Possible Assignment[b]
a	11.43	mAb Acidic 2	+ 7.0	Deamidation
b	12.90	mAb Acidic 1	+ 1.0	Deamidation
c	14.27	mAb Main	-	Unmodified mAb
d	14.87	mAb Basic 1	+ 3.0	Possible disulfide or conformational variant

[a] A representative glycoform is used to calculate Δ mass
[b] RDa detector mass accuracy spec is < 20 ppm for intact mAb based on NIST mAb standards

Table 2. Nonreduced Trastuzumab IEX-MS results and possible assignments for each peak

DISCUSSION

Until recently the investigation of charge variants required tedious fraction collection and buffer exchange, or a complex 2D-LC instrument setup, in order to acquire mass spectrometry data. With this online IEX-MS method no fractionation or buffer exchange is necessary to achieve high quality MS data. With the use of a dual pH and volatile salt gradient, we have both chromatographic separation of charge variant species as well as direct native MS detection for peak investigation.

Given its capability, this workflow could be highly useful in the biopharmaceutical industry, where charge variant analysis is widely employed for drug stability studies. Changes in the charge variant profile over time and/or stress conditions are critical to the development of a drug product, as some of these charge variants may have effects on drug potency and toxicity. The direct investigation of new or increasing peaks in the charge profile saves time and effort: possibly avoiding the need to send samples to specialized characterization labs. It also eliminates possible artificial degradation due to sample manipulation during fraction collection.

The method was first established with NIST mAb and Infliximab, as shown in Figures 2 and 3, respectively. The TOF settings were tuned for the optimal ionization of intact mAbs and subunits in native conditions. Source parameters were based on a balance of MS signal intensity and mass resolution along with consideration of what conditions best preserve the native state of the antibody and subunits. For NIST mAb and Infliximab, the prominent charge variants are related to the presence of C-terminal lysine. With this method, the C-terminal lysine additions are easily separated via IEX, and then confirmed with MS detection.

Trastuzumab was the chosen case study for charge variant monitoring in a stability experiment. Figure 4 shows the IdeS digest of T0 vs stressed Trastuzmab. There is a significant increase of 14.1% acidic variants in the Fab region (Peaks c and d), and 4.5% in the Fc region (Peak a). The increase in acidic variants for IdeS digests corresponds well with the increase in acidic variants observed in nonreduced analysis (18.7%), as shown in Figure 6. Figures 5 and 7 display the online MS data collected for each of the charge variant species, and Table 1 and 2 give possible assignments for each peak based on the mass observed.

One caveat to keep in mind is that some charge variants such as deamidation or conformational differences have little to no mass difference in comparison to the full antibody, which makes it difficult to confidently assign these variants by intact mass. In the case of Trastuzumab, the deamidation susceptibility is well characterized and expected as the acidic variant peaks observed. Direct IEX-MS is useful to give strong hypotheses for the near-isobaric variants, as well as rule out other possible variants.

Furthermore, the use of a chromatographic method has a distinct advantage over other charge-based separations, such as capillary electrophoresis or isoelectric focusing, for the ability to collect fractions for further testing. The variant peaks separated by this IEX method may be collected and analyzed via peptide mapping experiments to confirm the location of the modification or isolated for drug potency assays.

CONCLUSION

- **Successful establishment of IEX-MS method using a dual pH and volatile salt IEX gradient with optimized cation exchange resin**
- **Streamlined data acquisition with a compact high-performance TOF with increased user accessibility and performance standardization**
- **IEX-MS is ideal for high throughput monitoring of charge heterogeneity, with the added benefit of direct investigation of variants by native mass spectrometry**
- **IEX-MS successfully employed for monitoring of charge variants in pH-stressed Trastuzumab sample**
- **Further characterization (e.g. modification site confirmation, potency assays) is made possible by fraction collection**

References

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