Native AEX coupled to MS for the analysis of IgG4-based mAbs

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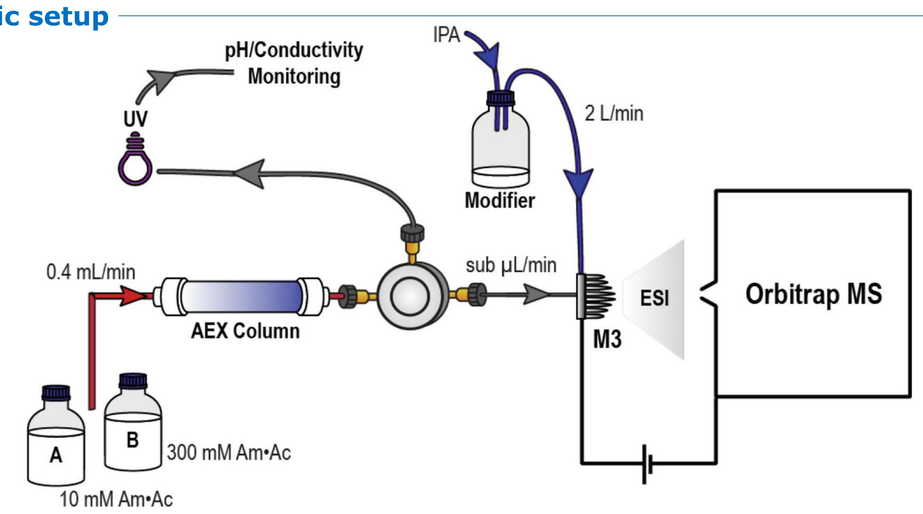
Introduction

Cation exchange chromatography (CEX) coupled to mass spectrometry is an excellent method for characterising the charge heterogeneity of IgG1-based monoclonal antibodies (mAbs). In contrast to IgG1-type mAbs, IgG4-based mAbs possess a pI <8 so that CEX is less suitable for their analysis. The successful application of an AEX method for charge heterogeneity analysis of IgG4-based mAbs coupled to MS was achieved using a BioPro IEX QF column from YMC [1]. The approach by Liu et al. is discussed here: Five different IgG4-based mAbs with different pIs (between 6.1–7.3) as well as the NISTmAb (pI = 9.2) were analysed using the strong anion exchange column (SAX) with non-porous particles, BioPro IEX QF. To enable MS analysis under native conditions, which require high salt concentrations, a special setup combined with nanoelectrospray ionisation mass spectrometry (NSI-MS) is needed. The developed method was successfully applied to mAbs with moderate pI, enabling the analysis of acidic and basic variants. The method was further improved to achieve higher resolution and enabled further investigation of glycosylated species and deamidated variants at Fc subunit level.

Model Case Study

- **AEX-MS method development for IgG4-based mAbs**
- **Method improvement by PNGase F (peptide:N-glycosidase F)-mediated deglycosylation is investigated**
- **Monitoring of Fc subunit variants by PNGase F treatment and IdeS digestion**
- **Analysis of temperature stressed mAb to investigate higher levels of deamidation**
- **BioPro IEX QF, a non-porous strong anion exchange column, was used as the stationary phase**

Schematic setup

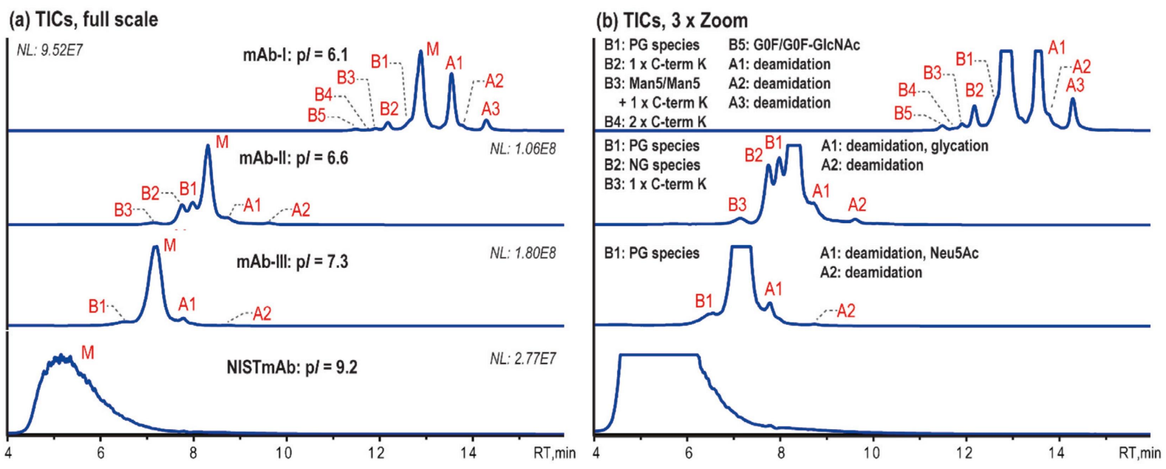


The combination of AEX and MS requires a special setup in which a stainless-steel T-piece after the column divides the flow. Most of the flow is directed to the UV detector, while the remaining sub-microlitre per minute flow is directed to the nanoelectrospray ionisation mass spectrometer (NSI-MS). NSI is used since it can tolerate high salt concentrations of up to 600 mM ammonium acetate. To further improve the spray stability, isopropanol is used as a dopant, modified desolvation gas.

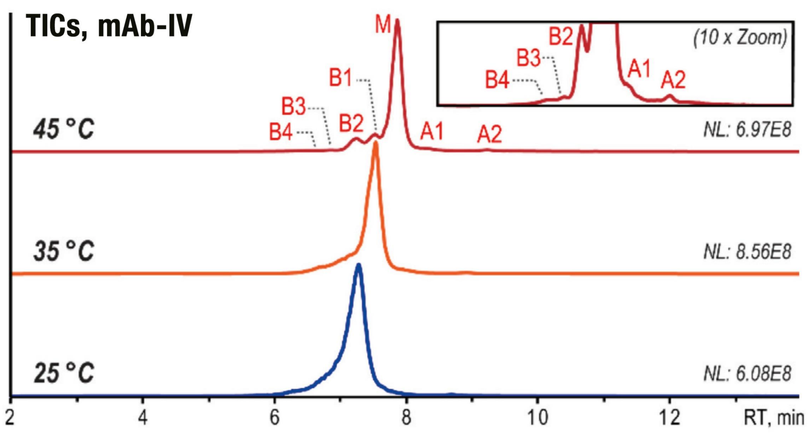
Native AEX-MS method development

Method conditions for all AEX-MS analyses

Column:	BioPro IEX QF (5µm) 100 x 4.6 mm ID
Eluent:	A) 10 mM ammonium acetate (pH 6.7) B) 300 mM ammonium acetate (pH 6.8)
Gradient:	0%B (0-2 min), 0-100%B (2-18 min), 100%B (18-22 min)
Flow rate:	0.4 mL/min
Temperature:	45 ºC intact mAb 25 ºC subunit analysis
Injection:	5 or 10 µg mAb sample
Detection:	NSI-MS (nanoelectrospray ionisation) UV
Sample:	In-house IgG4-based mAbs (Regeneron) NISTmAb
Post column setup:	Post column stainless-steel T-piece to direct the majority to the UV detector Remaining sub-microlitres per minute flow directed to the NSI-MS



The separation of various mAbs improved with lower pI, making the AEX-MS method developed suitable for IgG4-based mAbs with moderate pI. Even mAb-III with a pI of 7.3, which is higher than the mobile phase pH still showed sufficient separation indicating that the surface charge rather than the intrinsic charge provides the AEX-based separation. Similar acidic variants as in CEX-MS such as deamidation, glycation and sialic acid (Neu5AC)-containing species were observed. In contrast, the NIST-mAb with a pI of 9.2 showed insufficient separation, indicating that the AEX-MS method is less suitable for IgG1-based mAbs with higher pIs.



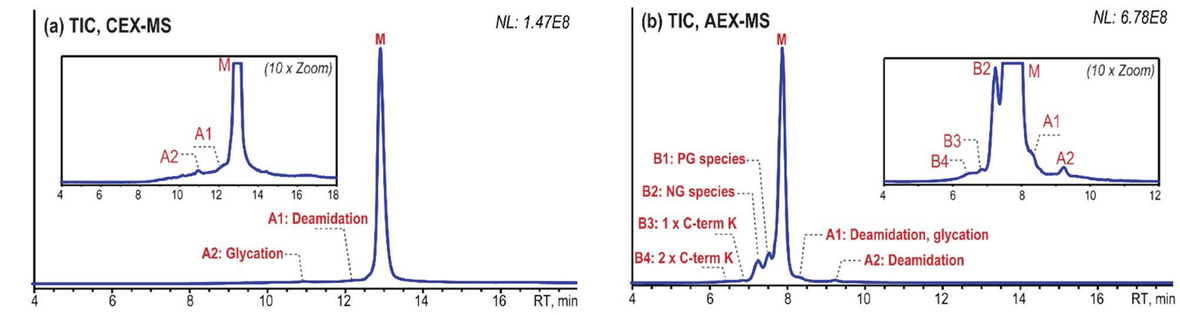
The analysis of the IgG4-based mAb-IV was greatly improved at 45 ºC compared to the other temperatures tested. Higher resolution of the IgG4 variants and sharper peaks were obtained. Under these conditions, four basic and two acidic peaks of mAb-IV were detected.

B1: PG species
B2: NG species
B3: 1 x C-Term K
B4: 2 x C-Term K
A1: deamidation, glycation
A2: deamidation

Comparison of the AEX-MS method with CEX-MS

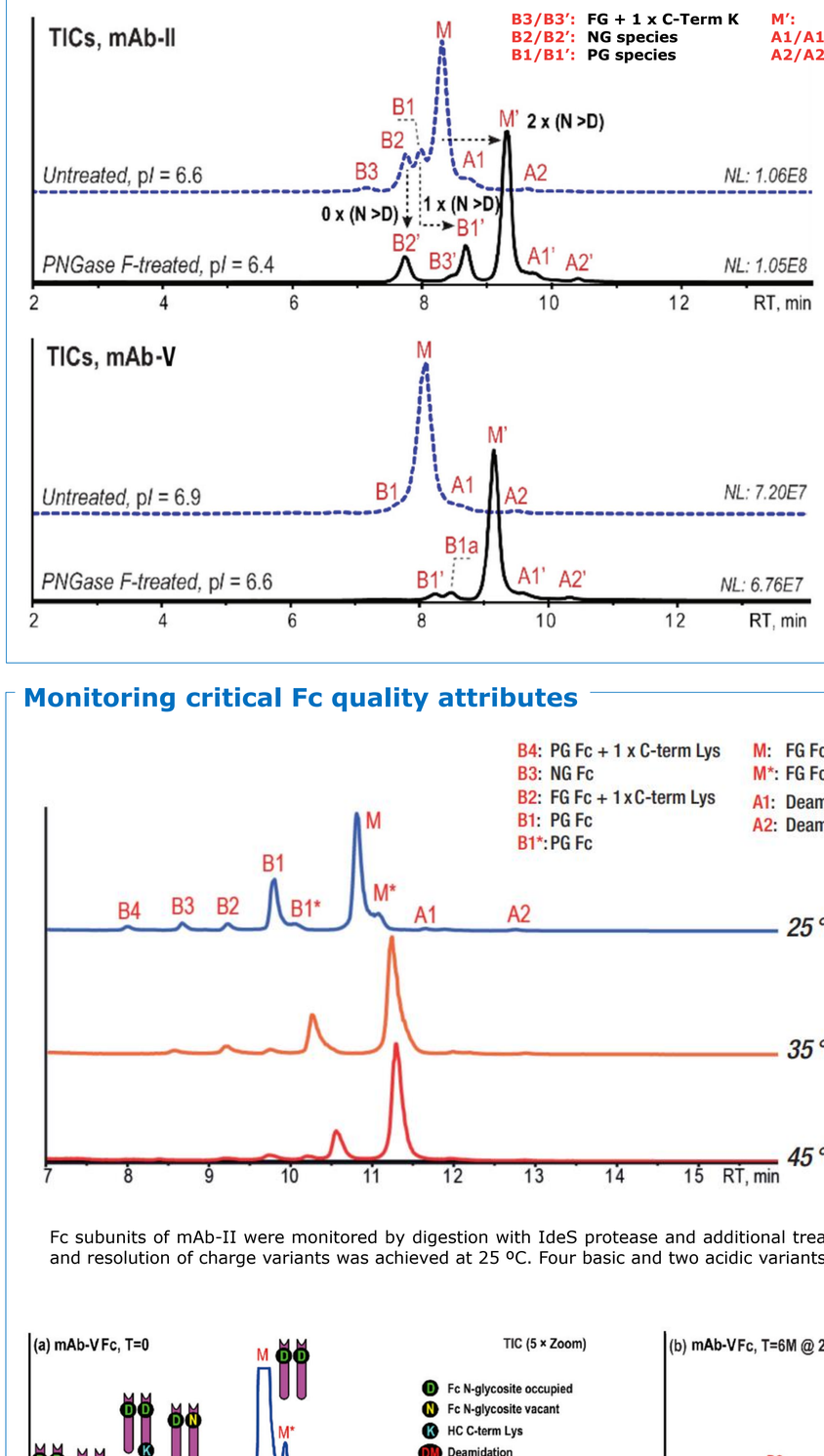
Method conditions for all CEX-MS analyses

Column:	BioPro IEX SF (5µm) 100 x 4.6 mm ID
Eluent:	A) 20 mM ammonium acetate (pH adjusted to 5.6 with 20 mM acetic acid) B) 150 mM ammonium acetate (pH 6.8)
Gradient:	0%B (0-2 min), 0-100%B (2-18 min), 100%B (18-22 min)
Flow rate:	0.4 mL/min
Temperature:	45 ºC
Injection:	10 µg mAb sample
Detection:	NSI-MS (nanoelectrospray ionisation) UV
Sample:	In-house IgG4-based mAb, pI=6.8 (Regeneron)
Post column setup:	Post column stainless-steel T-piece to direct the majority to the UV detector Remaining sub-microlitre per minute flow directed to the NSI-MS



With a CEX-MS method analysing the IgG4-based mAb-IV with a pI of 6.6. (a) no basic peaks and only two acidic peaks were observed and identified as deamidated and glycated variants. The AEX-MS method enabled the separation of four basic peaks and two acidic peaks (b). It also provided the possibility to separate site-specific deamidation variants. The overall separation using AEX-MS was better as the method provided sharper peaks and additional information about the base variants.

Further improvement of the basic variant separation

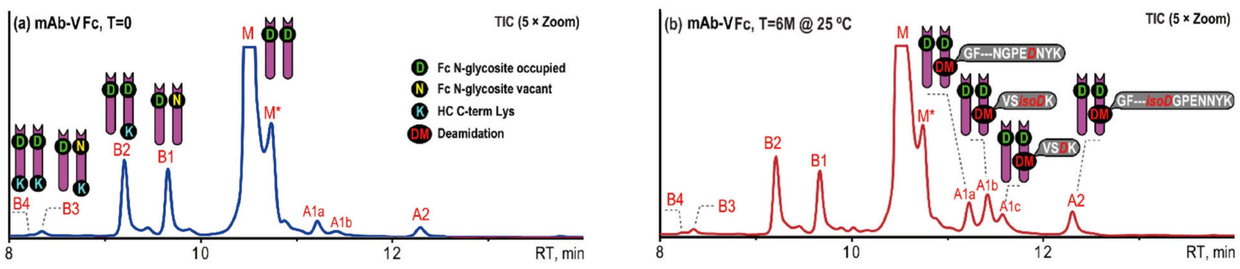


The pI of mAb-II and mAb-V was decreased to 6.4 for mAb-II and to 6.6 for mAb-V, by treatment with PNGase F (black), which converts the glycan-bearing asparagine to aspartic acid. As a result, the overall retention time for both mAbs and their variants was reduced and the peak sharpness was improved compared to the untreated sample (blue). By PNGase F treatment of mAb-V a further minor variant (B1a) was detected and identified as a partially glycosylated species.

B1/B1': FG + 1 x C-Term K
B1a: PG
M': FG
A1: deamidation, Neu5Ac, glycation, glucuronylation
A1': deamidation, glycation, glucuronylation
A2/A2': deamidation

Monitoring critical Fc quality attributes

Fc subunits of mAb-II were monitored by digestion with IdeS protease and additional treatment with PNGase F. Improved peak shape and resolution of charge variants was achieved at 25 ºC. Four basic and two acidic variants were identified.



Fc subunits of thermally stressed mAb-V (b) were successfully monitored. Thermal stress did not lead to changes in basic variants while acidic variants showed an increased number of peaks compared to the unstressed sample (a) indicating deamidation.

Deamidated Peptides	Relative Abundance				
	Main Fraction	A1a Fraction	A1b Fraction	A1c Fraction	A2 Fraction
GFYPSDIAVEWESNGQPE***D***NYK	0.1	***7.3***	4.1	1.8	2.1
VS(***isoD***)K	0.3	0.0	***28.5***	15.1	0.0
VS***D***K	0.2	0.0	2.3	***17.7***	0.1
GFYPSDIAVEWES***D***GQPENNYK	0.2	0.1	0.1	0.1	2.0
GFYPSDIAVEWES(***isoD***)GQPENNYK	1.0	0.1	0.7	0.9	***23.9***

The fractions of the deamidated variants of the mAb-V were identified by peptide mapping. The results demonstrate that the AEX-MS method developed can separate site-specific deamidation products at the Fc level, even at isoform resolution as shown previously by the chromatograms.

Conclusions

- AEX-MS analysis is very suitable for characterising charge heterogeneity in IgG4-based mAbs
- Compared to CEX, the AEX method showed overall **better separation** of the IgG4-based mAb with moderate pI
- **Improved resolution of the glycosylated variants** was further achieved by PNGase F-mediated deglycosylation
- AEX-MS methods are suitable for the **Fc critical quality attribute monitoring** of IgG4-based mAbs
- With the developed AEX-MS method the Fc critical quality attributes can be analysed **without peptide mapping**

Acknowledgment

Special thanks to Regeneron for providing the data.

[1] A. P. Liu, Y. Yan, S. Wang, N. Li, Coupling Anion Exchange Chromatography with Native Mass Spectrometry for Charge Heterogeneity Characterization of Monoclonal Antibodies, Anal. Chem. 2022, 94, 6355−62