Charge heterogeneity characterisation of an IgG4-based mAb using AEX coupled to MS

Cation exchange chromatography (CEX) is very well suited for characterising the charge heterogeneity of biomolecules. This also applies to most commercially available monoclonal antibodies (mAbs). They are often based on IgG1 and have a high isoelectric point (pI). Anion exchange chromatography (AEX), has so far only been used for relatively basic proteins.

In addition, IgG4-based mAbs are becoming increasingly important. Four FDA-approved mAbs are already based on IgG4 and at least six other candidates are in development [1]. This application demonstrates that the charge heterogeneity of an IgG4-based mAb with a pI of 6.6 (Regeneron's proprietary mAb) can be successfully evaluated using a strong anion exchange column (SAX), BioPro IEX QF, in conjunction with MS detection [2].

Table 1: Chromatographic conditions.

Column:	BioPro IEX QF (5µm) 100 x 4.6 mm ID
Part No.:	QF00S05-1046WP
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 °C intact mAb
	25 °C subunit analysis
Injection:	5 or 10 µg mAb sample
Detection:	NSI-MS (nanoelectrospray ionisation)
	UV
Sample:	Inhouse IgG4-based mAb, pI= 6.6 (Regeneron)
Setup:	Post column stainless-steel tee to direct the majority to the UV detector
	Remaining sub-microlitre per minute flow directed to the NSI-MS

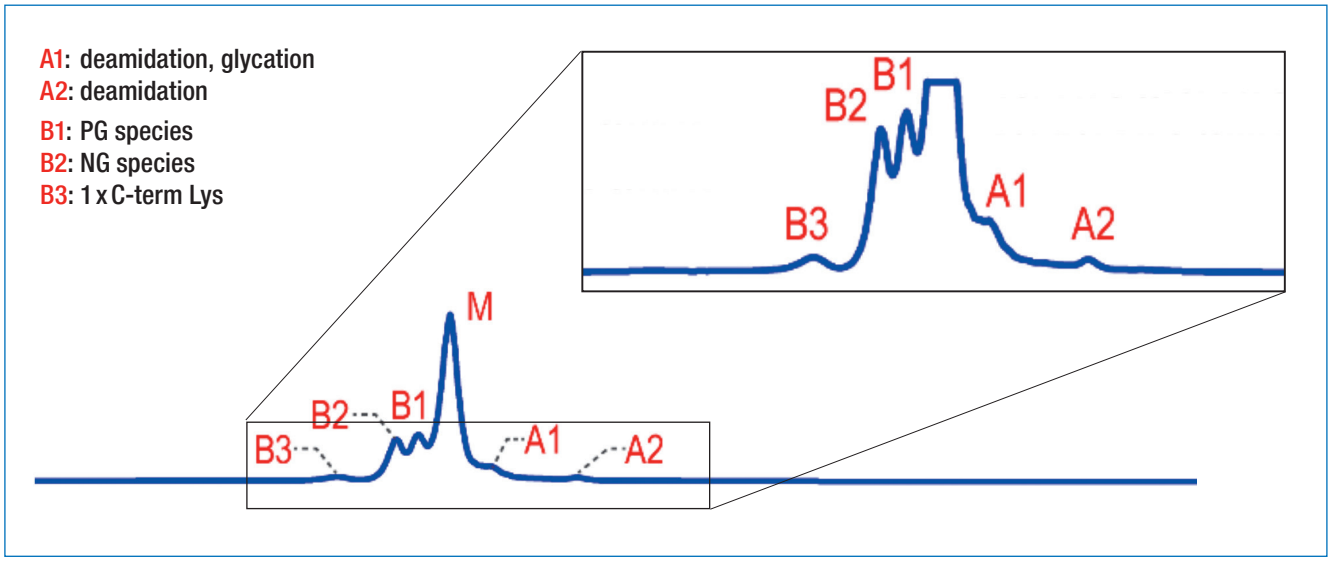

Figure 1: Native AEX-MS charge variant analysis, shown basic (B) and acidic (A) variant peaks as well as ...(5 µg mAb injection) [2].

Table 2: Summary of charge variant species observed by native AEX-MS analysis [2].

Charge Variant Peak	Charge Variant Identity	Predicted Intact Mass (Da)	Observed Intact Mass (Da)
B3	+1 x C-term Lys	148844.4[a]	148846.9
B2	Non-glycosylated mAb	145825.6	145822.8
B1	Partially glycosylated mAb	147270.9[b]	147268.8
M	–	148716.3[a]	148717.1
A1	Deamidation	148717.2[a]	148718.2
	Glycation	148878.4[a]	148885.0
A2	Deamidation	148717.2[a]	148718.4

[a] Predicted mass of charge variants containing N-linked G0F/G0F glycoforms.
[b] Predicted mass of charge variants containing N-linked G0F glycoforms.

With this AEX method three basic as well as two acidic charge variants can be separated from the main species (see Figure 1). Furthermore, this method seems to be highly sensitive to the Fc N-glycosylation macroheterogeneity as the fully glycosylated (FG) main species is separated from the partially glycosylated (PG) peak B1 and the non-glycosylated (NG) species B2, which elute earlier. This leads to the assumption that the surface charge rather than the intrinsic charge is decisive for the AEX separation. Further allocations of the charge variant peaks can be found in Table 2.

Improving resolution

mAbs with a lower pI are better separated in AEX mode. Therefore, the pI of the mAb was lowered by treatment with PNGase F-mediated deglycosylation. This reaction removes N-glycans and simultaneously converts the glycan-bearing asparagine (Asn) residue to aspartic acid (Asp), which all IgG4 mAbs contain in the Fc region in each of the two heavy chains. Therefore, up to two Asn to Asp conversions can be expected.

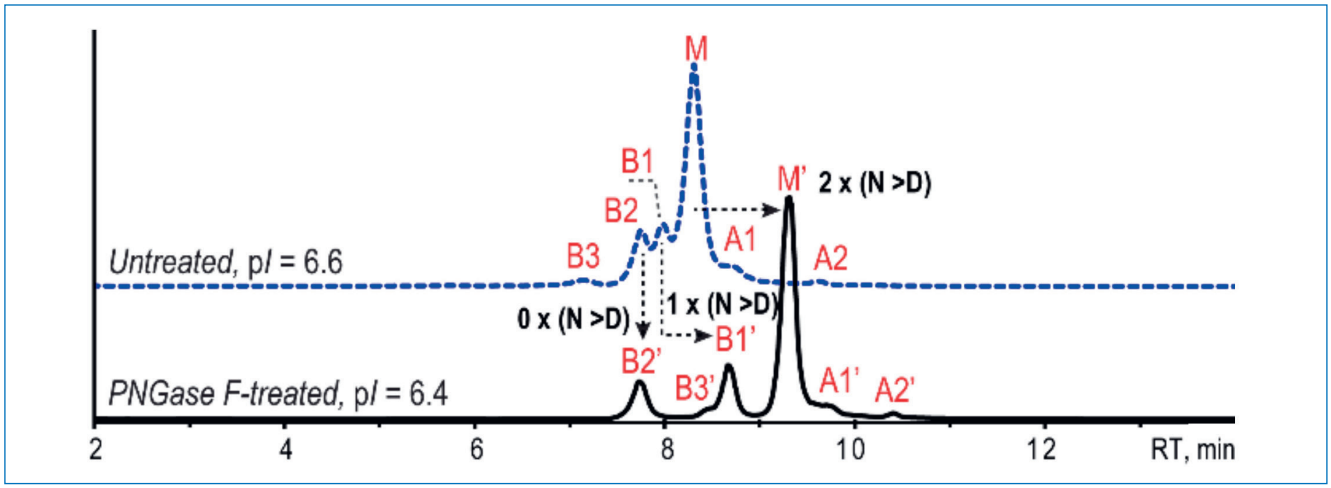

Figure 2: Native AEX-MS analysis before (blue) and after PNGase F-treatment (black) [2].

After the PNGase F-treatment the retention time of the FG main species is extended by about 1 min (see Figure 2) due to the increased acidity induced by now two Asp residues. The retention time of the PG species B1 is only shifted by about 0.5 min while the retention time of the NG species B2 remains unchanged. Due to lowering the pI from 6.6 to 6.4 the resolution is massively improved.

Fc critical quality attribute monitoring

This AEX-MS method can also be applied for subunit analysis of mAbs after IdeS digestion. As the pI of F(ab')2 fragments is relatively high, these fragments are retained poorly and are not considered any further.

The Fc fragments were treated with PNGase F-mediated glycosylation to profit from the improved resolution and further investigate the glycosylated species. For the Fc fragment analysis, a lower temperature of 25 °C shows improved peak shape and charge variant separation over the analysis at 45 °C (see Figure 3).

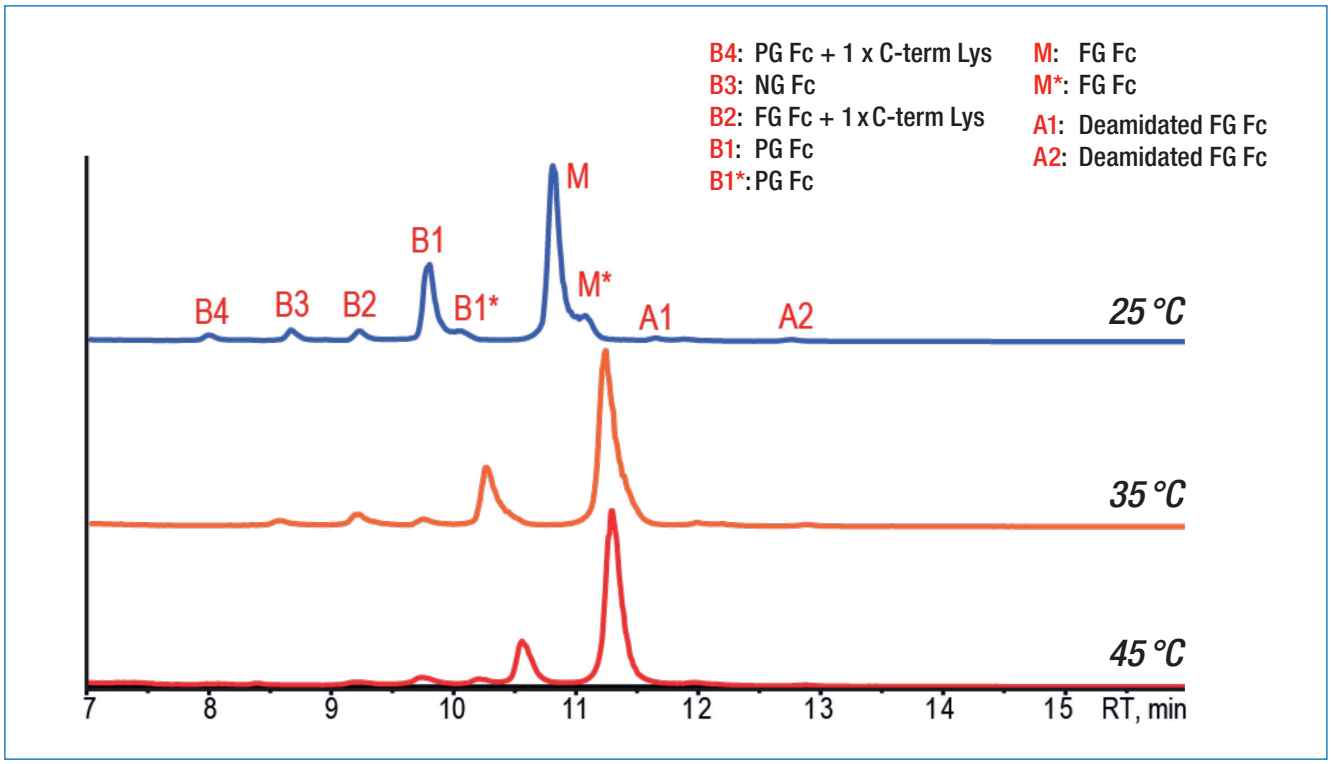

Figure 3: Native AEX-MS analysis of the PNGase F-treated and IdeS digested mAb at different temperatures: 25 °C (blue), 35 °C (orange) and 45 °C (red) [2].

Four basic and two acidic variants can be identified. The main peak as well as B1 show tailing shoulder peaks, with identical mass to the corresponding peak, which could be conformational isomers. In addition to the NG B3 and the PG B1 peak, B2 is identified as a fully glycosylated species with one unprocessed C-term Lys while B4 is identified as a partially glycosylated species with one unprocessed C-term Lys. These findings were confirmed by peptide mapping.

Charge Variant Peak	Charge Variant Identity	Theoretical Mass (Da)	Observed Mass (Da)
B4	PG Fc + 1 x C-term Lys	47670.1	47670.0
B3	NG Fc	47541.0	47541.0
B2	FG Fc + 1 x C-term Lys	47671.1	47671.0
B1	PG Fc	47541.9	47542.0
B1*			47542.0
M	FG Fc	47542.9	47543.0
M*			47543.0
A1	Deamidated FG Fc	47543.9	47544.1
A2	Deamidated FG Fc	47543.9	47544.0

Summary

AEX-MS analysis is a good alternative for the charge heterogeneity characterisation of IgG4-based mAbs. The resolution of the glycosylated variants can be further improved by PNGase F-mediated deglycosylation. AEX-MS methods are suitable for the Fc critical quality attribute monitoring of IgG4-based mAbs, while CEX remains the better option for F(ab')2 subunit analysis.

Literature

[1] Kaplon, H.; Reichert, J. M. MAbs 2021, 13, No. 1860476.
[2] A. 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, 6355–6362