Novel Ways to Introduce the Traditional Salt Based Chromatography Techniques of Size Exclusion and Ion Exchange Chromatography of Biopharmaceutical Proteins Into High Resolution Mass Spectrometry

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ABSTRACT

Thorough characterisation of Bio-therapeutic proteins is essential at all stages of development through to manufacture and final product quality control. Each monoclonal antibody (mAb) will have several different variant forms due to multiple post translational modifications that can occur during production, purification and storage. These modifications can often alter the charge distribution on the surface of the protein which can be characterised by charge variant analysis using ion exchange chromatography. Aggregation and fragmentation are other important modifications which are usually monitored by size exclusion chromatography taking advantage of the difference in size. Identification of structural variants is a critical challenge and Mass Spectrometry (MS) is used as an essential tool in the characterisation and identification of these protein variants. However, the techniques of ion exchange and size exclusion both require high salt eluents which is incompatible with MS. Structural variants exposed by these techniques must be collected separately off-line, then desalted before further characterisation by MS. Here we describe novel on-line coupling methods of ion exchange and size exclusion to the MS instrument to allow direct characterisation of mAb variants in the native form. Both analysis techniques have fast run times and greatly reduce analysis time and sample handling by avoiding fraction collection and sample desalting. The chromatographic resolution of charged variants using pH gradient elution with a novel volatile buffer preparation compares favourably with traditional salt elution. The proteins enter the Orbitrap-based MS system in the native state with a reduced charge distribution and an elevated mass to charge ratio. Variants found with this direct on-line coupling include deamidation, glycosylation and lysine truncation. The size exclusion analysis employs the use of a mechanically stable polymeric resin which does not carry the drawbacks of salt dependency in the eluent system that is inherent with silica resin technology.

INTRODUCTION

Charged variant analysis by ion exchange is traditionally done using salt gradient elution. However it has been shown that mAb charged variant analysis can be successfully achieved using pH gradient elution from the ion exchange column [1], The commercially available Thermo Scientific™ CX-1 pH gradient buffers [2] with the correct column produces a linear pH gradient which is easy to use. Although the salt concentration in the eluents is greatly reduced, the buffer components used here are still not MS compatible. In this study we developed a cocktail of MS compatible buffer components to buffer in the pH range for most mAb samples, achieving the required separation of mAb charged variants directly into the MS for analysis. There are many reasons why this has been difficult to perform, including careful selection of a high resolution, low capacity column, as the column itself will act as a buffer against any pH changes. Volatile buffers do not have the high buffering capacity of the CX-1 buffer system causing delays in pH equilibration on column. Extra care was taken to develop reproducible chromatography methods for several mAb products using this pH gradient buffer system. Ion suppression of the MS signal can be caused by elevated buffer concentrations so care was also taken to balance MS sensitivity with enough buffering capacity for pH control.
Polymer SEC column resins do not suffer from secondary interactions caused by reduction of salt in the eluent, allowing lower buffer concentrations in the eluents. The same buffer system was used for both SEC and Ion exchange giving good MS sensitivity.
Both these chromatography methods will introduce the proteins into the MS in the Native form. This has the advantage of a reduced number of charged states on the protein and better spatial resolution of these charge states. Signals from the different variant forms within one charged state have more chance of being separated from the variants of the neighbouring charge state.

MATERIALS AND METHODS

Equipment

Thermo Scientific™ Q Exactive™ Plus with BioPharma option, Thermo Scientific™ Vanquish™ Horizon UHPLC System, Thermo Scientific™ Acclaim™ SEC-300 column, 5µm, 4.6 x 300 mm, Thermo Scientific™ MAbPac™ SCX-10 column, 5µm, 2.1 x 50 mm. mAb samples from NIBRT.

On-line conductivity and pH monitoring was used during pH gradient optimization with the Thermo Scientific™ UltiMate™ 3000 PCM-3000 monitor

Data Analysis

Thermo Scientific™ BioPharma Finder 2.0, Thermo Scientific™ Chromeleon CDS 7.2, Thermo Scientific™ Xcalibur™ software v 2.2

RESULTS

Volatile pH Gradient Optimization

On-line monitoring of the pH was used during method development to aid in trouble shooting and to determine the real pH gradient that was being produced through the column with the buffering system. Each different mAb product has a different isoelectric point and so it is essential that each gradient method produces a pH in the column that is optimized around that value. In this way robust gradient methods were developed using a single volatile buffer cocktail for several different mAb samples. This was coupled directly into the MS for charged variant analysis and identification.

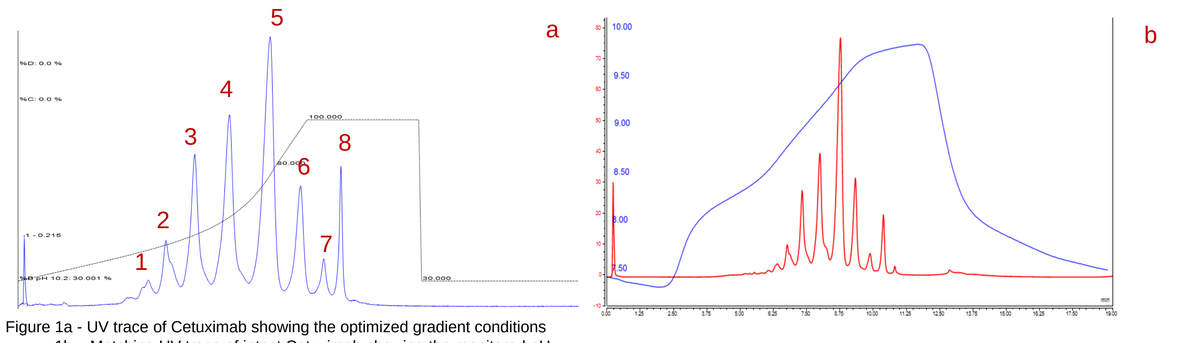

Figure 1a - UV trace of Cetuximab showing the optimized gradient conditions
1b – Matching UV trace of intact Cetuximab showing the monitored pH

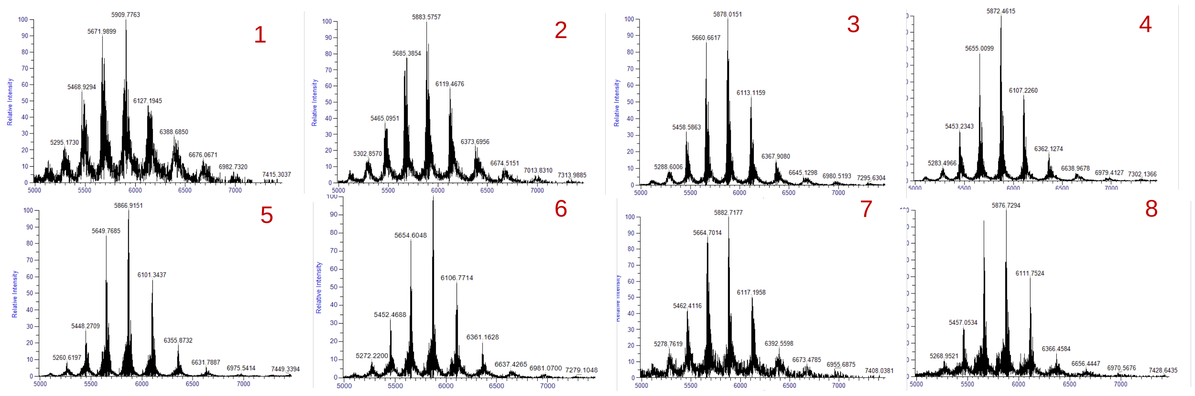

Figure 2 – Individual protein mass envelopes for each peak with the deconvoluted average mass

As the protein will be in its native folded state, the charges available on the surface of the protein are much lower in number, resulting in a reduced charge distribution on the protein and an elevated mass-to-charge ratio (*m/z*). This results in a higher spectral resolution between *m/z* peaks in the native state. Modifications that could overlap with different variants from different charge states can be resolved more easily. With Cetuximab the resolution of the multiple variant forms has not been compromised through using a volatile buffer system. At least 10 well resolved peaks can be seen, which will enter the MS source one at a time. the peaks 5,6 and 8 are lysine truncation variants which can be seen and identified from the mass difference corresponding to additional lysine residues. The peaks in front of the main peak 5 show a changing glycan pattern corresponding to increased levels of sialic acid residues in the glycan structure. The increase in negative charge from sialic acid gives less retention on the cation exchange column. All this information is achieved from one direct injection onto the LCMS system.

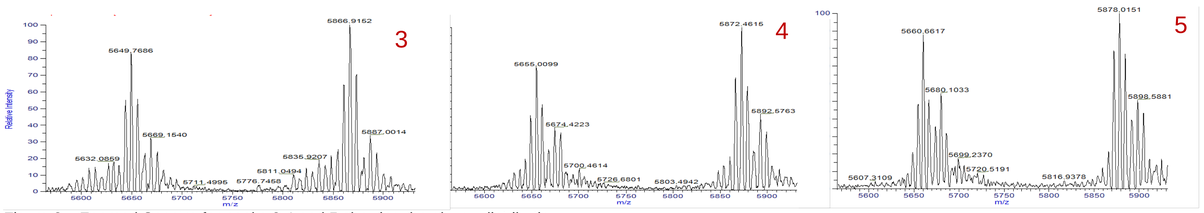

Figure 3 – Zoomed Spectra for peaks 3,4 and 5 showing the glycan distribution

To demonstrate a more global applicability of the volatile pH gradient buffer system, several other mAb samples where used and gradient methods optimized for each. Trastuzumab, Infliximab and bevacizumab results are shown in figure 4 as the UV trace with the programed gradient overlay.

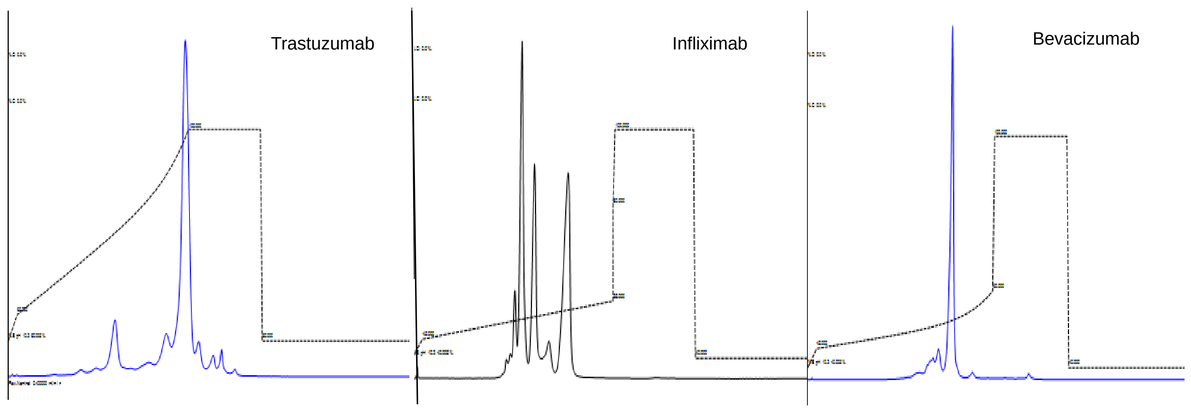

Figure 4 – Optimized chromatography for Trastuzumab, Infliximab and Bevacizumab

This shows that the chromatography is applicable to several additional mAb samples using this column / buffer system. The direct coupling to the Q Exactive™ Plus Hybrid Quadrupole-Orbitrap™ Mass Spectrometer allows for the acquisition of accurate mass data to identify the charge variants present in the sample. The gradients for Infliximab and Bevacizumab are very similar which relates to the similar isoelectric points of these two mAbs. Once a gradient has been optimized for one mAb, the same gradient should be applicable to other mAb products with similar isoelectric points (p*I*).

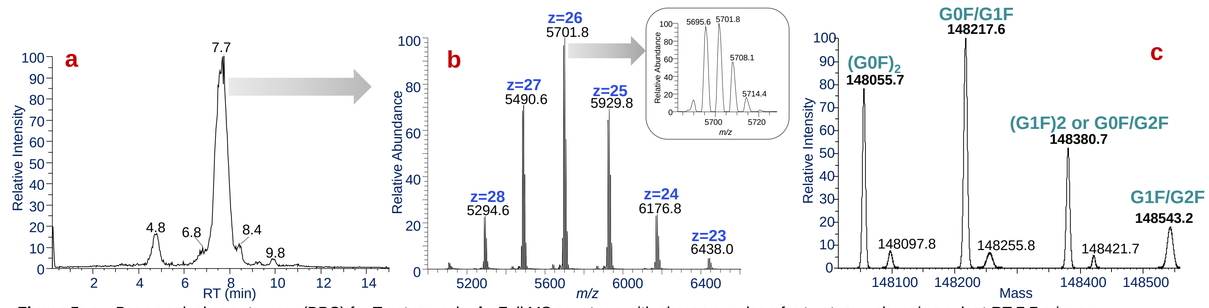

Figure 5: a – Base peak chromatogram (BPC) for Trastuzumab, **b:** Full MS spectrum with charge envelope for trastuzumab main peak at RT 7.7 min, **c:** Deconvoluted mass spectrum showing average masses for the four most abundant glycoforms.

Glycoform	Theoretical Av. Mass	Experimental Av. Mass	Δ Mass (ppm)
(G0F)2	148056.2	148055.7	3.4
G0F/G1F	148218.3	148217.6	5.0
G0F/G2F or (G1F)2	148380.5	148380.7	-1.5
G1F/G2F	148542.6	148543.2	-3.9

Trastuzumab in figure 5 shows two main peaks in the BPC with a clear, well resolved mass charge envelope. The deconvoluted average mass values correspond well to the theoretical values. The peak eluting at 4.8 minutes represents a deamidated form which averages experimentally at 0.8 Da bigger than the main peak corresponding to the theoretical mass difference of 0.98 for a deamidation event.

Adalimumab (Humira™, Abbvie Inc.) data is shown in figure 6. This mAb exhibits three major charged variant forms which can be identified as lysine truncation variants. The deconvoluted mass for all three variants show the mass difference corresponding to additional lysine residues. As lysine carries a positive charge the addition of each lysine to the end of the heavy chain increases the retention on the cation exchange column.

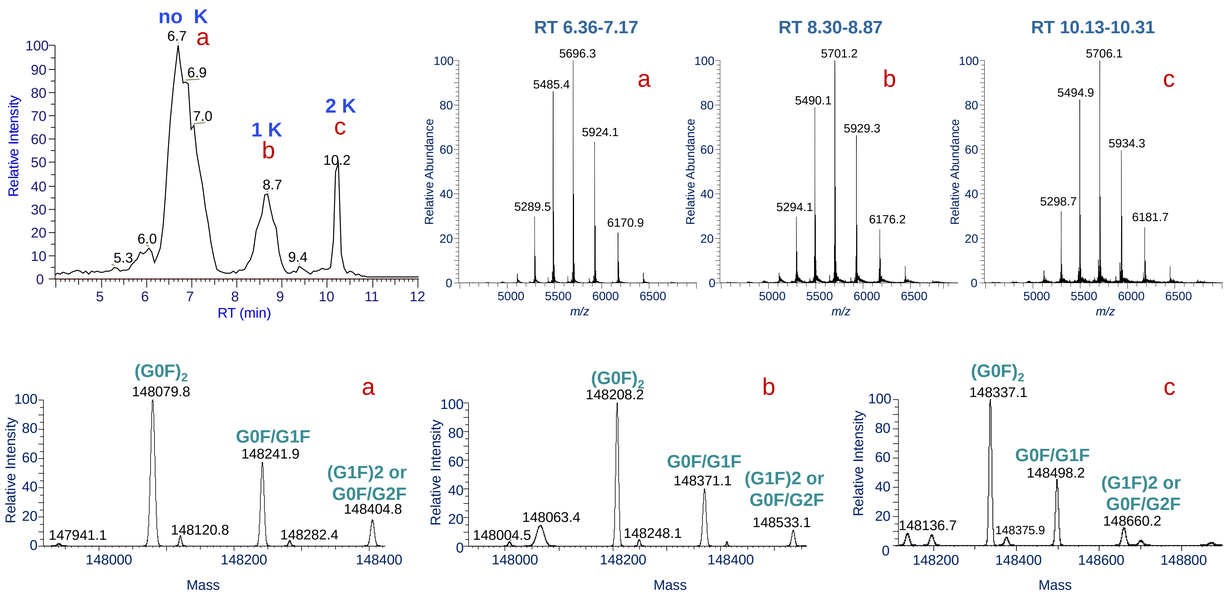

Figure 6 – BPC for Adalimumab showing three main peaks a, b, c. followed by the Full MS spectrum with charge envelope for each peak and the deconvoluted mass spectra for each peak showing the average masses for the three most abundant glycoforms.

Lys variant	Glycoform	Theoretical Av. Mass	Experimental Av. Mass	Δ Mass (ppm)
no K	(G0F)2	148080.2	148079.8	2.6
no K	G0F/G1F	148242.3	148241.9	2.9
no K	G0F/G2F or (G1F)2	148404.5	148404.8	-2.3
1K	(G0F)2	148208.4	148208.2	1.0
1K	G0F/G1F	148370.5	148371.1	-4.1
1K	G0F/G2F or (G1F)2	148532.6	148533.1	-3.1
2K	(G0F)2	148336.5	148337.1	-3.9
2K	G0F/G1F	148498.7	148498.2	3.2
2K	G0F/G2F or (G1F)2	148660.8	148660.2	4.1

The experimental deconvoluted mass values again correspond favorably with the theoretical values giving unambiguous peak assignments. Two of the earlier peak assignments for the Adalimumab ion exchange chromatography show the possible presence of fragments. This is confirmed by size exclusion chromatography using the Acclaim SEC 300 column with the same buffers.

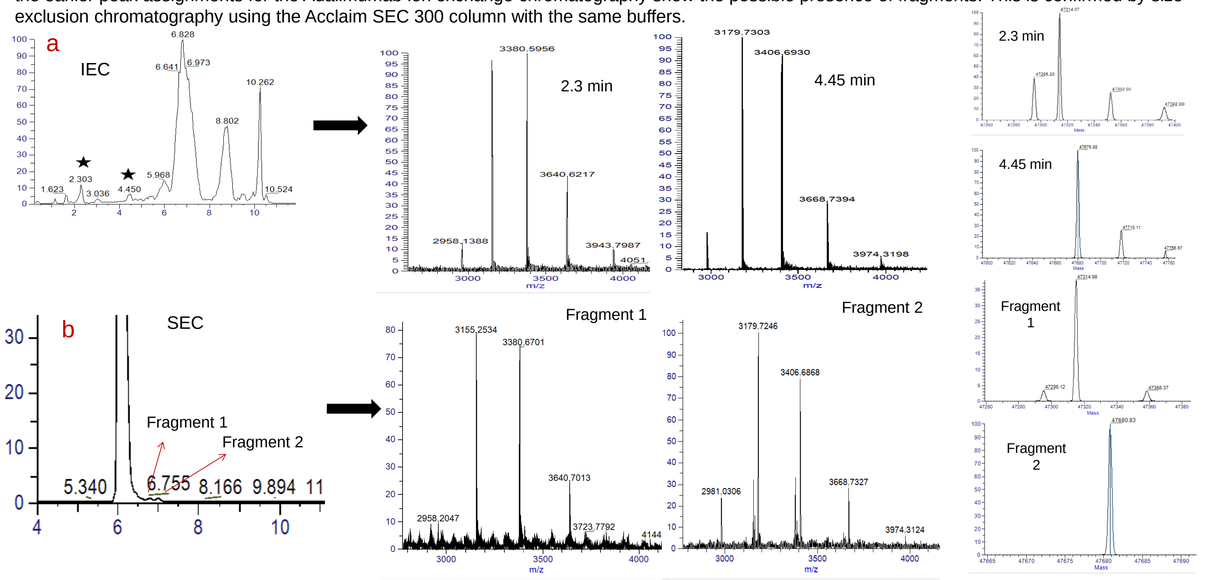

Figure 7a BPC for IEC of Adalimumab followed by the Mass spectra for the peaks at 2.3 and 4.4 minutes
7b BPC for the SEC of Adalimumab followed by the mass spectra for the fragment peaks eluting after the full sized mAb.

Size exclusion chromatography is another method which allows the introduction of protein samples into an MS source in the native folded state. The separation can be used to both desalt the sample and separate aggregates and fragments. The Acclaim™ SEC-300 LC Column is a polymeric mono-dispersed hydrophilic resin which tolerates the secondary binding effects of reduced salt in the buffer system much better than a silica based resin. The separation buffer can then be optimized more for MS sensitivity without the problems to the chromatographic integrity. This is important as SEC is not a concentration technique such as IEC and as such sample loading is more limited. The results confirm that the same fragments are seen in both SEC and IEC. The higher loading capabilities of the pH gradient ion exchange chromatography technique allows significantly more sensitivity to characterise low level variant species.

CONCLUSIONS

- Ion Exchange has been successfully interfaced directly to MS for charged variant analysis and direct identification.
- The method requires a volatile buffer cocktail with a carefully selected low capacity, high resolution ion exchange column.
- The system has global applicability demonstrated with several mAb samples using gradients optimized to the p*I of* the protein.
- Size exclusion chromatography can be used to confirm the native mass data.
- The low salt concentrations enabled by polymeric resin technology increases the sensitivity of native mass spectrometry.

REFERENCES

1. Farnan D, Moreno G.T. Analytical Chemistry 2009, 81(21), 8846–57

2. Thermo Scientific CX-1 pH Buffer product manual, P/N 065534-01 June 2013

3.TRADEMARKS/LICENSING