Options in Advanced Therapeutic Monoclonal Antibody Analysis

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Protein Biopharmaceuticals–Challenges in Characterisation
Monoclonal antibody (mAb) biopharmaceuticals form the largest part of a growing biotherapeutics drug market and produced a requirement to develop new analytical characterisation methods. These large biomolecules are very different from the world of small molecule drugs and require different types of characterisation with very different methodology. Small molecules produce a limited number of impurities compared to a mAb which theoretically can have almost 10,000 variant molecules due to the size and complexity.

Protein biopharmaceuticals include many types of protein from blood factors, hormones, therapeutic enzymes to whole and small domain antibodies. Whole mAb's however remain the most popular due to the effective treatment and diagnosis of a broad range of diseases. Due to the specificity of the mAb on its target there is also the advantage of reduced toxicity and side effects seen with this type of approach.

The number of development candidates for monoclonal antibody therapeutics is extensive creating the need for fast and efficient characterisation workflows during development and production of these products. These workflows differ significantly from the approaches and methods developed for small molecules. Detection, characterisation, quantification of impurities, structural variants and modifications, and monitoring stability is regarded as essential for demonstrating safety and efficacy of the bio-therapeutic and is required by FDA and other regulatory agencies.

Key words; Bio therapeutic analysis, Monoclonal Antibody, Chromatography, Mass Spectroscopy

Analytical Methodologies

Most pharmaceuticals have workflow platforms that have been developed to look at purity and efficacy. The most common analytical requirements would include methods to test for the presence of aggregates, fragments, glycosylation variants, truncation[1,2,3]and a series of post translational modification variants including oxidation, deamidation[4-8] and other chemical modifications that could occur during purification or storage. Amino acid oxidation in proteins is a common post-translational modification due to the oxidation of tryptophan or methionine residues. This can occur due to conditions found in production and on storage[4,5]. Oxidation is important because it can adversely impact the activity and stability of the product. Proteins and peptides containing Asparagine [Asn] adjacent to glycine are particularly susceptible to deamidation, converting Asn to aspartic acid or isoaspartic acid to different degrees [6-8]. The extent of deamidation is essential to process development and quality control. It can occur throughout the production and during storage so must be monitored at every stage. Yields and titre also need to be monitored from early stages in production. Some modifications such as glycosylation will occur during fermentation process and others during purification and production. After formulation into a product, shelf-life monitoring of bio-therapeutic products will monitor a variety of degradation products and typically involve several methodologies. In essence researchers are concerned with product quality and stability over the entire lifecycle of the product, from early manufacturing, through to storage and patient use. The amino acid sequence of the bio-therapeutic will be well known and so there will be an awareness of sites susceptible to modification.

Table 1 shows a condensed list of modifications and chromatography methods developed for the analysis and characterisation of protein based therapeutics. Each modification changes the large proteins in a different way which impacts on the type of chromatography method chosen for the analysis.

Column chromatography is not the only technique employed in the characterisation of protein bio-therapeutics. Isoelectric focussing methods for capillary electrophoresis have been used with good success in charged variant analysis [9]. Florescence and light scattering have been used for aggregate analysis [10, 11] to mention only a few. However one of the advantages of a column chromatography approach is the ability to collect fractions of unknown impurities for further identification by mass spectrometry.

mAb Capture

Protein A is a large 56 kDa protein that has found use in antibody research due to its high binding affinity to IgG. It binds the heavy chain at the Fc region of most immunoglobulin's with high specificity. Bound to a chromatographic support it is used heavily in mAb production and can also be employed as a means of titre analysis from fermentation clones or a method of downstream capture prior to further analysis by SEC or ion exchange [12]. Immobilised Metal ion Affinity Chromatography [IMAC] chromatography has also being used as a method of purification [13], but protein A affinity chromatography is by far the most common. Protein A capture on smaller trap columns allows the types of analysis developed at formulation level to be

Modification	Type of change	Column method
Aggregation	Size	SEC
Fragments	Size	SEC
Glycosylation	Conformational and charge	Ion exchange, Reverse Phase [RP], Hydrophilic Interaction Liquid Chromatography [HILIC]
Deamidation	Charge	Ion exchange or RP peptides
Lysine truncation	Charge	Ion Exchange
Disulphide related	Conformational	Ion exchange or RP peptides
Oxidation	Charge	RP peptides or Hydrophobic Interaction Chromatography [HIC]

Table 1: Relationship between the modification and the analytical method chosen

employed at the fermentation level as well. The elution from the affinity column is achieved at low pH and a neutralisation buffer can be used to bring the collected antibody quickly back to neutral pH. Speed of analysis is always an important factor, especially with high sample volumes. In the example in Figure 1 the capture step can be achieved well within 5 minutes, giving an estimate of titre and collection of the purified mAb for further analysis.

Aggregate Analysis by Size Exclusion Chromatography

One of the most common analytical methods is aggregation analysis [14, 15] by size exclusion chromatography [SEC]. Aggregation is a major problem in bio-therapeutics as these variants of the active pharmaceutical may not only change the effectiveness of the formulation but can also instigate an immune response to the bio-therapeutic, resulting in serious side effects. Aggregate formation may be a result of production, purification, formulation or handling. The analysis therefore needs to be done at every stage of manufacture. The SEC technique of separation by size has the necessary selectivity to see dimer and higher aggregation with the added advantage of also detecting the presence of proteolytic fragments or other size based variants. The high resolution columns required for this analysis are usually based on spherical porous [300 Å] silica particles. Modification of the resin with a hydrophilic boundary layer reduces non-specific binding to the stationary phase and can allow low salt eluents for direct coupling to mass spectrometry. Speed of analysis can also be implemented with faster flow rates at higher pressures and the use of shorter analytical columns. Figure 2 shows the high resolution and speed of a state of the art SEC column running high speed aggregate analysis of monoclonal antibodies. This can also be coupled to low level light scattering detection or MS [14]. The speed up of the analysis to less than 4 minutes, as seen by the elution of caffeine, is possible due to the high selectivity of the MAbPac SEC-1 [Thermo Scientific] column for monoclonal antibodies and the aggregates. Sufficient resolution is achieved on the shorter 150 mm column. This reduces the time of analysis with the reduced column volume and the increased flow rates possible through the shorter column. Resolution will always be compromised slightly at higher flow rates but the resolution is still sufficient and the time gains are significant for high sample volumes. Throughput can be increased further using a dual pump with two columns

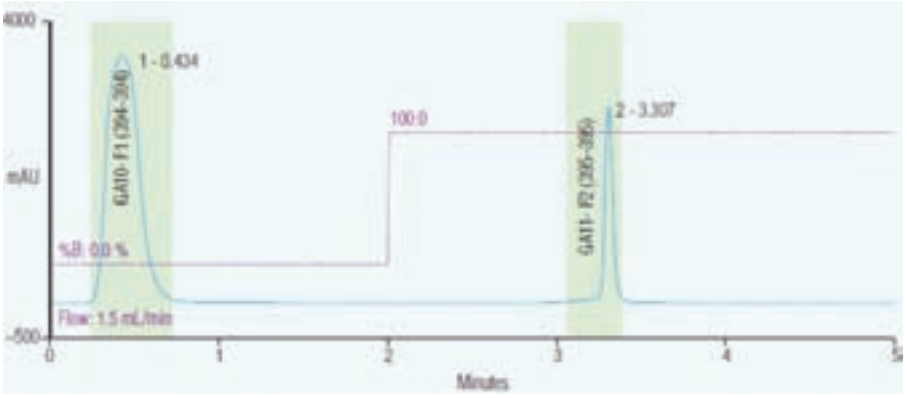

Figure 1: Protein A Capture of a polyclonal antibody [POROS® A 50 mm x 4.6mm i.d.]. Wash for 2 minutes with 10mM sodium phosphate, 150 mM NaCl, pH 7.0. Elute for 3 minutes with 50 mM Glycine-HCl, 150 mM NaCl, pH 2.5.

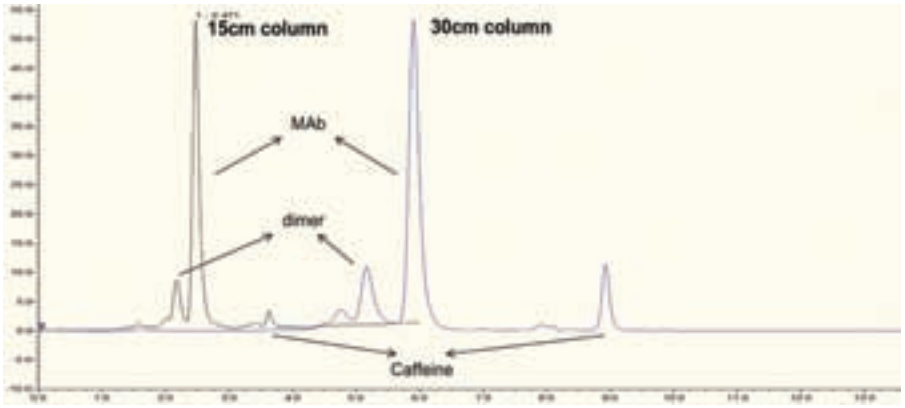

Figure 2: Size exclusion of a high aggregation mAb sample using a 150mm x 4mm i.d. and a 300mm x 4mm i.d. MAbPac SEC-1 50 mM sodium phosphate, 300 mM NaCl, pH 6.8 eluent with a flow rate of 0.5ml/min for the 150mm column and 0.4ml/min for the 300mm column.

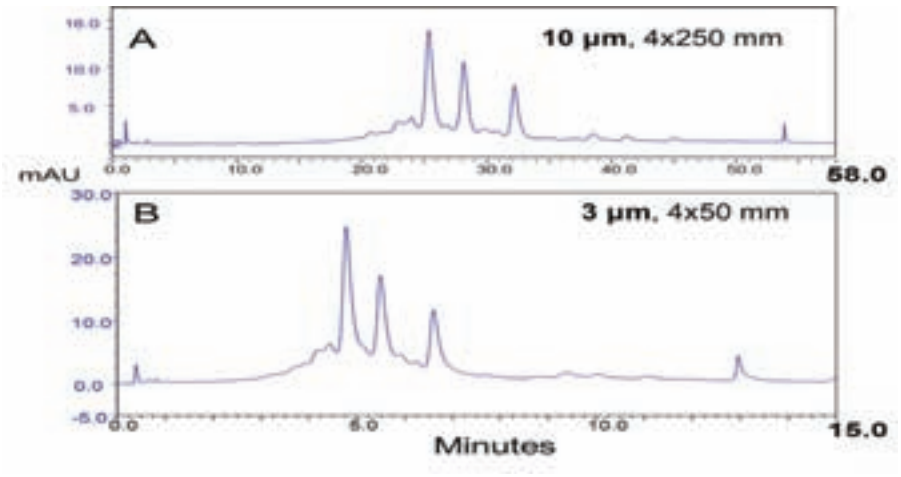

Figure 3: MAbPac SCX columns in the formats shown are compared with the same mAb sample. Eleuent A is 20 mM MES + 60 mM NaCl pH 5.6. Eluent B is 20 mM MES + 300 mM NaCl pH 5.6. Gradient from 20 to 35% B in 50 min for A and 10 min for B. flow rate 1.0ml/min for A and 0.6ml/min for B. Detection at 280nm.

where the injection is interlaced between each column [15]. Analysis of the reduced heavy and light chains or the Fc and Fab fragments produced following papain digestion is also possible within the selectivity of these SEC columns [16].

Variant Analysis with Cation Exchange

The purity and consistency of bio-therapeutic proteins must be established at each stage of manufacture. Contrary to small molecules, proteins are subject to a wide variety of enzymatically produced modifications such as glycosylation. In addition they are also prone to chemical modifications such as oxidation, aspartic isomerisation and deamidation. These post translational modifications can result in many different isoforms which may or may not affect the function of the product. Analysis of these multiple isoforms requires a high resolution technique. As many of the modifications change the charge or conformation of the protein, high resolution ion exchange chromatography [IEC] has proved a valuable technique for this analysis [17-20]. mAb's tend to have a high isoelectric point [PI] and bind well to cation exchange columns due to the positive charge on the protein at pH values bellow the PI. The first column generally accepted in the bio-pharmaceutical industry for routine use in these analyses was the ProPac® WCX-10 [Thermo Scientific]. This column gives baseline separation of lysine truncation variants and good resolution of other acidic and basic isoforms [17]. C-terminal lysine's are often absent in proteins produced from mammalian cells due to the activity of basic carboxypeptidases. 4-morpholineethanesulfonic acid (MES)-based mobile phases tend to be used as they were found to give much better resolution than phosphate based buffers for this and other protein variant separations[17]. The results for these analyses are well suited to quality control monitoring of closely related protein isoforms. The run time with this 10µm pellicular resin format is around 50 minutes. To speed up and further improve this analysis an SCX column was specifically developed, available in 3µm resin size and shorter column lengths. The resin is pellicular for fast separations with a hydrophilic boundary layer to prevent unwanted interactions of the proteins with the column. The results using this column technology are shown in Figure 3. The reduced resolution of a shorter column is compensated by the smaller particle size resulting in equivalent resolution in a quarter of the time, increasing throughput dramatically.

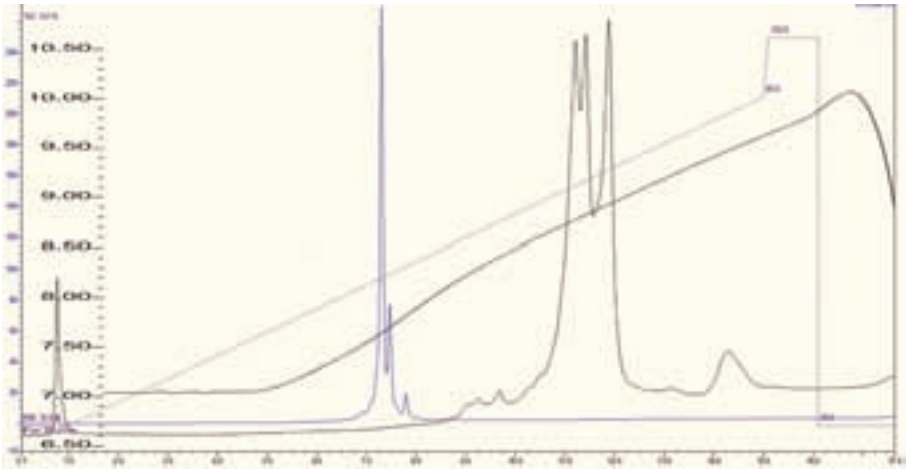

Figure 4: pH gradient analysis of two different mAb samples with different isoelectric points. pH is monitored on-line and produced using 10mM Tris HCl, 10mM Carbonate twin buffer system. Flow rate 300µl/min with a MAbPac SCX, 3µm particle size, 50mm x 4mm i.d.

pH Gradients as a Universal Chromatography Method for Charged Isoforms

Shorter columns are also useful when employing pH gradient separations. As elution from the column is controlled by removing the charge on the protein with pH, interaction with a long column length is not as critical. Re-equilibration of pH and elution is also much faster with the shorter columns in this mode of separation. The lure of pH separation is the potential for a universal chromatography method for all mAb samples [21]. Antibodies are produced against different antigenic determinants and so a spectrum of mAb's will have a range of isoelectric points. This forces a new method development project for each individual mAb under characterisation by ion exchange, often focussed on the best pH for separation. As a pH gradient moves from low pH [loading] to a higher pH on the cation exchange column the protein will meet its isoelectric point, become neutral, and elute. The method can therefore be used as a universal screening tool for a broad range of mAb samples. An example of two different mAb samples is shown in Figure 4 running the same pH gradient method. The second mAb to elute has a higher isoelectric point than the first but both separate well using the same universal method. When running pH gradients or blending specific pH eluents with a quaternary gradient pump it is beneficial to monitor the pH on-line so that a record of the actual pH is recorded for method transfer and accurate method write up. The pH produced during a gradient is very dependent on the choice of the correct buffers. A gradient over a relatively small pH range like the one in Figure 4 requires only 2

buffers that give buffering capacity over that region. A larger pH range may require 3 or 4 buffer components to get accurate linear gradients produced over the column.

Reverse Phase Analysis

mAb samples can also be analysed with reverse phase chromatography which will give an orthogonal analysis to ion exchange with the separation based on hydrophobicity rather than charge and is compatible with mass spectroscopy [22]. This analysis can be achieved on intact mAb or on Fc and Fab fragments resulting from papain digestion. It is also possible to run the analysis at the heavy and light chain level after reduction of the disulphide bonds. The smaller fragment analyses are regarded as more amenable to reverse phase and there are good C4 columns available for this type of analysis as shown in Figure 5. The Fab fragment chromatographs as a sharp symmetrical peek showing several smaller variant peeks separated easily from the main peek. An advantage of reverse phase separations is the ability to directly connect to mass spectrometry for accurate confirmation of the molecular weight [22]. C4 columns have also been used with intact mAb and with these conditions at elevated temperatures give relatively good peek shape and resolution. Larger proteins however tend to give carryover problems with the next injection. As much as 20% carryover can be seen with particularly large sticky protein samples. This problem is largely removed with the use of Polystyrene based monolithic columns such as the ProSwift RP 4-H 250mm x 1mm i.d. column which gives very little carryover and good resolution of the larger proteins.

Native Molecular Weight Protein Variant Analysis

Some variant analyses rely on the use of enzymatic digestion to the peptides to look for sites of oxidation or deamidation in the peptides following reverse phase HPLC and mass spectroscopy. Glycoform analysis normally requires the enzymatic release of oligosaccharides, fluorescent labelling and a separation with HILIC chromatography. These are all viable and well tested methods and giving reliable results. The time it takes to prepare the sample for analysis however makes the run times irrelevant and they cannot really be seen as simple high throughput methods. Much of this information can be gained much more quickly and easily with direct injections of the

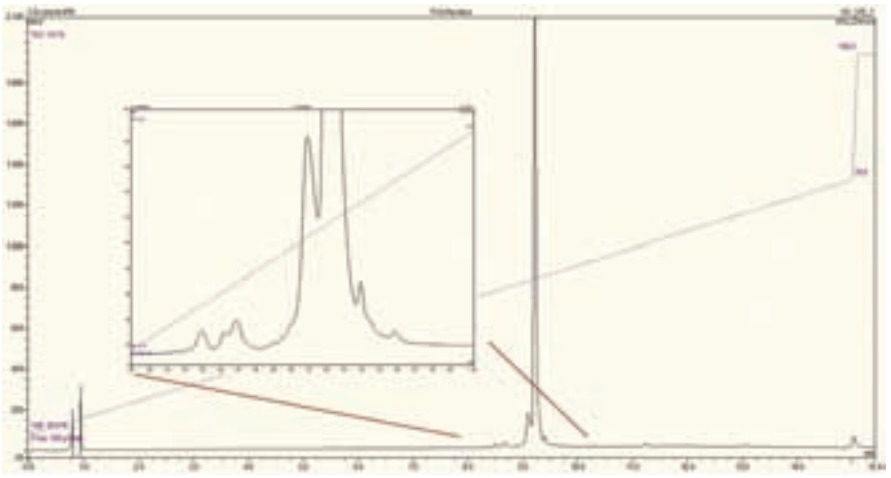

Figure 5: Analysis of an Fab fragment using an Accucore C4 , 100mm x 2mm i.d. Gradient of 10 to 70% acetonitrile, 0.1% Trifluroacetic acid in 15 minutes. 300µl/min at 70C.

variants using a monolithic ProSwift RP column onto a high resolution LCMS system [Figure 6].

High resolution mass spectrometers [HRMS] can be used for the analysis of intact or fragment mAb samples [23-26]. With resolving power of 140,000 to 240,000 the mass differences for intact mAb variants such a deamidation, truncation, oxidation and even glycoforms are well within the mass accuracy of Orbitrap based HRMS systems.

Examples of how very high resolution mass Spectroscopy can be used for this purpose, including baseline separation glycoforms are appearing in the literature [25]. In this example after data analysis of the deconvoluted spectrum, 10 glycoforms in total were identified including two deglycosylated forms. Other examples using a resolution of 240,000 on a high resolution Orbitrap showed the incomplete reduction of the disulphide bonds in a light chain sample [26]. This was seen as a 4 Dalton lower shift in the accurate mass predicted by the target amino acid sequence. The high resolution achievable allows the detection of something as small as a disulphide reduction on a biomolecule as large as an intact mAb. As this can be done without fragmentation and MS alone it provides the possibility of using lower specification mass spectrometers and setting up relatively easy and simple methodologies using direct HRMS detection. This much simplifies the use of high resolution MS and allows rapid detection and quantification of a series of mAb variants that would otherwise require lengthy experimental procedures. Confirmation of product quality and identity can be achieved in a fast and simple experiment this way. If further information is required MS/MS can be done using HCD fragmentation.

Conclusions

The analysis of therapeutic biomolecules requires an array of experimental techniques that are quite different from the techniques employed with small molecule drug analysis [27].

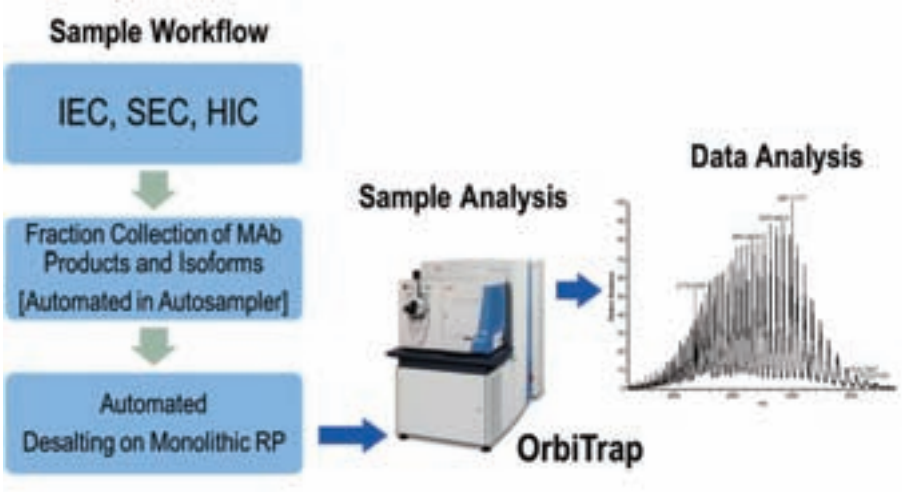

Figure 6: An automated mAb analysis workflow using2D chromatography and high resolution intact mass spectrometry to produce variant isoform data from an intact mAb sample.

Standard experimental workflows can be set up for the characterisation from a choice of methodologies. Several different platforms can be used to supply the results required by the FDA and other regulatory agencies demonstrating the safety and efficacy of the bio-therapeutic.

Another aspect of the differences in methodologies from a small molecule perspective is the relatively common use of corrosive salts in the chromatography eluents. Size exclusion, Protein A capture as well as ion exchange and hydrophobic interaction chromatography all use high levels of salt containing eluents. These are all commonly used techniques in the characterisation of bio-therapeutics and have an impact on the instruments used for the analysis. In place of standard stainless steel HPLC systems the use of titanium alloys provides biocompatible systems that ensures robust and reliable operation, even with high salt and pH buffers typically required for protein separation.

Combinations of the techniques described here can prove to be powerful tools in the characterisation of the micro-heterogeneity present in mAb products. Ion exchange chromatography shows highly selective resolution of charged variants but is less compatible with MS detection due to the salt content of the eluent. The initial positive identification of each variant peak in the chromatogram must be achieved through other techniques. Using a combination of ion exchange and reverse phase HPLC/MS can easily provide this. Introducing the individual fractions from the ion exchange separation onto a monolithic reverse phase column can desalt and inject the fraction onto the HRMS system within a few minutes. Intelligent systems with autosamplers which can collect and re-inject samples onto different columns can be used for this purpose and other combinations of methods [28]. Capture [Protein A] and re-injection of mAb's to a second dimension analytical column [SEC, SCX, HIC [29] or RP] allows automated characterisation directly from fermentations at an early stage in development.

The recent introduction of HRMS methods for intact mAb characterisation is an example of how the methodologies are still evolving in the field of bio-therapeutic protein analysis.

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