Chromatography Solutions

Technical note #029

Avantor® ACE® UltraCore BIO columns for the middle-up/down analysis of monoclonal antibodies

INTRODUCTION

Monoclonal antibodies (mAbs) are firmly established as proven therapeutic drugs, with 95 approved for use in the US and a further 17 investigational antibody therapies in regulatory review as of February 2021 [1]. This class of biomolecules has found success due to better tolerance, limited side effects, high efficacy, and specificity. Large therapeutic proteins such as mAbs require extensive characterisation to ensure the safety and efficacy of the drug product, in line with ICH Q6b regulatory requirements [2]. Due to the high structural complexity of these molecules, this will typically require workflows that include a variety of chromatographic approaches including reversed-phase, ion-exchange and HILIC. Within this workflow, analyses to characterise the amino acid sequence of the intact protein (top-down approach), as well peptide sub-units released through hydrolytic cleavage (bottom-up approach), will often be employed for structural sequencing. Due to the lengthy preparation and potential for artefact formation in a bottom-up approach and the limited sequence coverage of the top-down approach, middle-down and middle-up are emerging approaches that are sometimes employed [3]. Both use selective enzymatic hydrolysis, along with chemical reduction, to partially hydrolyse the target protein into well-defined sub-units. In the middle-up approach, mass measurement of the fragments is performed, whilst in middle-down, MS/MS sequencing is carried out. Both of these utilise liquid chromatography (LC) to separate the individual fragments, prior to analysis by mass spectrometry (MS). This Technical Note discusses how Avantor® ACE® UltraCore BIO columns can be utilised to provide the rapid and high-resolution LC separations required for both these approaches.

MAB STRUCTURE AND PARTIAL DIGESTION

Most therapeutic mAbs are immunoglobulin G1 (IgG1) based molecules [4] and consist of two identical heavy polypeptide chains (Hc) of approximately 50 kDa each and two light chains (Lc) of approximately 25 kDa each (shown in blue and green respectively in Figure 1). The four polypeptide chains are linked together via disulfide

bonds to give the characteristic schematic Y-shaped structure shown in Figure 1.

From a functional perspective, the mAb structure can be defined as being comprised of two identical antigen binding fragments (Fab), which define the antigen binding specificity of the mAb and the crystallisable fragment (Fc). The Fc and Fab fragments are linked via the flexible hinge region. Through controlled enzymatic digestion in non-denaturing conditions, it is possible to cleave the mAb at the hinge region of the heavy chain to produce the Fc and $F(ab')_2$ fragments, with the latter being comprised of the two Fab fragments. The use of IdeS (IgG-degrading enzyme of Streptococcus pyogenes) has been shown to conveniently and rapidly cleave specifically between two adjacent glycine residues below the hinge region and is commonly used [5]. Chemical reduction of these fragments (for example by incubation with guanidine hydrochloride and TCEP) can then be used to yield the light chain (Lc), Fc/2 and Fd fragments. Characterisation of these various fragments can then be achieved by LC-MS.

RPLC ANALYSIS

Reversed-phase LC (RPLC) is typically used for the separation of the various mAb fragments discussed, as it can provide high-resolution separations of closely related hydrophobic variants and is readily coupled to MS due to the mobile phase systems and flow rates employed. The fragments released by the approaches in Figure 1 range in mass from approximately 25 to 50 kDa, whilst the intact mAb is around 150 kDa. It is therefore beneficial to use a column that can provide high-resolution separation of the intact protein for top-down analysis, as well as the proteolytically generated sub-units. To successfully analyse large molecular weight peptides and proteins, it is necessary to use a wide pore RPLC column with a pore size of at least 300 Å. Using too small a pore size (for example widely available 100 Å phases) will result in poor accessibility of the protein into the stationary phase, due to the large analyte hydrodynamic radii, leading to poor retention of the analyte and loss of resolution. Generally, diffusion into the pores slows significantly as the pore size falls below 10 x the size of the analyte [6].

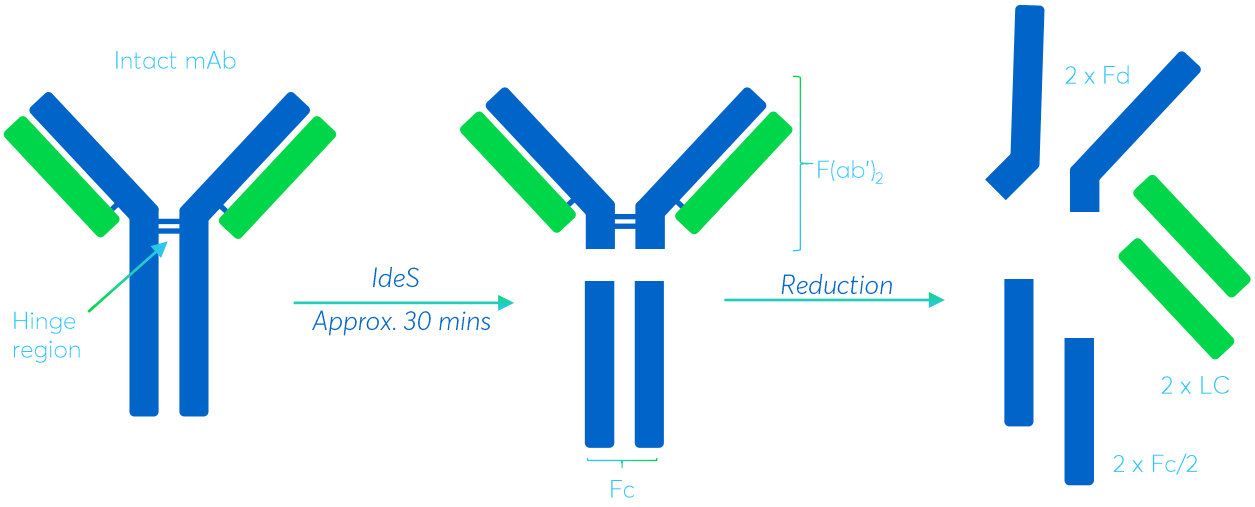
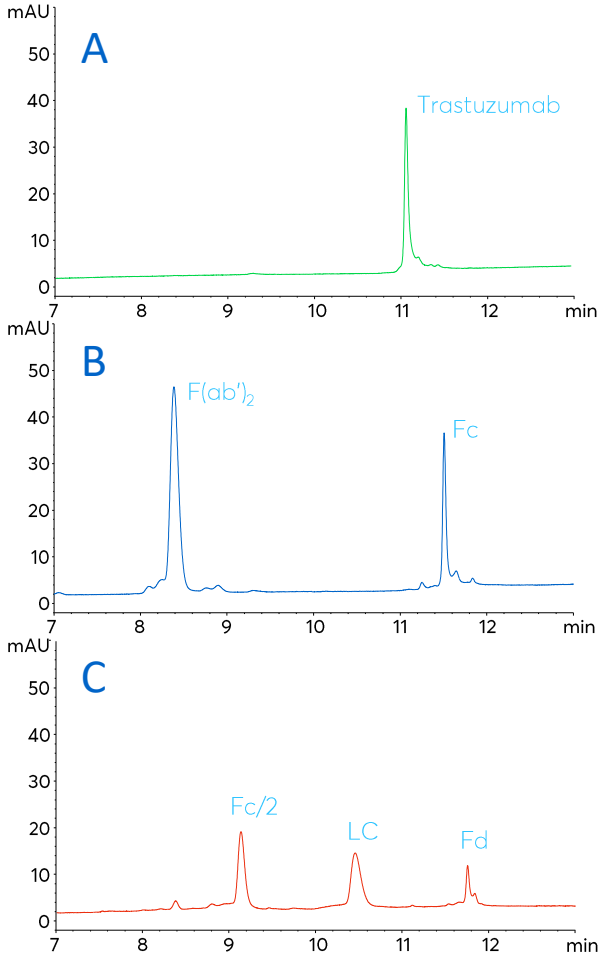


Figure 1: Generalised mAb structure (heavy chains coloured blue and light chains coloured green) and reaction scheme for proteolysis using IdeS, followed by reduction. Adapted from reference [3].

Generating high-efficiency separations can also prove challenging due to the slow molecular diffusivity of large proteins. Many RPLC separations are carried out using stationary phases manufactured from fully porous silica particles. For proteins, the fully porous nature of these particles, combined with slow analyte diffusion, can result in slower mass transfer and an overall reduced kinetic performance, giving rise to broader chromatographic peaks and lower-resolution separations. The use of solid-core stationary phases can help to overcome this. The solid core of such particles leads to a reduction in mass transfer, in addition to favourable improvements in intra-column flow characteristics and longitudinal diffusion, providing better separations for large proteins (please refer to reference [7] for more details).

The Avantor® ACE® UltraCore BIO range of columns is manufactured using solid-core technology and is available with pore sizes of 300 or 500 Å, specifically for the analysis of large peptides and proteins. C4, C18 and Phenyl2 bonded phases are also available to help optimise separation selectivity. Figure 2 demonstrates how the Avantor® ACE® UltraCore C18 300 Å column can be successfully utilised to provide the chromatographic separation required for middle-up/down strategies. The chromatogram A shows retention of the intact mAb under investigation (trastuzumab, 145.5 kDa). The UltraCore column provides good peak shape for the intact mAb, with low level impurity peaks clearly observable. Chromatogram B shows the separation of the products produced by digesting trastuzumab with IdeS. The F(ab')$_2$ and Fc fragments are readily separated.



Columns:	Avantor® ACE® UltraCore BIO C18-300
Dimensions:	100 x 3.0 mm
Mobile Phases:	A: 0.1% TFA in H_2O B: 0.1% TFA in MeCN
Gradient:	

Time (mins)	%B
0	25
20	50
21	95
23	95
24	25
34	25

Flow Rate:	0.43 mL/min
Injection:	20 µL
Temperature:	60 °C
Detection:	UV, 214 nm

Figure 2: Separation of A: trastuzumab, B: trastuzumab IdeS digestion and C: reduced IdeS digestion on an Avantor® ACE® UltraCore BIO C18-300 column. IdeS digestion was carried out at room temperature using a FragIT™ digestion kit (Genovis). Subsequent reduction was carried out by incubation in 4 M guanidine hydrochloride and 50 mM TCEP for 45 min at 56 °C [8].

In addition, the high resolution of the separation means that low-abundance variants are clearly observed. The final chromatogram shows the results obtained on reduction of the IdeS digest proteins with guanidine hydrochloride and TCEP. The separations are all carried out using identical conditions and utilise a gradient with 0.1% TFA as a mobile phase additive. An elevated temperature of 60 °C is used to help improve molecular diffusivity, as discussed above, and can also help reduce the number of conformational states for the target protein, thereby achieving excellent peak shape and performance for these high molecular weight analytes.

CONCLUSION

The use of middle-up/down approaches for the MS-driven structural analysis of mAbs is a powerful tool. A prerequisite for high-quality LC-MS characterisation is a robust, high-resolution RPLC separation to achieve resolution of critical mAb fragments and closely related variants. The use of an LC stationary phase manufactured from solid-core silica particles can prove highly advantageous for the analysis of proteins and peptides due to improved kinetic performance with large molecular weight analytes. The Avantor® ACE® UltraCore BIO range of wide-pore columns has been specifically designed to tackle challenging large molecule separations and features two pore-size options, along with three stationary phase chemistries. This Technical Note has highlighted the key aspects of the middle-up/down approach and demonstrated how to achieve the required separation using an Avantor® ACE® UltraCore BIO C18-300 column.

REFERENCES

1. The Antibody Society, "Antibody therapeutics approved or in regulatory review in the EU or US," [Online]. Available: https://www.antibodysociety.org/resources/approved-antibodies/. [Accessed 17 February 2021].
2. CH Harmonised Tripartite Guideline, "Specifications: Test Procedures and Acceptance Criteria for Biotechnological/Biological Products Q6H," 1999. [Online]. Available: https://database.ich.org/sites/default/files/Q6B%20Guideline.pdf. [Accessed March 2021].
3. L. Fornelli, D. Ayoub, K. Aizikov, A. Beck and Y. Tsybin, "Middle-Down Analysis of Monoclonal Antibodies with Electron Transfer Dissociation Orbitrap Fourier Transform Mass Spectrometry," *Analytical Chemistry*, vol. 86, pp. 3005-3012, 2014.
4. Y. An, Y. Zhang, H.-M. Mueller, M. Shameem and X. Chen, "A new tool for monoclonal antibody analysis. Application of IdeS proteolysis in IgG domain-specific characterization," *mAbs*, vol. 6, no. 4, pp. 879-893, 2014.
5. M. Ryan, D. Petrone, J. Nemeth, E. Barnathan, L. Björck and R. Jordan, "Proteolysis of purified IgGs by human and bacterial enzymes in vitro and the detection of specific proteolytic fragments of endogenous IgG in rheumatoid synovial fluid," *Molecular Immunology*, vol. 45, no. 7, pp. 1837-1846, 2008.
6. U. D. Neue, HPLC Columns, Theory, Technology and Practice, Milford: Wiley-VCH, 1997.
7. Avantor® ACE® Technical Note #0028 "Analysis of intact proteins using Avantor® ACE® UltraCore BIO columns".
8. S. Carillo, R. Perez-Robles, C. Jakes, M. R. da Silva, S. M. Martín, A. Farrell, N. Navas and J. Bones, "Comparing different domains of analysis for the characterisation of N-glycans on monoclonal antibodies," Journal of Pharmaceutical Analysis, vol. 10, p. 23, 2020.