Monoclonal Antibody Analysis on a Reversed-Phase C4 Polymer Monolith Column

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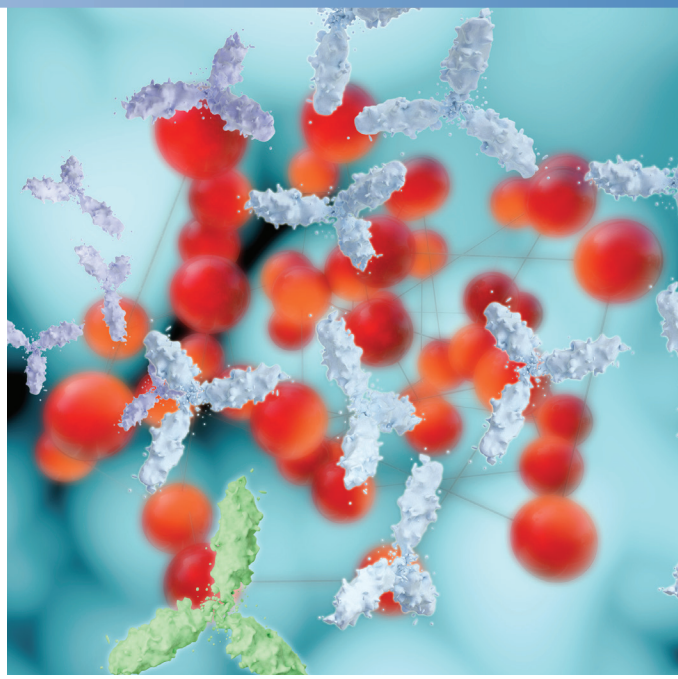
Key Words
Protein, ProSwift, C4 RP-5H, butyl, reversed phase, proteomics, mass spectrometry, high throughput, biopharmaceutical, capillary, monolith, biomolecules, monoclonal antibody, mAb, Fab, glycosylation, carry-over

Goal
To demonstrate the separation of a monoclonal antibody (mAb) and an antigen-binding fragment (Fab) from variants or degradation products, on a reversed-phase C4 (butyl) monolith column using UV detection. The separation is conducted on a 500 µm x 10 cm Thermo Scientific™ ProSwift™ C4 RP-5H column using conventional water/acetonitrile based eluents. This simple, straightforward method can be modified to optimize chromatographic separations for analysis time, resolution, and MS detection of mAbs, mAb fragments, and other protein-based drugs possessing multiple structural variants.



Introduction
Monoclonal antibodies (mAbs) are a rapidly growing area of biopharmaceutical therapeutics that have been shown to be efficacious in the treatment of a wide range of diseases including cardiovascular, autoimmune disorders, and cancers.[1] The nature of recombinant mAb production results in a range of biochemical modifications that can influence the safety and efficacy of the mAb.[2] For these reasons it is critical to understand the structural variants that exist and to what extent they alter the properties of the drug. A variety of methods exist for the separation of mAbs from their structural variants, including ion exchange and hydrophobicity-based HPLC.[3,4] HPLC methods that allow tandem operation with mass spectrometry (MS) are of particular interest due to the ability of the user to directly characterize the individual variants detected. With the use of MS-compatible eluents, reversed-phase chromatography is well suited to tandem LC-MS methods for the separation and detection of structural variants from the main antibody.

Many reversed-phase HPLC columns are based on silica or polymeric media functionalized with a hydrophobic, alkyl or phenyl group. Highly porous polymeric and silica media offer high loading capacity but suffer from band broadening at high flow rates, limiting their use in high-throughput applications. In contrast, monolithic media has been shown to provide increased peak resolution with increased flow rate.[5] The *in situ* polymerization process used to make ProSwift C4 RP-5H columns creates a continuous polymer bed possessing a network of interconnected pores. The porous nature of the monolithic solid phase provides the low backpressure, enabling the use of high flow rates for improved peak resolution and fast, high-throughput analysis. The poly(ethylene dimethacrylate-co-butyl methacrylate) chemistry of the polymeric monolith makes it applicable for reversed-phase separation of a wide range of biomolecules. In comparison to conventional phenyl and alkyl functionalized silica-based separation media, the methacrylate backbone and butyl functionality of the ProSwift C4 RP-5H column is less hydrophobic. This property results in reduced carryover between sample runs, making these columns especially suited for the analysis of high molecular weight proteins including mAbs. The polymeric solid phase is physically robust

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and can be used at temperatures up to 90 °C. The combination of low column backpressure and column stability at high temperatures provides a wide range of conditions for optimizing the gradient for protein separation using conventional reversed-phase eluents. A well-designed gradient is particularly important when separating proteins with only minor variations in structure (e.g., glycosylation) for mass spectrometry analysis.

In this application note we illustrate the separation of both a therapeutic monoclonal antibody and a Fab fragment from various other species present in the samples. By using optimized gradient conditions, multiple minor components can be observed both before and after the main peaks based on their differences in hydrophobicity. Extension of the methods demonstrated here to other mAbs and mAb fragments can be used to improve the separation of structural variants for use with other detection methods, such as mass spectrometry, to determine protein structure.

Experimental

Chemicals and Reagents

- Fisher Scientific LC-MS grade water (P/N W/011217)
- Fisher Scientific LC-MS grade acetonitrile (P/N A/0638/17)
- Thermo Scientific™ Pierce™ Trifluoroacetic acid, LC-MS Grade (P/N 28901)
- Thermo Scientific National Mass Spec Certified 2 mL clear vial with blue bonded PTFE silicone cap MSCERT4000-34W
- ProSwift C4 RP-5H Capillary Monolith Column, 500 µm ID × 10 cm, 10-32 (P/N 164931)
- Thermo Scientific Immobilized Papain, Agarose Resin (P/N 20341)
- Thermo Scientific™ NAb™ Protein A Plus Spin Kit, 0.2 mL (P/N 89948)

Sample Preparation

The Fab and Fc fragments were generated from the monoclonal antibody by papain digestion using Thermo Scientific Immobilized Papain. The Fab fragment was enriched using the NA Protein A Plus Spin Kit. Both the digestion and Fab purification procedures were performed according to the manufacturer's protocol.

Sample for Figure 1

Therapeutic mAb 1
0.5 mg/mL diluted in mobile phase A
Injection volume: 2 µL

Sample for Figure 2

Fab fragment from therapeutic mAb2
Approximate concentration of 0.2 mg/mL diluted in mobile phase A
Injection volume: 8 µL

Separation Conditions

Instrumentation	Thermo Scientific™ Dionex™ UltiMate™ 3000 LC system, consisting of the following:
	DGP-3600 pump dual ternary gradient pump (P/N 5040.0066)
	WPS-3000PL RS Well Plate Autosampler (P/N 5826.0020)
	TCC-3000RS Rapid Separation Thermostatted Column Compartment (P/N 5730.000)
	VWD-3400RS Rapid Separation Variable Wavelength Detector (P/N 5074.0010) with 2.5 µL cell (P/N 6074.0300)
Column	ProSwift C4 RP-5H Capillary Monolith Column, 500 µm ID × 10 cm, 10-32 (P/N 164931)
Mobile phase A	97.5/2.5 (v/v) water/acetonitrile + 0.1% trifluoroacetic acid (TFA)
Mobile phase B	10/90 (v/v) water/acetonitrile + 0.08% TFA
Flow rate	150 µL/min
Temperature	85 °C
Gradient	Table 1

Table 1. LC gradient conditions for Figure 1 and Figure 2.

Time (min)	A	B
0	85	15
0.1	85	15
15.0	45	55
15.1	0	100
16.0	0	100
16.1	85	15
22.0	85	15

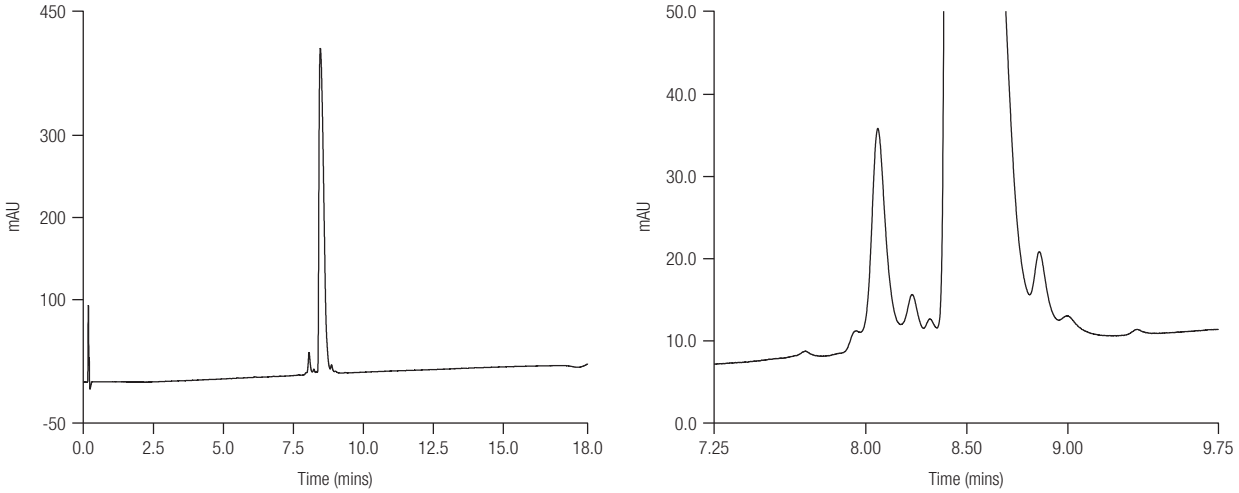
Data Processing

Thermo Scientific™ Dionex™ Chromeleon™ 6.8 Chromatography Data System

Results and Discussion

Structural variants of mAbs often arise due to post-translational modifications including, but not limited to, incomplete disulfide bond formation, glycosylation, N-terminal pyroglutamine cyclization, C-terminal lysine processing, deamidation, isomerization, and oxidation.[2] These structural modifications can change the overall hydrophobicity or charge of the mAb and thus alter the interactions of the mAb with chromatographic media. Cationic and anionic exchange columns can often be used to separate charged variants of mAbs due to differences in the number of cationic or anionic charges resulting from mAb modification. For instance, cation exchange columns can readily be used to separate mAb variants due to the extent of C-terminal lysine processing (e.g., 0, 1, or 2 lysine residues appended to the C-terminal ends of the Fc domain).[6] However, modifications such as glycosylation may not alter the charge of the molecule but will affect the hydrophobicity. Thus, orthogonal methods such as ion exchange will not effectively separate these particular mAb variants. Reversed-phase methods rely on differences in analyte hydrophobicity to achieve separation with less hydrophobic species eluting earlier than more hydrophobic species. For this reason, reversed-phase separation is a more general method since many mAb modifications will alter the local hydrophobicity of the mAb to a certain extent while not all modifications will alter the charge.

Figure 1 shows the separation and detection of a therapeutic mAb from other components using a 500 µm × 10 cm ProSwift C4 RP-5H column. The ProSwift C4 RP-5H monolith was selected due to the lower hydrophobicity of the butyl functionality and polymethacrylate backbone compared to the alternative polymeric phenyl functionalized, divinylbenzene-based media. A flow rate of 150 µL/min was used to improve peak resolution. To reduce the column backpressure, the column was heated to 85 °C; this also improved mass transfer, peak shape, and further reduced carryover between runs. To improve the detection of minor components, a high sample load was used. The left chromatogram in Figure 1 shows the main mAb peak with a PWHH of only 11 seconds and the detection of minor peaks both before and after the main peak. To better illustrate the separation of these minor components, the right chromatogram shows an enlarged region from 7.25–9.75 minutes. Under these separation conditions, five minor peaks are observed before the main mAb peak and three minor peaks are observed after the main mAb peak. Excellent peak shape is observed for all components in this sample using a reversed-phase method on the ProSwift C4 RP-5H column.



Column: **ProSwift C4 RP-5H**
Format: 500 µm × 10 cm
Eluents: A: water/acetonitrile (97.5/2.5) + 0.10% trifluoroacetic acid
B: water/acetonitrile (10/90 v/v) + 0.08% trifluoroacetic acid

Gradient:

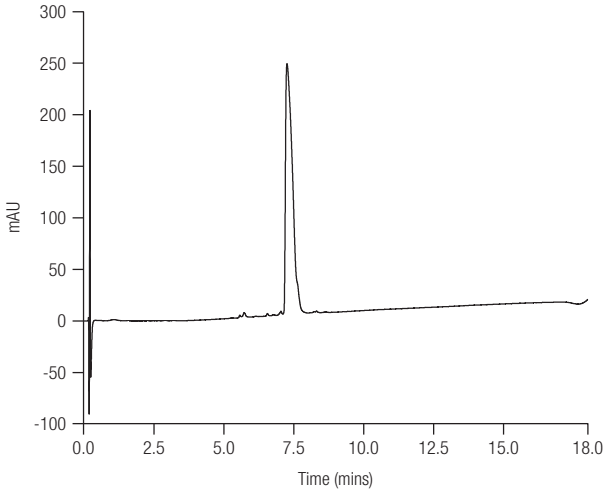
Time	%A	%B
0.0	85	15
0.1	85	15
15.0	45	55
15.1	0	100
16.0	0	100
16.1	85	15
22.0	85	15

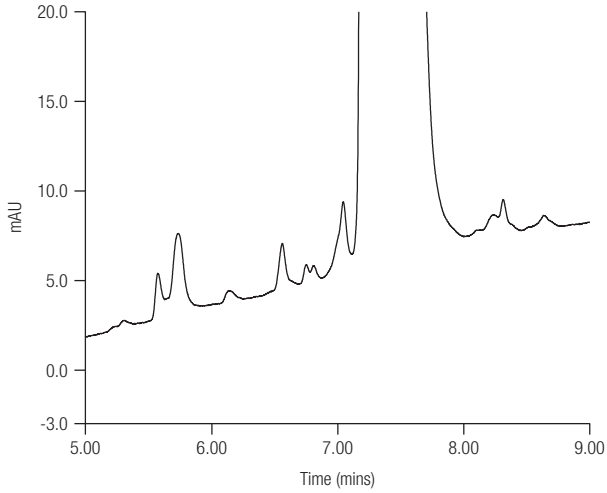
Flow Rate: 150 µL/min
Inj. Volume: 2.0 µL
Temp.: 85 °C
Detection: UV at 214 nm
Sample: mAb, 0.5 mg/mL

Figure 1. Monoclonal antibody analysis on a 500 µm × 10 cm ProSwift C4 RP-5H capillary monolith column, showing (left) the full mAb peak and (right) an enlarged region of the chromatogram at the base of the main mAb peak.

In addition to the intact antibody, understanding the structure and modification of the various mAb fragments (e.g., light chain and heavy chain, Fab, Fc, etc.) is important to the development of effective biopharmaceuticals. For example, the analysis of the Fab domain is of particular relevance as it is the region of the antibody that binds to the target antigen of interest. Mutations in the Fab structure can potentially result in a non-binding mAb or otherwise reduced efficacy. Using the same analysis conditions as in Figure 1, Figure 2 shows the chromatogram of the Fab fragment of a therapeutic mAb. To prepare the Fab sample, immobilized papain was used to enzymatically cleave the Fab fragment from the Fc region of the monoclonal antibody. Next, a NAb Protein A Plus Spin Kit was used to remove the Fc region from the sample. As the Fc region binds to the Protein A, it was removed, leaving the Fab and other non-Protein A-binding species to be collected. As before, the left chromatogram shows a narrow peak for the main Fab fragment with a PWHH of 17 seconds. The enlarged region shown in the right chromatogram reveals multiple components still present in addition to the main Fab fragment. Seven minor peaks are observed before the main Fab peak and three minor peaks are observed after the main peak. As demonstrated for the intact antibody above, the ProSwift C4 RP-5H monolith column provides excellent resolution of the components from the main Fab peak.

For both of the analyses shown here, separation is achieved due to differences in hydrophobicity of the sample components, with more hydrophilic components eluting earlier and more hydrophobic components eluting later than the main mAb or Fab peak. When combined with mass spectrometry, these methods can be used to determine whether the observed minor components are degradation products or structural variants (e.g., glycosylation structure, deamidation, oxidation, etc.). Structural information regarding protein modification is critical to the design of mAbs and other protein-based therapeutics and for proper clone selection for production in many biopharmaceutical processes. Optimization of separation conditions using UV detection prior to characterization by MS can often be faster when working with multiple components samples.





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Eluents: A: water/acetonitrile (97.5/2.5) + 0.10% trifluoroacetic acid
B: water/acetonitrile (10/90 v/v) + 0.08% trifluoroacetic acid
Gradient:

Time	%A	%B
0.0	85	15
0.1	85	15
15.0	45	55
15.1	0	100
16.0	0	100
16.1	85	15
22.0	85	15

Flow Rate: 150 µL/min
Inj. Volume: 8.0 µL
Temp.: 85 °C
Detection: UV at 214 nm
Sample: Fab

Figure 2. Fab fragment analysis on a 500 µm × 10 cm ProSwift C4 RP-5H capillary monolith column showing (left) the full Fab fragment peak and (right) an enlarged region of the peak at the base of the main Fab peak.

Conclusion

- The ProSwift C4 RP-5H column provides excellent peak shape and resolution of minor components in whole mAb and mAb fragment samples.
- The use of elevated linear velocity with relatively shallow gradients provides excellent resolution of closely eluting species.
- Reversed-phase chromatography enables the separation and detection of minor variants and degradation products of mAbs and mAb fragments based on differences in hydrophobicity offering an alternative to ion exchange and HIC-based methods.
- Detection by UV can provide a fast route to separation optimization.

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