Monoclonal Antibody Analysis on a Reversed Phase C4 Polymer Monolith Column

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Overview

Purpose: To demonstrate the separation of a commercial 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.

Methods: The separation of a therapeutic monoclonal antibody was optimized using a C4 capillary monolith column. The antibody was then fragmented and the antigen binding fragment analyzed.

Results: The optimized separation resolves several additional components in the monoclonal antibody separation that may be attributed to post translational modifications or degradation products. Analysis of blank injection post antibody analysis show very little carry over observed compared to a leading silica based C4 column.

Introduction

Monoclonal antibodies (mAbs) are a rapidly growing area of biopharmaceutical therapeutics. The nature of recombinant mAb production results in a range of biochemical modifications that can influence the safety and efficacy of the mAb. 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. 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. Due to 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 groups. 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; whilst in contrast monolithic media has been shown to provide increased peak resolution with increased flow rate.

The Thermo Scientific™ ProSwift™ C4 RP-5H column exists as 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. A 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.

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Methods

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 Thermo Scientific™ NAb™ Protein A Plus Spin Kit. Both the digestion and Fab purification procedures were performed according to the manufacturer's protocol.

Liquid Chromatography (or more generically Separations)

Instrumentation: Thermo Scientific™ Dionex™ UltiMate™ U3000 System with DGP 3600 Pump
Column: ProSwift C4 RP-5H Monolithic Nano Column, 500 µm ID × 10 cm, 10-32 (P/N 164931)
Gradient Conditions: for Figure 1 and Figure 2
Mobile phase A: 97.5/2.5 (v/v) H_2O/CH_3CN + 0.1% TFA
Mobile phase B: 10/90 (v/v) H_2O/CH_3CN + 0.08% TFA
Flow rate: 150 µL/min
Temperature: 85 °C

Time (min)	%A	%B
0	85	15
0.1	85	15
15	45	55
15.1	0	100
16	0	100
16.1	85	15
22	85	15

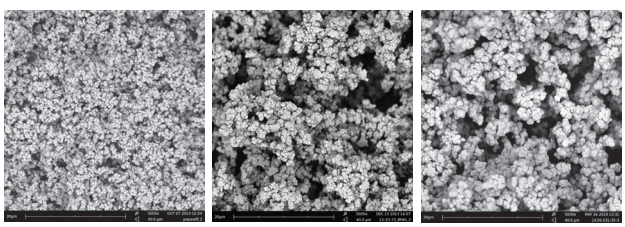
Data Analysis Software: Thermo Scientific™ Dionex™ Chromeleon™

Results

Pore Structure

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. Compared to the PepSwift monolith columns the through pores of ProSwift are larger and this wide pore structure allows the use of high linear velocities at room temperature without generating high backpressure.

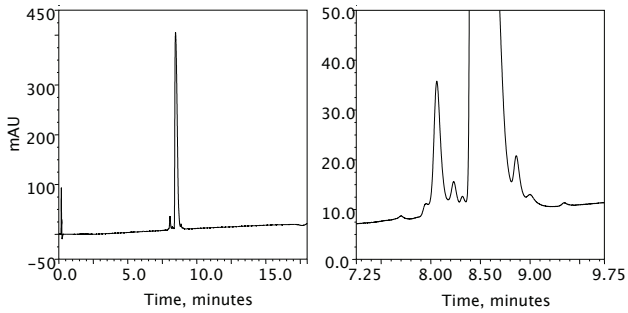
FIGURE 1. Scanning electron microscope images of a PepSwift (left), ProSwift RP-4H (center), and ProSwift C4 RP-5H column.



Analysis of a Therapeutic mAb

Figure 2 shows the separation and detection of a therapeutic mAb from other components. In order to improve peak resolution a flow rate of 150 µL/min was used. The increased flow rate allows each component to rapidly exit the column after elution from the surface. Due to the absence of a secondary pore structure diffusion related mass transfer is minimized.

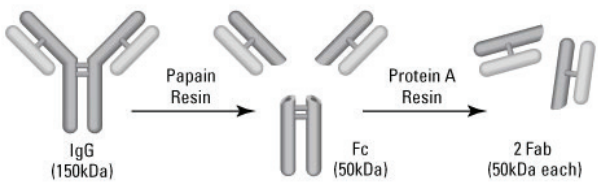
FIGURE 2. Monoclonal Antibody analysis on a 500 µm x 10 cm ProSwift C4 RP-5H monolith column showing (left) the full mAb peak and (right) an enlarged region of the chromatogram at the base of the main mAb peak



To further reduce the column backpressure the column was heated to 85 °C; this also improves mass transfer, peak shape and further reduces carryover between runs. To improve the detection of minor components a high sample load was used. The left chromatogram in Figure 2 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. This is better illustrated in the expanded right chromatogram. 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.

Preparation of Fab Fragment

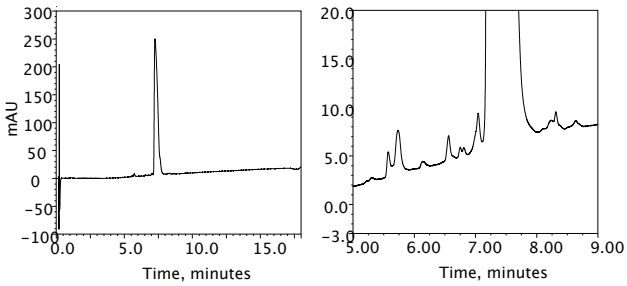
In addition to the intact antibody, understanding the structure and modification of the various mAb fragments (e.g., light chain and heavy chain, Fab, Fc, etc.) is important to the development of biopharmaceuticals.
Analysis of the Fab domain is of particular interest as it is the region of the antibody that binds to the target antigen. Mutations in the Fab structure can result in a non-binding mAb or otherwise reduced efficacy. Figure 3 shows an example scheme to fragment the antibody using enzymatic digestion. To prepare the Fab sample, immobilized papain enzymatically cleaves the Fab fragment , then Protein A removes the Fc region from the Fab and other non Protein A-binding species.



Analysis of mAb Fragments

Figure 4 shows the chromatogram of the Fab fragment. 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.

FIGURE 4. Fab fragment analysis on a 500 µm x 10 cm ProSwift C4 RP-5H 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.

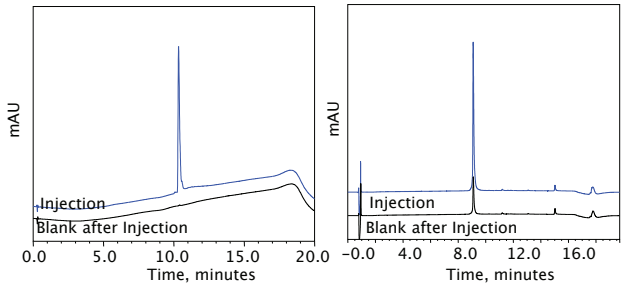


Carryover Analysis

In order to avoid cross contamination between sample injections or to validate product analysis protocols the minimum allowable carryover (MAC) is typically determined. With particularly hydrophobic or "sticky" proteins ,sample carryover can be troublesome or require extensive cleaning protocols.

Figure 5 demonstrates how the ProSwift C4 RP-5H column packing compared to a leading silica based C4 material. It can clearly be seen the improvement in sample recovery and reduced carryover when observing the following gradient run with no sample injection.

FIGURE 5. Left – Column: ProSwift C4 RP-5H (500 µm x 10 cm); Mobile Phase: A: H_2O + 0.05% TFA, B: H_2O/CH_3CN (20/80 v/v) + 0.04% TFA; Gradient: 10–70% B in 15 min; Flow Rate: 100 µL/min; Inj. Vol: 1 µL; Temp: 70 °C; Detection: UV at 214 nm; Sample: mAb – 0.3 mg/mL in DI water + 0.05% TFA. Right – Column: Competitive Silica-C4150 Å (2.1 x 100 mm); Mobile Phase : A: H_2O/CH_3CN (97.5/2.5 v/v) + 0.1% TFA, B: H_2O/CH_3CN (10/90 v/v) + 0.08% TFA; Gradient: 10–70% B in 15 min; Flow Rate: 300 µL/min; Inj. Vol: 1 µL; Temp: 70 °C; Detection: UV at 214 nm; Sample: mAb – 0.5 µg/mL in Eluent A



Conclusion

- When combined with mass spectrometry, these methods can be used to determine whether the observed minor components are degradation products or structural variants
- Monolith technology allows fast separations of large molecules
- High linear velocity combined with shallow gradients provides increased resolution
- Less hydrophobic co-polymer results in lower carryover

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