New Polymeric Capillary Columns for the Separation of Whole Proteins

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Overview

- Thermo Scientific™ ProSwift™ RP-4H monolith columns possess large through pores that allow the column to operate at much lower backpressures than conventional non-porous, packed columns
- At a constant gradient time, the flow rate can be increased to increase protein peak capacity and shorten sample cycle time to significantly reduce overall analysis time for high throughput applications
- Capillary ProSwift RP-4H monolith columns can be readily used in combination with MS to detect and characterize hundreds of proteins in complex cell lysate samples

Introduction

Chromatographic Media for Protein Separations

The separation of proteins is of great importance in the areas of clinical research, systems biology, biomarker discovery, and proteomics. The limits of protein separation and detection in these applications are constantly being approached resulting in the need to develop more advanced chromatographic media and MS instrumentation to keep pace with this constantly evolving field. Many conventional methods for protein analysis rely on bottom-up proteomics workflows, which involve the enzymatic digestion of proteins with subsequent LC-MS analysis to separate and identify the resulting peptides. Computational analysis is then used to re-stitch these fragments back together to determine the original protein structures and make-up of the sample. Recently, there has been rapid growth in the area of top-down proteomics, which entails the separation and MS characterization of intact proteins derived from cell lysate and tissue samples. For continued growth of top-down methods, the challenges associated with separation of these complex, often unrefined, protein samples must be addressed through the development of new chromatographic media.

A monolith column consists of a continuous polymer rod composed of larger through pores, which result in low backpressure at elevated flow rates, and smaller pores for efficient analyte separation without the drawback of poor diffusive mass transfer. These properties make these materials uniquely suited for the analysis of intact proteins, in contrast to conventional packed columns, which can suffer from high backpressure, column fouling, and poor loading depending on the size and surface characteristics of the particulate media. For monoliths, the low column backpressure enables the user to operate at higher flow rates to adjust the gradient length resulting in increased protein resolution and thus improved separation for MS detection. Furthermore, higher flow rates lead to reduced method times allowing one to analyze a greater number of samples in a shorter amount of time for high throughput applications.

Here, we demonstrate the benefits of using monolithic columns for the purposes of MS detection and characterization of cell lysate samples containing hundreds proteins. First, we show the effect of eluent flow rate on protein peak resolution and analysis time when using a constant gradient time. Second, the monolith columns are used to analyze molecular weight fractions derived from cell lysates of a mammalian cell line. Application of the methods shown here can be used to optimize the user’s workflow in order to maximize the number of proteins identified in a given sample set while minimizing the total time necessary for high throughput applications.
FIGURE 1. Pore distribution of the ProSwift RP-4H monolith; 5000x SEM Image (left) and mercury intrusion porosimetry plot (right).

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**Experimental**

**Method**

ProSwift RP-4H Monolithic Nano Column, nanoViper
- 200 µm x 25 cm (P/N 164923)
- 100 µm x 50 cm (P/N 164921)

Thermo Scientific™ Dionex™ UltiMate™ RSLCnano system with UV Detection

Thermo Scientific™ Orbitrap Elite™ Mass Spectrometer

Thermo Scientific™ Dionex™ Chromelone™ Chromatography Data System Software

Thermo Scientific™ Xcalibur™ Software

Thermo Scientific™ ProSightPC 2.0 Software

**Peptide/Protein Mixture**

1 µL Sample Loop

Angiotensin II, Substance P, Lysozyme, Myoglobin, L-Lactic Dehydrogenase each 3 µg/mL in water +0.1% TFA

**Cell Lysate Fractions**

5 µL injection loop

HeLa Cell lysate molecular weight fractions 11–23 kDa and 20–52 kDa
Results

Protein Separation on Monolith Columns

Monolith Structure and Flow Rate Effects

The SEM image in Figure 1 illustrates the presence of both large and small pores in the structure of the ProSwift RP-4H monolith. The larger pores enable the use of higher flow rates for convective mass transfer of the analyte to the functional surface. The narrow distribution of smaller pores evidenced in the porosimetry plot promote efficient separations of the proteins. Even at higher flow rates, the presence of the larger pores results in a low column backpressure as can be observed in the column pressure versus linear eluent velocity in Figure 2. This property of the monolith column allows operation at room temperature when used with high pressure downstream components related to MS.

FIGURE 2. Column pressure at different temperatures measured over a range of linear velocities on the ProSwift RP-4H capillary column.

Effect of Eluent Flow Rate on Protein Resolution and Sample Throughput

Figure 3 demonstrates how simply increasing the flow rate on the ProSwift RP-4H monolith column can increase the peak capacity. Retaining the original gradient at increase flow rate provides increased resolution and faster elution of the desorbed protein means they elute from the column with less influence of diffusion. This results in narrower peaks to provide a greater peak capacity (47, 72 and 95 at 2, 4 and 8 µL/min respectively) over the same gradient time. Additionally, the decrease in gradient delay at higher flow rates results in an overall shorter analysis time. When analyzing a large number of samples, these small changes in separation time can significantly reduce the amount of time required to complete analysis. The overall effect is approximately a 1.25X decrease in analysis time by increasing the flow rate from 2 µL/min to 8 µL/min.
New Polymeric Capillary Columns for the Separation of Whole Proteins

The separation of proteins is of great importance in the areas of clinical research, molecular biology, and structural biology. The challenges associated with separation of large molecules, such as intact proteins, are ongoing and have led to the continued growth of top-down methods, which entail the separation and detection of proteins from intact cells or tissues. The analysis of intact proteins is crucial for understanding their structure, function, and interactions. Conventional methods, such as high-performance liquid chromatography (HPLC) and capillary electrophoresis (CE), have limitations in terms of separation efficiency, detection limits, and sample throughput. Therefore, there is a need for new technologies that can overcome these limitations.

Monolith columns offer several advantages over conventional packed columns. They possess a combination of larger through pores for eluent flow and smaller pores for analyte separation that make them well suited for the separation of intact proteins. This dual pore structure results in low backpressure at elevated flow rates, and smaller pores for contrast to conventional non-monolith media leading to degradation of column performance over time and column fouling, and poor loading depending on the size and surface characteristics of the analyte. Monolith columns are less prone to fouling than conventional HPLC media, which can suffer from high backpressure, decreased loading capacities, and reduced reproducibility. ProSwift™ RP-4H monolith columns possess large through pores with subsequent LC-MS analysis to separate and identify the resulting peptides.

Reproducibility of Separation on Monolith Columns

Fouling is a common problem associated with the analysis of protein samples on conventional HPLC media leading to degradation of column performance over time and reduced column capacity. This is typically evidenced by a reduction in peak retention time. The constant peak retention times for a peptide/protein mixture in Figure 4 shows excellent reproducibility over 500+ runs for the ProSwift RP-4H monolith column.

FIGURE 3. The effect of increased flow rate using a constant gradient time on a ProSwift RP-4H 200 µm x 25 cm column.

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FIGURE 4. Run reproducibility for a ProSwift RP-4H monolithic column, 200 µm x 25 cm

1. Angiotensin II
2. Substance P
3. Lysozyme
4. Myoglobin
5. L-Lactic Dehydrogenase

The ProSwift™ ProSwift™ RP-4H monolith columns are less prone to fouling than conventional non-monolith media leading to degradation of column performance over time and column fouling, and poor loading depending on the size and surface characteristics of the analyte. Monolith columns possess a combination of larger through pores for eluent flow and smaller pores for analyte separation that make them well suited for the separation of intact proteins, in contrast to conventional packed columns, which can suffer from high backpressure, decreased loading capacities, and reduced reproducibility.
Mass Spectrometry Analysis on Capillary Monolith Columns

Analysis of Molecular Weight Fractions from HeLa Cell Lysates

The ProSwift RP-4H capillary columns are specifically designed to be used at flow rates suitable for MS detection of proteins. Figure 5 shows MS detection and characterization of protein molecular weight fractions derived from HeLa cell lysates following separation on a ProSwift RP-4H 100 µm x 50 cm column. At an operational flow rate of only 1 µL/min, the monolith column provides excellent resolution of individual peaks in these samples containing 100’s to 1000’s of proteins. This results in clear signals for MS detection and characterization of the many gene products (proteins encoded by distinctly different genes) and proteoforms (protein forms resulting from the same gene after a combination of alternative splicing events and post-translational modifications) separated by the gradient. For the 11–23 kDa sample, 158 gene products and 332 proteoforms were identified, and, for the 20–52 kDa sample, 59 gene products and 273 proteoforms were identified using ProSightPC 2.0 software to search against a Homo sapiens taxonomy subset of the Swiss Prot database. For both samples, the identifications obtained from the ProSwift RP-4H separation were significantly greater in comparison to identifications obtained using existing resin-based separation protocols (e.g., DVB or C4). These results show that the monolith ProSwift RP-4H columns are well suited to separating proteins in both low and high molecular weight ranges at the low flow rates required for MS detection.

The data also illustrates the reverse phase functionality of the column media without the influence of size exclusion since both low and high molecular weight proteins elute at both early and late stages of the gradient. Controlling the properties of the gradient (eluent composition and flow rate, gradient time, temperature, etc.) allows fine tuning of the separation for the specific application in order to optimize resolution, detection sensitivity, and analysis time.

**FIGURE 5.** LC-MS analysis of molecular weight fractions from HeLa cell lysates using a ProSwift RP-4H 100 µm x 50 cm column. Left: 11–23 kDa, Right: 20–52 kDa

<table>
<thead>
<tr>
<th>Column: ProSwift RP-4H 100 µm x 50 cm</th>
<th>Eluents: A: water/acetonitrile (95/5 v/v) + 0.2% formic acid</th>
<th>B: water/acetonitrile (5/95 v/v) + 0.2% formic acid</th>
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<tr>
<td>Gradient: 5 – 55% B in 50 minutes</td>
<td>Flow Rate: 1 µL/min</td>
<td>Inj. Vol: 5 µL</td>
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<td></td>
<td>Temp: Room temperature</td>
<td>Detection: Thermo Scientific™ Orbitrap Elite with Thermo Scientific™ Xcalibur™ Software</td>
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- 12.0 kDa
- 17.2 kDa
- 17.8 kDa
- 17.3 kDa
- 14.4 kDa
- 11.4 kDa
- 12.0 kDa
- 20.9 kDa
- 18.9 kDa
- 21.8 kDa
- 16.8 kDa
- 22.8 kDa
- 17.3 kDa
- 20.7 kDa
- 31.5 kDa
- 35.8 kDa
- 42.6 kDa
- 45.9 kDa
- 46.8 kDa
- 47.7 kDa
- 51.8 kDa
- 45.9 kDa
- 46.8 kDa
- 51.8 kDa
Conclusion

Protein Separation and Analysis on Monolithic Columns

- Monolith columns possess a combination of larger through pores for eluent flow and smaller pores for analyte separation that make them well suited for the separation of protein samples using elevated flow rates while maintaining a low column back pressure with efficient convective mass transfer.

- Increasing eluent flow rate on monolith columns can result in narrower peaks to give a higher peak capacity for a given gradient run and reduce the overall analysis time for high throughput applications.

- ProSwift RP-4H capillary monolith columns are less prone to fouling than conventional porous particle-based media and can be used for hundreds of runs without degradation of column performance.

- ProSwift RP-4H capillary monolith columns are well-suited for the separation of samples containing hundreds of proteins for the purposes of MS detection and characterization.

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