

Superior Protein Separations with 1 mm i.d. Poly(styrene-*co*-divinylbenzene) Monolithic HPLC Columns

INTRODUCTION

The preparation of monolithic separation media for high-performance liquid chromatography (HPLC) composed of a single, continuous, macroporous polymer support has been reported since the late 1960s. Due to its unique material properties, polymer-based monolithic columns have become an attractive alternative to packed columns, particularly for the LC separations of complex bimolecular samples, such as proteins, monoclonal antibodies, oligonucleotides, and peptides.

In this application note (AN), the LC performance of 1 mm i.d. poly(styrene-*co*-divinylbenzene) monolithic columns for reversed-phase, gradient-elution separations of intact proteins is evaluated. The effects of column temperature, flow rate, and gradient time on peak width and peak capacity are discussed. High column efficiency at optimized LC conditions is demonstrated with the reversed-phase separation of a protein sample containing ribonuclease A, myoglobin, and carbonic anhydrase, and the separation of a complex mixture of intact *E. coli* proteins is presented.

EQUIPMENT

Dionex UltiMate® 3000 Proteomics MDLC system consisting of:

- Membrane degasser
- ×2 dual-gradient pump
- Thermostatted flow manager,
- Well-plate sampler
- UV detector

Chromeleon® Chromatography Data System was used to control the system.

A ProSwift® RP-10R (1 × 50 mm) monolithic column was used for the reversed-phase protein separations.

RESULTS AND DISCUSSION

Optimizing the Monolithic Bed Structure

Polymer-based monolithic stationary phases typically offer high-efficiency protein separations due to the absence of mesopores in the polymer backbone. Consequently, mass transfer is driven by convection instead of diffusion. In addition, the macropore and globule size of the ProSwift RP-10R monolithic column was tuned to maximize separation efficiency. The column was operated at volumetric flow rates (typically around 60 $\mu\text{L}/\text{min}$) to minimize sample dilution, thus maximizing detection sensitivity. Figure 1 shows the typical polymer globular structure of the ProSwift RP-10R monolithic column. It is evident that the monolithic material is well-attached (covalently) to the surface of the capillary wall. This increases the robustness of the column during prolonged use and does not require the frits used in packed columns.

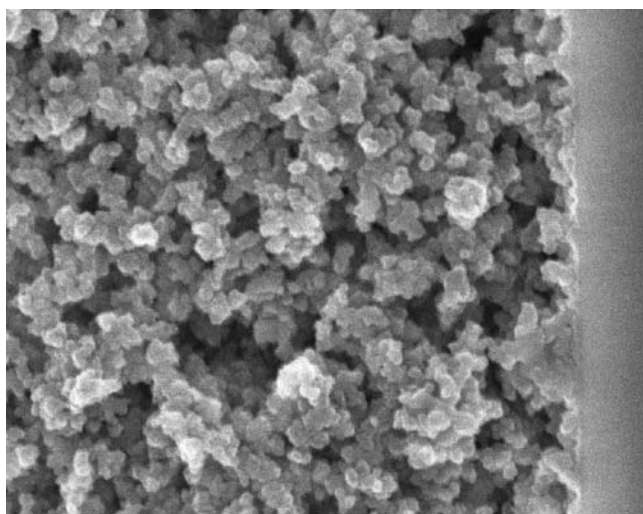


Figure 1. Scanning electron micrograph of the polymer morphology of the 1 mm i.d. ProSwift RP-10R monolithic column.

High-Efficiency Protein Separations

Peak capacity in gradient LC is defined as the maximum number of peaks that can be separated with a resolution of 1 and elute in the applied gradient window. The effects of LC conditions, such as column temperature, flow rate, and gradient time on peak capacity were investigated for gradient LC separations of intact proteins. At elevated column temperatures, diffusion is enhanced, leading to faster mass transfer and narrower peaks. Since the poly(styrene-*co*-divinylbenzene) monolithic material exhibits excellent temperature stability, the column was operated at 80 $^{\circ}\text{C}$. With increasing gradient time, an increase in peak capacity was observed. At optimized LC conditions (flow rate in the optimum of the van Deemter curve and a column temperature of 80 $^{\circ}\text{C}$), a steep initial increase in peak capacity from 34–300 was obtained upon increasing the gradient time from 5 to 20 min. At longer gradient times and shallower gradients, the peak capacity levelled off to a maximum of 475 at a gradient time of 120 min. This effect is caused by a linear increase in peak width of intact proteins with longer gradient time. Although longer gradient times may yield higher peak capacities, the costs of analysis time become unfavorable, and two-dimensional chromatography may be used as an alternative.

Figure 2 is an example of a high-efficiency separation of a complex protein mixture (*E. coli*) containing more than 4400 proteins. Whereas commonly used columns for protein separations are able to differentiate between 50 proteins, the ProSwift RP-10R monolithic column easily generates a peak capacity of approximately 400 in an analysis time of 60 min. In addition, running the column at volumetric flow rates from 50 to 100 $\mu\text{L}/\text{min}$ ensures excellent compatibility with electrospray interfacing prior to mass-spectrometric (MS) detection.

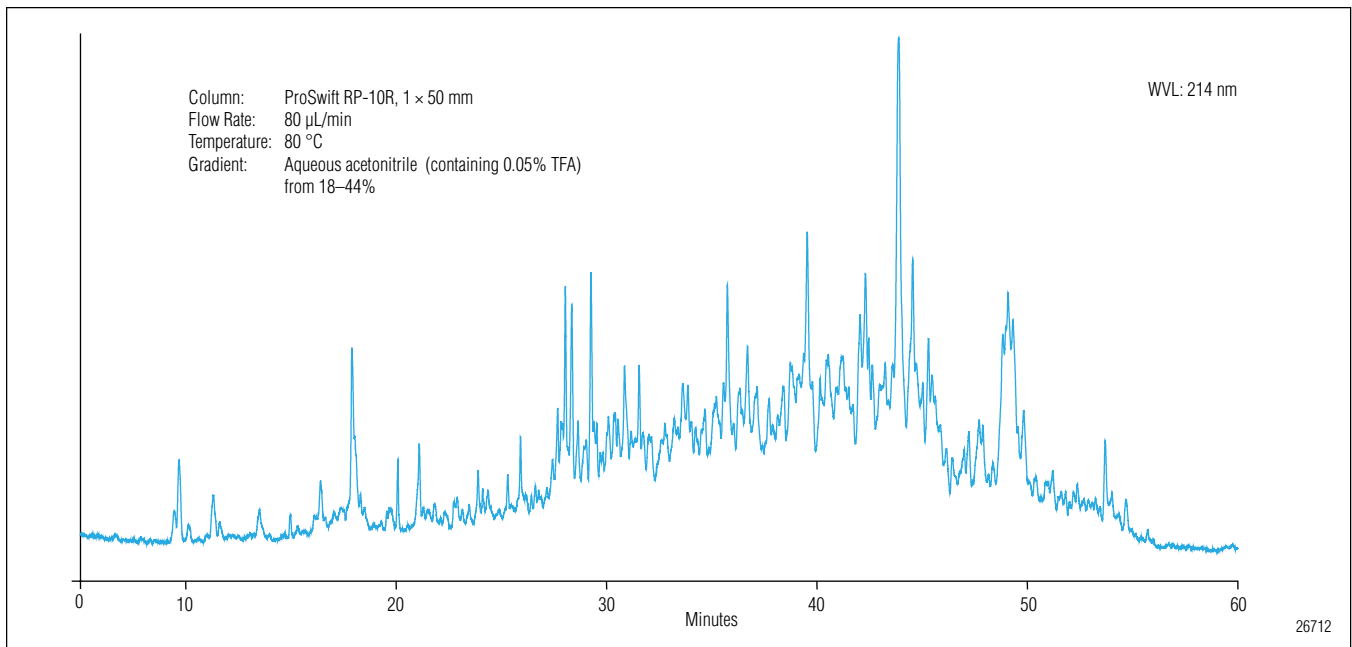


Figure 2. High-efficiency separation of *E. coli* proteins applying a 60 min gradient.

Ultrafast Bimolecular Gradient Separations

For the separation of moderately complex protein samples, the ProSwift RP-10R column is typically operated at a flow rate of 60 $\mu\text{L}/\text{min}$, a temperature of 80 $^{\circ}\text{C}$, and applying gradient times of 10 min. Figure 3A shows a separation of a simple protein test mixture containing ribonuclease A, myoglobin, and carbonic anhydrase. To speed up the separation, the column was operated at a high flow rate of 100 $\mu\text{L}/\text{min}$, applying 80 $^{\circ}\text{C}$ column temperature and a short gradient (Figure 3B). The separation was obtained within 1 min, yielding peak widths at half of the peak height of only 1 s.

CONCLUSIONS

The ProSwift RP-10R monolithic column provides superior high-resolution and high-speed separations of intact proteins. After optimizing column temperature, flow rate, and gradient time, a peak capacity of 475 was achieved within an analysis time of 2 h. In addition, high-throughput 1 min separations were demonstrated with peak widths at half height of only 1 s.

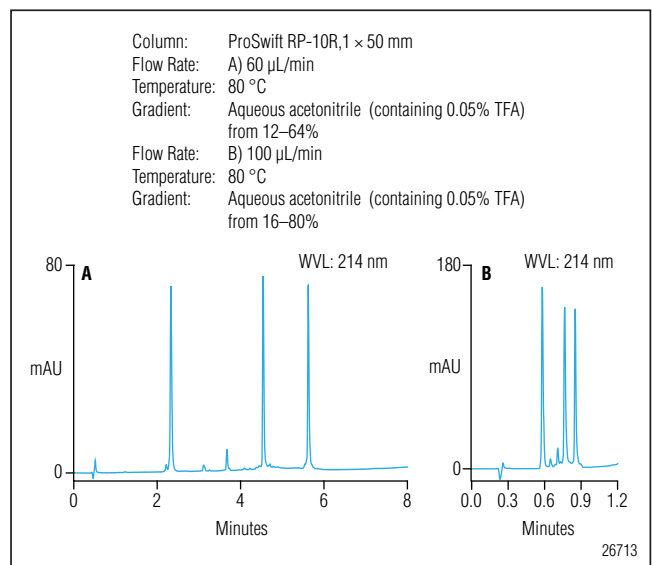


Figure 3. Fast LC separation of ribonuclease A, myoglobin, and carbonic anhydrase applying a 10 (A) and 1 min (B) gradient.

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