Optimized Superficially Porous Particles for Peptide and Protein Analysis

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Abstract

For several years we have been designing and producing superficially porous (Fused-core®) particles for HPLC columns. The characteristics of these particles were specifically created to separate certain solutes optimally, usually based on molecular size. The original 2.7 µm superficially porous particles were created with an average pore size of 90 Å, which was suitable for small molecule analytical separations. This particle technology has been expanded to include wider pore sizes and larger particle sizes that are specifically designed for larger biomolecules. Novel particle designs with specially selected bonded phases for peptide and protein separations are described. This presentation includes fast separations of peptides and intact protein mixtures, as well as examples of very high resolution separations of larger proteins and associated variants and contaminants. Columns with bonded phases for these particles demonstrate high temperature stability, which is ideally suited for the conditions that are often used for analytical and small scale preparative biomolecular separations. Protein recovery and sample loading investigations are included. The optimized shell thickness of the new particles represents a compromise between a short diffusion path versus adequate retention and mass load tolerance. Examples of high molecular weight protein separations highlight the advantages of using columns of superficially porous particles with wider pores. Some comparisons with conventional totally porous particles are also shown.
<table>
<thead>
<tr>
<th>Fused-Core Particle</th>
<th>Particle Size (µm)</th>
<th>Pore Size (Å)</th>
<th>BET Surface Area (m²/g)</th>
<th>Shell Thickness (µm)</th>
<th>% Porosity</th>
<th>Pore Volume (cm³/g)</th>
<th>Separation Utility</th>
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</thead>
<tbody>
<tr>
<td>Halo</td>
<td>2.7</td>
<td>90</td>
<td>135</td>
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<td>75</td>
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<td>HALO-5</td>
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Sample Loading Study for HALO Protein C4

Columns: 4.6 x 100 mm; Gradient: 39-49% B in 10 min.
Mobile phase: A – 0.1% trifluoroacetic acid
B – acetonitrile with 0.1% trifluoroacetic acid; Temperature: 60 °C
Flow rate: 0.5 mL/min.; Injection volume: 5 µL

The particle with 0.20 µm shell thickness offers a compromise between sample loadability and retention, as well as an optimized diffusion path for large MW biomolecules.
HALO® Wide-Pore Fused-Core Particles

HALO Protein

- Shell with 400 Å pores
- 3.0 µm
- 3.4 µm
- Solid Core

HALO-5 Peptide

- Shell with 160 Å pores
- 0.6 µm
- 3.3 µm
- 4.6 µm
- Solid Core
SEM of Images of HALO Protein Particles

FIB (Focused Ion Beam) sliced particle
Comparison of Bonded Phases

Columns: 2.1 x 100 mm HALO Protein
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: PDA @ 280 nm
Temperature: 60 °C

Flow rate: 0.5 mL/min
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 35-66.3% B in 5 min.

Sample: In order
1. Cytochrome c 12.4 kDa
2. Lysozyme 14.3 kDa
3. α-chymotrypsin 25 kDa
4. Catalase 250 kDa (4 x 60 kDa)
5. Enolase 46.7 kDa
6. Carbonic anhydrase 29 kDa
7. β-amylase 200 kDa (4 x 50 kDa)
Column Stability Study

Column: 2.1 x 100 mm; Mobile phase gradient: 25-40% acetonitrile/0.1% aqueous trifluoroacetic acid in 10 min; Temperature: 90 °C; Flow rate: 0.5 mL/min; Detection: 215 nm

• The HALO Protein C4 bonded phase is stable up to 90 °C, showing very little loss of retention.
Protein Separations: Effect of Temperature

- Protein peak shape and recovery improve with increased temperature of analysis.
Protein Separations: Fused-Core compared to Totally Porous

- Separation is 3 times faster at the same back pressure on the HALO Protein column compared to the same sample run on a sub-2-µm totally porous particle column.
Protein Recovery Studies

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<th>Protein</th>
<th>Recovery</th>
</tr>
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<tbody>
<tr>
<td>Cytochrome c</td>
<td>100 (5.8 SD)</td>
</tr>
<tr>
<td>Catalase</td>
<td>92 (18 SD)</td>
</tr>
</tbody>
</table>

- Proteins were fraction collected from a 4.6 x 100 mm HALO Protein C4 column run at 60 °C under gradient conditions with water/ACN/0.1% TFA mobile phase. Blanks were obtained by replacing the column with a union.
- Lyophilized proteins were reconstituted using 3 M Urea/1% Triton X-100/0.25% acetic acid.
- Protein recoveries were measured using QuantiPro™ BCA Assay Kit for 0.5-30 μg/mL protein (Sigma-Aldrich, St. Louis, MO).
- Samples were incubated at 37 °C for 100 min.
- Each sample was run in duplicate.
- Absorbance values were measured at 562 nm.
- HALO Protein C4 shows good recovery of proteins.
**Protein Separations: Effect of Pore Size**

- **Columns:** 2.1 x 100 mm
- **Instrument:** Shimadzu Nexera
- **Injection Volume:** 1 µL
- **Detection:** 280 nm
- **Temperature:** 60 °C

**Mobile Phase A:** water/0.1% TFA
**Mobile Phase B:** 80/20 ACN/water/0.1% TFA
**Gradient:** 40-47% ACN in 10 min.
**Flow rate:** 0.3 mL/min

**Peak Identities (in order):**
1. Catalase 250 kDa [~60 kDa subunit]
2. α-Chymotrypsinogen A 25.0 kDa
3. β-Galactosidase 465 kDa [116 kDa subunit]
4. β-Amylase 200 kDa [~50 kDa subunit]

- **Peak widths in minutes provided above each peak.**
- **The 400 Å pores of HALO Protein enable sharp peaks for high MW biomolecules.**
The extremely low back pressure of the HALO-5 Peptide ES-C18 column enables fast, efficient proteomic separations with a low potential for plugging.
Separation of Reduced IgG1 using TFA Mobile Phase

Column: 2.1 x 100 mm HALO Protein C4
Instrument: Shimadzu Nexera
Injection Volume: 1 µL
Detection: 280 nm
Temperature: 80 °C

Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/water/0.1% TFA
Gradient: 33-40% B in 10 min.
Flow rate: 0.25 mL/min

Sample: 0.5 mg/mL IgG1 treated with 100 mM DTT in 8 M Guanidine HCl at 50 °C for 35 min.
Sample reduced only.

High Resolution Analysis of mAb IgG1 Light and Heavy Chains with LC/MS

2.1 mm ID x 100 mm HALO Protein C4; 0.4 mL/min.; A: 0.5 % formic acid with 20 mM Ammonium Formate
B: 45% AcN/45% IPA/ 0.5 % formic acid with 20 mM Ammonium Formate; Gradient: 29-32% B in 20 min.; 80 °C
Detection: 280 nm Abs; Shimadzu LCMS-2020, ESI +4.5 kV, 2 pps, 500-2000 m/z

<table>
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<tr>
<th>ID</th>
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<tbody>
<tr>
<td>LC1</td>
<td>23,204</td>
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<tr>
<td>LC2</td>
<td>23,192</td>
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<tr>
<td>LC3</td>
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<tr>
<td>HC4</td>
<td>50,680</td>
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<tr>
<td>HC5</td>
<td>28,862</td>
</tr>
</tbody>
</table>

LC = light chain
HC = heavy chain

Masses deconvoluted using MagTran
Conclusions

- Fused-core particles with 400 Å pores are effective for efficiently separating proteins without restricted diffusion.
- Protein separations can be run approximately 3 times faster on columns of Fused-core particles compared to columns of sub-2-µm particles at the same back pressure.
- Fused-core particles have performance advantages over totally porous particles for separating peptides and proteins.
- Columns of 400 Å particles are both efficient and stable up to 90 °C.
- With the low back pressure afforded by 5-µm 160 Å Fused-core particles, columns of these particles are less prone to overpressurizing due to plugging and longer columns can be run for high resolution separations of proteomic samples.
- With the correct choice of mobile phase, high resolution LC-MS data can be obtained for mAb separations using 400 Å Fused-core particles.
Acknowledgment

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