Improving Biomolecule Separations with Superficially Porous Particles of Silica

Barry Boyes$^{1,2}$, Tim Langlois$^{1}$, Brian Wagner$^{1}$, Stephanie Schuster$^{1}$ and Joe DeStefano$^{1}$

1. Advanced Materials Technology, Inc.
Wilmington, Delaware, USA
bboyes@advanced-materials-tech.com
2. Complex Carbohydrate Research Center
University of Georgia
Athens, Georgia, USA

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ABSTRACT OF THE DISCLOSURE

This invention relates to an improvement in chromatography and chromatographic columns. A novel packing of superficially porous refractory particles for use in chromatography has been prepared consisting of a plurality of discrete macroparticles with impervious cores and having irreversibly joined thereto a coating of a series of sequentially adsorbed like monolayers of like colloidal inorganic microparticles. The coating is characterized by being uniform and of predetermined thickness. In preferred embodiments, the cores would be ceramics, preferably glass spheres, and the coating would consist of monolayers of colloidal refractory particles, preferably silica, in a structure of predetermined thickness and porosity.
The Early Days - Practice

Liquid Chromatography

Diuron

Monuron

Gas Chromatography

1.4 mm x 500 cm
The Early Days – Practice at E.I. DuPont Company

PermaPhase ODS Reversed Phase
2.1 x 1000 mm; 50°C; 1.5 mL/min
5-100% MeOH @3%/min

JA Schmit, RC Williams, RA Henry
The Middle Days – “Best is the enemy of better.”
Superficially Porous Particles (SPP/90Å): 2006/7

- Low back pressure due to the particle design (solid core with a porous shell)
- No need for specialized HPLC equipment
- Not necessary to filter samples and mobile phase since frits are not as small as needed for sub-2-µm
- High resolution is maintained at high flow rates (flat C-term in van Deemter plot)

Wide Pore SPP Can Fit the Needs for Protein Science

What is needed for high performance separations of larger (Bio) molecules?

• Pore size must “fit” molecule size
  - Restricted diffusion limits efficiency and load capacity
  - Peak capacity effects by kinetic and retention limitations
• Particle morphology must optimize surface area/volume
  - Shell thickness determines diffusion path and surface area
  - Must have “Right” size and desirable particle distribution
• Surface chemistry appropriate to samples

“Everything is a compromise.”
Halo Peptide Fused-Core Particle Analysis

Electron Micrographs of Halo Peptide

Halo Peptide (160 Å)
mean = 2.85 µm, SD = 0.14

Standard Halo (90 Å)
mean = 2.82 µm, SD = 0.14
Halo Peptide Column Efficiency

Columns: 4.6 x 100 mm; Particle size: 2.7 μm
Mobile Phase: 50% ACN/50% water/0.1% TFA

At 2 mL/min ~2.7 fold lower

Solute: 1-chloro-4-nitrobenzene

β-amyloid (1-38)
MW: 4100 Da
90 Å

β-amyloid (1-38)
MW: 4100 Da
160 Å

Leu-Enk
MW: 555 Da
160 Å

Cells: 4.6 x 100 mm; Particle size: 2.7 μm;
Mobile Phase: Leu-Enk: 21% ACN/79% Water/0.1% TFA
β-amyloid (1-38) 160 Å : 29% ACN/71% Water/0.1% TFA
β-amyloid (1-38) 90 Å : 27% ACN/73% Water/0.1% TFA

High Speed Separation of apo-Transferrin Tryptic Digest

- **0.75 mL/min**
  - 5-60% B in 30 min.
  - P < 287 bar

- **1.0 mL/min**
  - 5-60% B in 20 min.
  - P < 376 bar

- **1.5 mL/min**
  - 5-60% B in 15 min.
  - P < 548 bar

- **Column**: 2.1 x 100 mm ES-C18 160 A
  - A: Water/ 0.1% TFA
  - B: 80% ACN / 0.1% TFA
  - Temp: 60 °C

- **Detection**: 215 nm
- **Sample**: Apotransferrin tryptic digest
- **Injection volume**: 15 uL
Superficially Porous (Fused-Core®) Wide Pore Particles: 400 Å

- Example above is 3.4 µm particle/400 Å pore size
- Many variations in shell thickness, pore size and particle size have been studied
- Theory to support “best properties” is complex, with limited tests using proteins, particularly with larger proteins
- Look for compromise in diffusion path for high MW molecules (to maintain small C-term), load tolerance, usability, speed and efficiency

Fragments for mAb Structure: IdeS Digest

http://www.genovis.com/fabricator


Halo Protein C4 400 Å, 2.1 mm ID x 150 mm; 5 mM DFA; 28-38% AcN in 20 min; 0.35 mL/min, 80 ºC; Orbitrap Velos Pro (30,000 Res) 500-4000 m/z, +3.8 kV, 275 ºC capillary
IgG H and L Chain Separations

Column: HALO 400Å C4, 2.1 x 150 mm; Flow rate: 0.4 mL/min; Temp: 75 °C
Mobile Phase A: water/10 mM DFA; Mobile Phase B: AcN/ 10 mM DFA;
Gradient: 28.5-31.2%B 8 min; 31.2-45.8% in 12min
Instrument: Shimadzu Nexera/Abs (220nm); Orbitrap Velos Pro, 15k Res, ESI 3.8 kV
Injection Volume: 10 µL of mAb (5 µG) in 0.1% TFA Reduced and IAm alkylated Cys

Trastuzumab
L – 23,728 Da
H – 49,997 Da + Glycans (G₀, G₀F, G₁F, G₂F)

S/M MSQ8 ADC Mimic
L₀ – 23,284 Da; L₁ – 23,895
H₀ – 49,585 Da + Glycans (G₀F, G₁F ) + n(611 Da)
Superficially Porous (Fused-Core®) Wide Pore Particles: 1000 Å

- 2.7 μm particle with 0.5 μm thick shell and 1000 Å pores
- Densely bonded C4 phase with endcapping
- Outstanding high temperature and low pH stability
- Surface area ~ 22 m²/g
- Designed for larger proteins

Protein Separation on Wide Pore SPP vs FPP

- Improvement in peak width and retention with larger pore SPP
- As protein size increases, peak widths decrease with increasing pore size
- Similar results in TFA and DFA as mobile phase acidic modifiers

2.1 mm ID x 150 mm C4 columns
20-50% AcN/0.1% DFA in 24 min
Flow: 0.5 mL/min
Temp: 60°C
1.5 μL (0.15-0.2 ug each)

1. RNase A 13.7 kDa
2. α-Lactalbumin 14.2 kDa
3. Enolase 93.1 kDa
4. Carbonic Anhydrase 30.0 kDa
mAb IgG Separation on Wide Pore SPP vs FPP

High Efficiency Separation of Trastuzumab

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Injection Volume: 2 µL (1 µg); Temp: 80 °C

- Large improvement in peak width and *increased* retention with pore size for SPP, moderate additional improvement in peak width with 1000 Å pores
mAb IgGs Separation on Wide Pore SPP vs FPP

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 20 min; Injection Volume: 2 µL (1 µg); Temp: 80 °C

FPP : 0.334 min
SPP : 0.108 min
Flow Rate Effects on Peak Volume for mAb IgG

Fixed Volume Gradient Conditions (4.8 mL); Peak Volume = PW_{1/2} x Flow Rate
Trastuzumab 0.5 µg; 29-35% AcN in 0.1% DFA; 80°C;

- Mass transfer is improved for the large pore SPP particles with higher MW protein.
- Trastuzumab and Silumab exhibited similar results
Load Effects on Peak Width for SPP and FPP for mAb IgG

2.1 mm ID x 150 mm C4 columns; Trastuzumab 0.7 – 140 µg;
27-37% AcN (0.1% DFA) in 10 min; 80°C

- For larger molecules, large pore SPP particles tolerate large sample masses effectively.
- Performance loss is progressive, occurring around 20-50 µg on column
- At all load levels 1000Å pore size SPP performed best for this mAb
IgG2 Disulfide Variant Separation

Column: HALO 100Å C4, 2.1 x 150 mm; Flow rate: 0.2 mL/min; Temp: 60 °C
Mobile Phase A: 88/10/2 water/ACN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/ACN/water/0.1% TFA; Gradient: 20-28% B in 32 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab in 0.1% TFA; Detection: 280 nm; Temp: 60 °C

IgG2 Separation

Column: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Injection Volume: 4 µL of 0.5 mg/mL mAb; Detection: 280 nm; Temp: 80 °C
Mobile Phase A: 95/5 water/N-propanol/0.1% DFA; Mobile Phase B: 70/20/10 N-propanol/AcN/water/0.1% DFA;
Gradient: 14-24% B in 20 min; Instrument: Shimadzu Nexera, Velos Pro Orbitrap

UV Denosumab

TIC Denosumab

Denosumab, glycosylated

Denosumab, deglycosylated

Deconvoluted Mass vs Retention (130-160 kDa)
IgG2 Separation

Column: HALO 1000Å C4, 2.1 x 150 mm; Flow rate: 0.1, 0.2, 0.4, or 0.6 mL/min; Mobile Phase A: 88/10/2 water/ACN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/ACN/water/0.1% TFA; Gradient: 20-28% B in time scaled to flow rate; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab in 0.1% TFA; Detection: 280 nm; Temp: 60 °C

Flow rate, mL/min

Peak volume (PW1/2) µL

Peak 6
Peak 1
Summary and Future Work

- Improving protein separations is both materials and chemistry.
- Superficially porous particle silica packing materials have met the promise of supplying superior separations for large (and small) molecules. Fused-Core with enlarged pore sizes (400 and 1000 Å) have particular utility for protein analyses, are highly robust, and routinely allow faster protein separations with higher efficiency.
- Patience and persistence can pay off, eventually. Dr. Kirkland demonstrates this well with this technology, which required significant effort between concept and practice.
- We continue to build on this legacy, developing new materials and methods (MP and SP) to enable larger biomolecules (>100 kDa) LC and LC/MS analysis, and to improve materials targeted to lower molecular weight analytes, using a variety of LC modes.

“Every experiment tells you something.”
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“You Sell the Sizzle, not the Steak”

“You Biology Guys need a lot of Help”

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