Selectivity Manipulation for LC/MS Analysis of Protein Variants

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Outline

- Superficially porous particles (SPPs) with 1000Å pores are a new class of HPLC materials for high resolution separations of large biomolecules, demonstrating narrow peak widths, increased retention, and high efficiency compared to sub-2µm fully porous particles (FPPs).
- Sample loading for IgGs are compared between the 1000Å SPPs and 300Å FPPs, with the 1000Å SPPs showing improved performance.
- Large pores reduce restricted diffusion with larger molecules, evident for separations of IgG1 and IgG2 samples, which show modest change in peak volume at elevated flow rates, allowing fast analyses.
- New bonded-phase surfaces for 1000Å SPPs are shown, expanding the range of separation selectivity options.
- The combination of bonded-phase and mobile phase manipulations can allow higher resolution LC and LC/MS analysis of intact proteins.

Introduction

Superficially porous particles (SPPs) demonstrate very high performance capabilities as packed columns for LC separations. Recent refinements of SPPs for separations of large biomolecules (>50,000 MW) show the advantages of very large pore size, 1000Å, for large proteins and protein complexes using reversed-phase operation. High resolution protein LC/MS is dependent on operating variables, showing particular sensitivity to column temperature and mobile phase composition. Nevertheless, suitable conditions are shown for specific classes of analytes (such as, monoclonal IgG1, IgG2 and other biotherapeutic proteins) that exhibit high separation performance for these large biomolecules. These high efficiency separations lead to confident assignment of protein structures.

Materials and Methods

Columns of HALO 1000Å with C4, ES-C18 and Diphenyl bonded phases were produced at Advanced Materials Technology, Inc. (Wilmington, DE). SEM images were obtained using a Zeiss (Jena, Germany) Auriga 60 High Resolution Focused Ion Beam & Scanning Electron Microscope at the University of Delaware (Newark, DE).

Mobile phase modifiers were obtained from Pierce (TFA) and Synquest Laboratories or MilliporeSigma (DFA). Acetonitrile (ACN) and n-propanol (nProp) was MS grade from JT Baker. Standard proteins were from MilliporeSigma. Monoclonal antibodies were commercially obtained or generous gifts of highly purified biotherapeutic grade products.

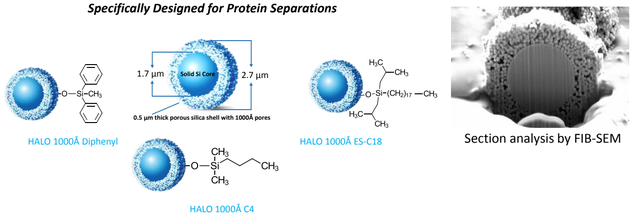
Analytical protein separations used the Shimadzu Nexera LC-30 components (40 µL or 180 µL mixer), with the SPD 20A UV or SPD-M30A PDA detector. An Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI interface was operated at 3.8 kV potential for electrospray. Intact protein MS spectra were recorded in the Orbitrap, using 15,000 resolution scans, an AGC setting of $2x10^6$, and 60-80V in-source CID dissociation.

Deconvolution of MS spectra used MagTran v1.02 (based on ZScore [Zhang and Marshall; JASMS 9 (1998) 225]), or Thermo Scientific Protein Deconvolution v 4.0. Chromatographic peak widths are reported as full width at half height ($PW_{1/2}$).

Improved Superficially Porous Particles for Separations of Proteins

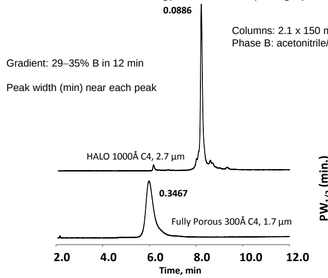
HALO 1000Å Phases

Specifically Designed for Protein Separations

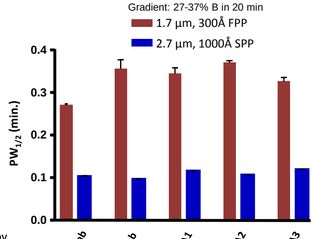


Section analysis by FIB-SEM

- New 1000Å SPP silica materials have been created for highly efficient protein separations. The schematic shows the characteristics for the 2.7 µm particle diameter SPPs.
- Protein separations show improved band widths and resolution, even when compared to much smaller diameter (sub-2µm) fully porous particles (FPP), without the disadvantage of high column backpressures.
- Larger pore sizes improve mass transfer limitations, leading to higher efficiency (narrowed bands) for proteins, permitting higher speed separations.
- HALO 1000Å technology includes multiple highly stable bonded-phase options to enhance selectivity.



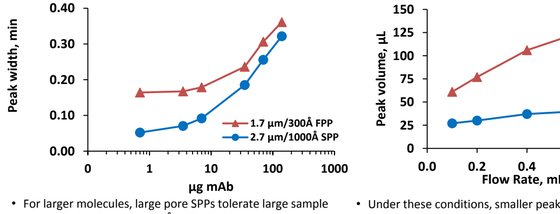
Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: as specified; Detection: 280 nm; Temp: 80 °C



- Highest resolution of IgG1s (Silumab) demonstrated by the 2.7 µm particle with 1000Å pores. This is not unique to this specific antibody, as shown in the graph.

Sample Load Effects on Peak Width for IgG1

Columns: 2.1 x 150 mm; Flow rate: 0.5 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 10 min; Instrument: Shimadzu Nexera; Injection Volume: 0.1,0.5, 1, 5, 10, or 20 µL of 7mg/mL trastuzumab; Detection: 280 nm; Temp: 80 °C



- For larger molecules, large pore SPPs tolerate large sample masses better than the 300Å FPP, at all loads.
- Even though the SPP material has lower surface area (22 m^2/g) than FPP (>80 m^2/g), the *fully-effective surface* is accessible to intact proteins.

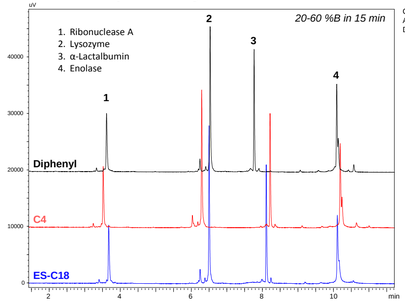
Flow Rate Effects on Peak Volume for IgG1

Columns: 2.1 x 150 mm; Flow rate: 0.1, 0.2, 0.4, 0.6, or 0.8 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 29-35% B; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

- Under these conditions, smaller peak volume shows improved mass transfer kinetics
- Large pore SPPs are best at all flow rates and do not show marked decrement with increasing flow rates, as FPP do.

Selectivity Options Using Bonded Phases for HALO 1000Å SPPs

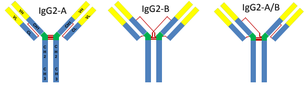
Separation of Standard Proteins



Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H_2O/0.1% TFA; Mobile Phase B: ACN/0.1% TFA; Gradient: 20-60 %B in 15 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

- Comparisons of alkyl chain (C4, ES-C18) and Diphenyl 1000Å SPP silica materials show useful selectivity differences to allow separations choices, when using a specific set of operating conditions (acid modifier, temperature and organic solvent).
- Comparing retention of proteins between these bonded phase columns, with retention in small molecule separations, shows poor correlation between these very different sample types.
- The new HALO 1000Å Diphenyl SPP uses a unique bonding approach, addressing a traditional problem in lifetime and reproducibility.

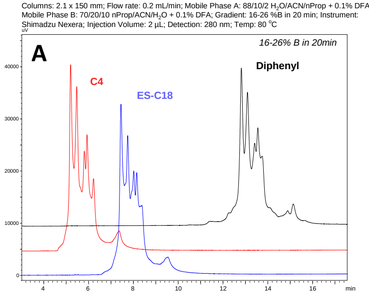
Separation of IgG2



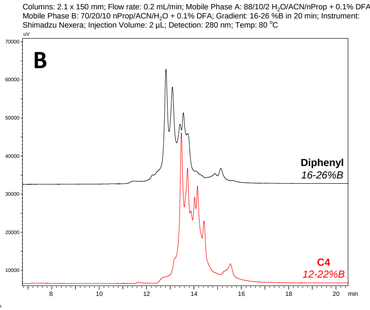
The denosumab IgG2 mAb demonstrates disulfide bridge isoforms that can be resolved by HPLC. with assignments (Panel C, below): P1 and P2,2b are IgG2-B enriched; P3 and P4 are IgG-2A/B, and P5 and P6 are IgG2A, with the presumed identity of P6 as IgG-A*. The assignments are based on non-reduced Lys-C digestion mapping, and employing terminologies of previously employed (Dillon, et al., 2008, *J.Biol.Chem*, 283, 16206; Wypych, et al., *J.Biol.Chem*, 283, 16194; Wang, et al., 2010, *J.Sep.Sci.* 33, 2671).

- Panel A is an initial comparison of the 3 bonded-phase materials, for potential to resolve isoforms,
- Panel B adjusts retention to the middle of the gradient range of comparison, examining resolution and appearance of additional peaks,
- Panel C represents fine tuning of the gradient slope to effect resolution of protein isoforms (disulfide bridge variants).

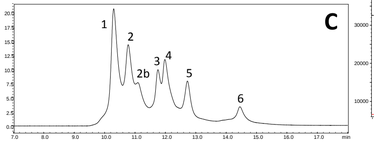
Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H_2O/ACN/nProp + 0.1% DFA; Mobile Phase B: 70/20/10 nProp/ACN/H_2O + 0.1% DFA; Gradient: 16-26 %B in 20 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C



Columns: 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 H_2O/ACN/nProp + 0.1% DFA; Mobile Phase B: 70/20/10 nProp/ACN/H_2O + 0.1% DFA; Gradient: 16-26 %B in 20 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL; Detection: 280 nm; Temp: 80 °C

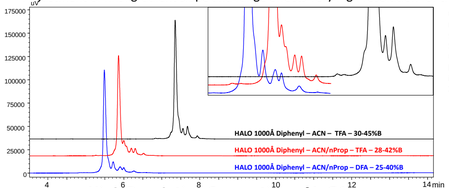


Column: Halo 1000Å C4, 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 water/ACN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/ACN/water/0.1% TFA; Gradient: 20-28% B in 32 min, Temp: 60 °C



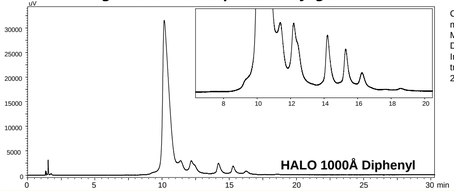
Selectivity Options for 1000Å SPPs With Mobile Phases

- Diphenyl 1000Å SPP column is compared for the effects of organic modifier and acidic modifier on the separation of the minor protein variants of the IgG1 mAb trastuzumab.
- Resolution of the variants trailing the main IgG component are improved by the combination of bonded-phase (Diphenyl), temperature, an organic modifier of mixed ACN/n-propanol, and difluoroacetic acid (DFA) as acid. Adjustment of final gradient slope and range effects very high resolution for these variants.



Column: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: H_2O + specified 0.1% acid; Mobile Phase B: Specified organic solvent + 0.1% acid; Gradient: as specified in 15 min; Injection Volume: 2 µL of 2 mg/mL trastuzumab in aq. 0.1% TFA; Detection: 280 nm; Temp: 80 °C

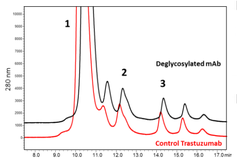
High Resolution Separation of IgG1

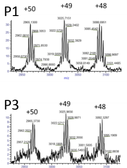


Column: 2.1 x 150 mm; Flow rate: 0.25 mL/min; Mobile Phase A: H2O/0.1% DFA; Mobile Phase B: 50/50 ACN/nProp/0.1% DFA; Gradient: 29-33 %B in 29 min; Injection Volume: 2 µL of 2 mg/mL trastuzumab in aq. 0.1% TFA; Detection: 280 nm; Temp: 60 °C

LC/MS of mAb with HALO 1000Å

- To investigate the structures of the resolved variants, high resolution separations were conducted on intact mAb, as well as PNGase F deglycosylated mAb. High quality mass spectra of the intact variants was obtained by online coupling ESI with the Orbitrap mass spectrometer. Extracts of the raw spectra used for mass analysis of Peaks labeled 1 and 3 are shown, and the attached Table presents mass differences between Peak 1 (trastuzumab), and the Variants 2 and 3, as determined for both intact mAb and after glycan removal.





Glycosylated/Deglycosylated Deconvolution Results

Peak #	G0F/G0F		G1F/G0F		G1F/G1F, G2F/G0F		Deglycosylated PNGase F	
	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)	Theoric (Da)	Measured (Da)
P1_GLY	148057	148052	148219	148220	148381	148381	145167	145166
P2_GLY		148061		148202		148367		145155
ΔMass		11		18		14		11
P3_GLY		148059		148224		148378		145161
ΔMass		7		4		3		5

Reasonable agreement in mass differences are observed before and after glycan removal. The data obtained contribute to the ongoing definition of the resolved variant structures.

Conclusions

- High resolution reversed phase separations were demonstrated for a variety of proteins, including IgG1 and IgG2 mAbs, using columns of novel 1000Å SPP silica packing materials.
- 1000Å SPPs exhibit superior efficiency for large protein separations. Despite having a lower total surface area than FPP, large pore SPPs exhibit better sample loading for large biomolecules, and maintain small peak volumes at increased flow rates.
- The superior 1000Å SPP materials show subtle, but useful, selectivity differences between the newly created ES-C18 and Diphenyl bonded-phase column packings.
- A strategy for varying bonded phase, temperature and mobile phase modifiers was demonstrated for resolution of closely related mAb IgG variants.

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