High Efficiency Wide Pore Superficially Porous Particles for LC/MS of Larger Proteins

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Presented at ASMS 2017 - ThP 601

Outline

- Superficially porous particles (SPPs) with 1000 Å pores demonstrate narrow peak widths, increased retention, and high efficiency compared to sub-2µm fully porous particles (FPPs).
- Sample loading is compared between the 400 and 1000 Å SPPs and 300 Å FPP, with the 1000 Å SPPs showing improved performance.
- Flow rate effects are evaluated using both IgG1 and IgG2 samples with modest change in peak volume at elevated flow rates (fast analyses).
- High resolution separations are demonstrated under LC-MS conditions for IgG2 with the 1000 Å SPPs.

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, 1000 Å, 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 separations performance for these large biomolecules. These high efficiency separations lead to confident assignment of protein structures, including description of post translational and chemical modifications – see Poster ThP 539 for examples of analytical approaches for such modifications.

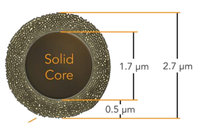
Materials and Methods

Columns of HALO 400Å and HALO 1000Å C4 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) or Synquest Laboratories (DFA). Acetonitrile was MS grade from JT Baker. Proteins were from MilliporeSigma.
Monoclonal antibodies were commercially obtained.
Analytical protein separations used the Shimadzu Nexera LC-30 components (40 µL or 180 µL mixer), with the SPD 20A UV detector and MS-2020 quadrupole MS operated in series at +4.5 kV capillary potential (A special low volume flow cell was obtained from Shimadzu Scientific for this effort, to minimize band dispersion effects) or the SPD-M30A PDA detector. An Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI interface operated at 3.8 kV potential was used to collect MS. Intact protein MS spectra were recorded in the Orbitrap, using 15,000 resolution scans and 60-80V in source CID. 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 half height ($PW_{1/2}$).

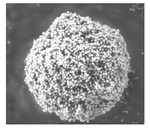
Improved Superficially Porous Particles for Separations of Proteins

- New 1000 Å SPP silica materials have been created for protein separations. The schematic below shows the characteristics for the 2.7 µm diameter SPPs. These materials allow highly efficient packed columns, exhibiting fast protein separations , even for very high molecular weight proteins and polypeptides (for example, IgG and myosin).
- Comparisons of protein separations show improvements in band widths and resolution, even when compared to much smaller diameter (sub-2µm) particles, without the disadvantage of high column backpressures.
- Larger pore sizes improve mass transfer limitations, leading to higher efficiency (narrowed bands) for larger proteins. Compared with 300 Å pores, and below, we see improvements at 50 kDa and above.

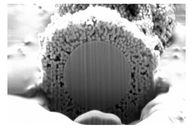
HALO® Fused-Core 1000 Å Protein Particle



Shell with 1000 Å pores



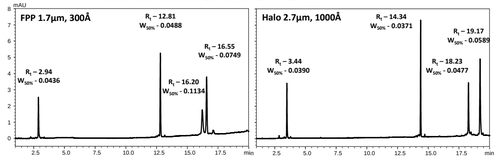
SEM



Section analysis by FIB-SEM

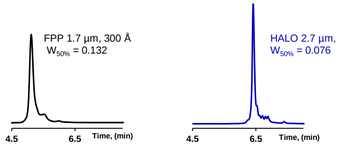
Large Pore SPP Improve Protein Separation Efficiency (Peak Width)

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: 20-50% B in 24 min; Instrument: Shimadzu Nexera; Injection Volume: 1.5 µL (0.15-0.2 µg each); Detection: 280 nm; Temp: 60 °C
Peak Identities: Ribonuclease A; α-Lactalbumin; Enolase; Catalase

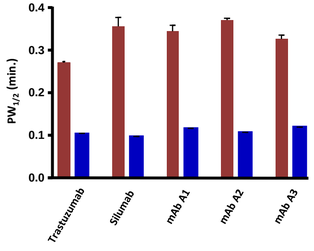


Smaller IgG1 Peak Widths with 1000 Å SPP Compared to 300 Å FPP

Columns: 2.1 x 150 mm; Flow rate: 0.4 mL/min; Mobile Phase A: water/0.1% TFA; Mobile Phase B: acetonitrile/0.1% TFA;
Gradient: 32-38% B in 12 min; Injection Volume: 2 µL (1 µg); Detection: 280 nm; Temp: 80 °C



- Improved resolution of an IgG1 (trastuzumab) is demonstrated by the 2.7 µm particle with 1000 Å pores. This is not unique to this antibody.



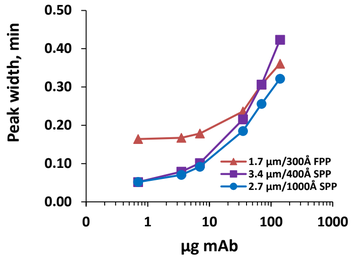
- For all mAbs tested, the peak widths measured at 50% peak width were smaller with the 1000Å SPPs compared to the 300Å FPPs

1.7 µm, 300 Å FPP
2.7 µm, 1000 Å SPP

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; Instrument: Shimadzu Nexera; Injection Volume: 2 µL (1 µg); Detection: 280 nm; Temp: 80 °C

Sample Load Effects on Peak Width for IgG1

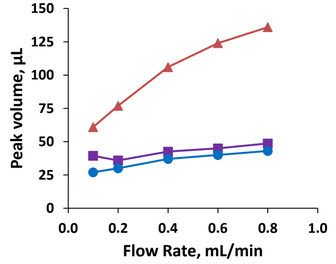
Columns: 2.1 x 150 mm; Flow rate: 0.5 mL/min; Mobile Phase A: water/0.1% DFA; Mobile Phase B: acetonitrile/0.1% DFA; Gradient: 27-37% B in 10 min; Instrument: Shimadzu Nexera; Injection Volume: 0.1,0.5, 1, 5, 10, or 20 µL of 7mg/mL trastuzumab; Detection: 280 nm; Temp: 80 °C



- For larger molecules, large pore SPPs tolerate large sample masses effectively
- Performance loss is progressive, occurring at 20-50 µg on column
- At all load levels, 1000Å pore size SPPs performed best

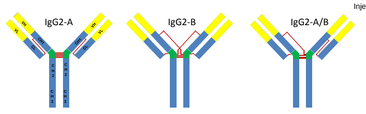
Flow Rate Effects on Peak Volume for IgG1

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

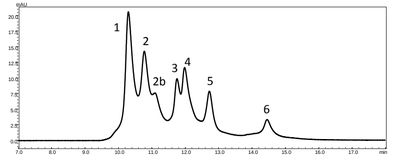


- Under these conditions, smaller peak volume indicates improved mass transfer
- Large pore SPPs are best at all flow rates and do not show marked decrement with increasing flow rates, as FPP do.

High Resolution IgG2 Separations using 1000 Å SPPs

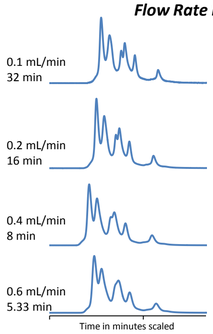


Column: HALO 1000Å C4, 2.1 x 150 mm; Flow rate: 0.2 mL/min; Mobile Phase A: 88/10/2 water/AcN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/AcN/water/0.1% TFA; Gradient: 20-28% B in 32 min; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab in 0.1% TFA; Detection: 280 nm; Temp: 60 °C

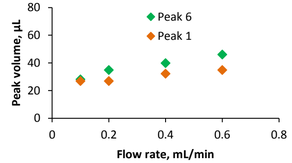


- Very high resolution separations are achieved with the 1000Å SPPs for a complex IgG2 such as denosumab
- Disulfide bridge isoforms are partially resolved; assignment is: P1 and P2,2b are IgG2-B enriched; P3 and P4 are IgG-2A/B, and P5 and P6 are IgG2A, with the presumed identity of P6 as IgG-A*. The 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).

Flow Rate Effects on Peak Width (Volume) for IgG2



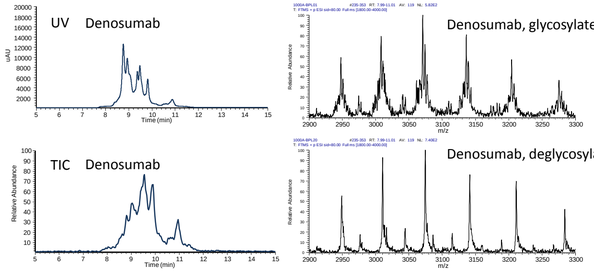
Column: HALO 1000Å C4, 2.1 x 150 mm; Flow rate: 0.1, 0.2, 0.4, or 0.6 mL/min; Mobile Phase A: 88/10/2 water/AcN/n-propanol/0.1% TFA; Mobile Phase B: 70/20/10 n-propanol/AcN/water/0.1% TFA; Gradient: 20-28% B in time scaled to flow rate; Instrument: Shimadzu Nexera; Injection Volume: 2 µL of 2 mg/mL denosumab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



- Averages of 2 are plotted for all points except the flow rate at 0.1 mL/min
- As was observed for IgG1, increased flow rate shows little to no decrement on the peak volume for IgG2 when 1000 Å pore SPPs are used

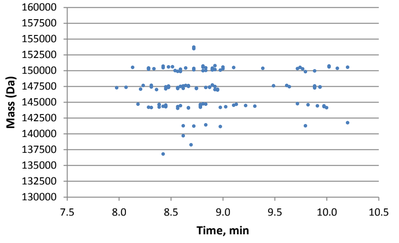
LC/MS of IgG2 using 1000 Å SPPs

Column: HALO 1000Å C4, 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



- Informative intact mass spectra are obtained using the described LC/MS conditions, for native or deglycosylated IgGs, including the example of the IgG2 above. Deconvoluted MS data is shown in the panel below, for approximate assessment of glycosylation condition. Sub-µg quantities are sufficient for routine analysis on analytical scale columns.
- The quality of MS data can be strongly dependent on the IgG preparation, so various additives and mobile phase modifiers have been explored. The interactions between favorable MS and separations conditions are complex, and will be the focus of a later presentation.

Deconvoluted Mass vs Retention (130-160 kDa)



- The masses for the glycosylated denosumab were deconvoluted using Protein Deconvolution 4.0
- Differences in masses correspond to different glycan and post-translational modifications, which do not appear to vary significantly with the disulfide isoforms for this IgG2.

Conclusions

- High resolution reversed phase separations were demonstrated using IgG1 and IgG2 mAbs with columns of 1000 Å SPPs
- 1000 Å SPPs exhibit favorable efficiency for large protein separations, compared to 300 Å fully porous particles, for IgG1 and IgG2 mAbs
- 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. This effect possibly reflects improved pore volume accessibility with such SPP materials

Acknowledgements: Tim Langlois, Conner McHale, and Bob Moran for advice and technical assistance. This work was supported in part by National Institute of General Medical Sciences, [GM116224 to BEB]. The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institute of Health.