Improving Protein LC/MS Analysis

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Outline

- Protein separations are improved by the use of superficially porous particles (SPP) of an appropriately enlarged pore size
- Highly efficient protein separations can be obtained in shorter times than previously available
- Mobile phase acidic modifiers are compared. Formic acid (FA) exhibits relatively poor LC performance and trifluoroacetic acid (TFA) the best. ESI-MS intensities show the opposite trend, with FA the best.
- Several novel acid modifiers are compared, exhibiting improved LC performance, with MS performance better than TFA.
- Difluoroacetic acid (DFA) may be a good compromise as a modifier, exhibiting useful advantages for separation and online MS performance.

Introduction

Protein therapeutics and protein reagents continue to find expanded use in research and health care. This contributes to a highly active growth in protein analysis by LC and LC/MS. Many of the proteins of interest are quite large, for example monoclonal antibodies and other multi-subunit proteins, and these present special problems in terms of resolution and separation speed. Present methods for separating and characterizing proteins include various chromatographic separation approaches such as ion-exchange, size-exclusion, hydrophilic interaction, hydrophobic interaction, and reversed-phase. The latter method is especially attractive for many applications because of the capability for efficient and fast separations, using conditions that can be integrated with subsequent analytical tools, most importantly, with MS detection. Improvements in protein separations using conditions that take advantage of ongoing improvements in MS instrumentation are needed. We have previously described the use of superficially porous silica particle (SPP) materials for small and moderate size molecules, and most recently have extended this approach to much larger molecules, including proteins [Schuster, Wagner, Boyes and Kirkland; J. Chromatogr. A 1315 (2013) 118]. Examples of high resolution protein separations with such particles are shown herein. In the course of conducting this analysis of stationary phase materials for protein separations, we have identified significant opportunities to improve resolution, while addressing limitations of typical LC conditions (eg., use of TFA), for application to MS analysis.

Materials and Methods

Columns of HALO Protein C4 were produced at Advanced Materials Technology Inc. (Wilmington, DE). These materials employ superficially porous Fused-Core® silica particles of 3.4 µm diameter, a shell thickness of 0.2 µm, and pore size of 400 Å. Analytical protein separations used the Shimadzu Nexera LC-30 components (40 µL mixer), with the MS-2020 quadrupole MS operated in series at +4.5 kV capillary potential. Capillary column separations used the Dionex RSLC 3000 with a trap column, connected to the Orbitrap VelosPro MS (ThermoScientific, Inc.), with the low flow IonMax ESI interface at 3.8 kV potential. Deconvolution of MS spectra used MagTran v1.02, based on ZScore [Zhang and Marshall; JASMS 9 (1998) 225]. Chromatographic peak widths are reported as half height ($PW_{1/2}$). To assess protein charge state effects of modifiers, the average ionization state was calculated:

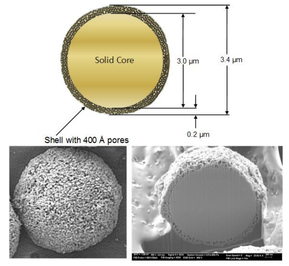
$$q_{avg} = \frac{\sum_{i=1}^{N} q_i * w_i}{\sum_{i=1}^{N} w_i}$$

wherein, N is the number of charge states, q_i the charge on the *i* charge state, and w_i is the intensity for that charge state.

Improved SPP Particles for Separations of Proteins

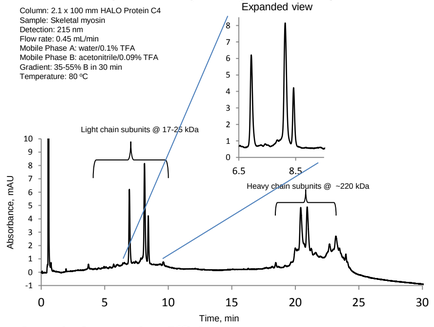
- New large pore (400 Å) SPP silica materials have been created for protein separations. The schematic below shows the characteristics for the 3.4 µm diameter SPP particle. These materials allow highly efficient packed columns, exhibiting fast protein separations , even for very high molecular weight proteins and polypeptides (for example, 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.

HALO® 400Å Fused-Core Particle

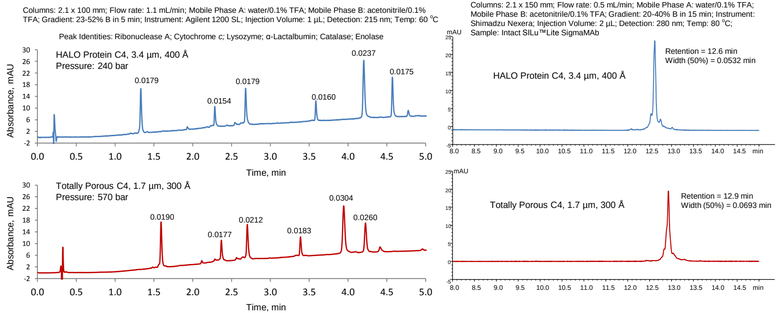


SEM Section analysis by FIB-SEM

Subunit Separation of a Large Protein by RP-HPLC



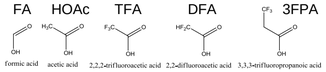
Large Pore SPP Improve Protein Separation Efficiency



- The SPP HALO Protein C4 column exhibits higher performance protein separations compared to a totally porous sub-2µm column.

Acidic Mobile Phase Modifiers for LC/MS Analysis

- TFA is the acidic mobile phase modifier of choice for protein and peptide separations, showing good peak shape and high column efficiency
- TFA can be a bad choice for LC/MS, due to ESI suppression (low signal), background problems (chemical noise), and system persistence (requiring extensive cleanup of an LC/MS for eliminating carryover)
- Formic and acetic acid are widely adopted for LC/MS applications, with variable performance for protein separations, but excellent ESI/MS compatibility
- Alternative acidic modifiers that allow good peak shape, recovery, selectivity, and detection capabilities (absorbance and ESI signal intensities) are needed – some compared for fitness to task are shown below



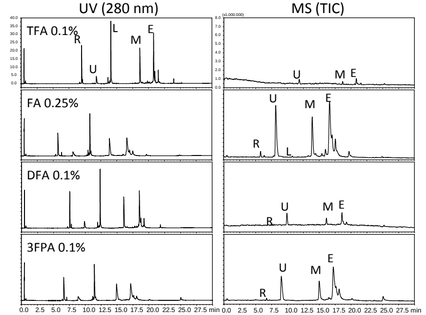
Acidic Modifiers for Protein RP-HPLC/MS

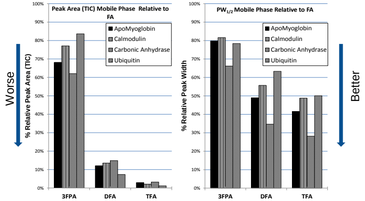
2.1 x 100 mm Halo Protein 400 C4

Gradient: 15-55% AcN 30 min
Flow: 0.35 mL/min
Temp: 50ºC

25 pmol each protein
R – Ribonuclease A
U – Ubiquitin
L – Lysozyme
M – apo-Myoglobin
E – Enolase

MS-2020 Single Quad
400 – 2000 m/z 3 pps
3.8 kV ESI





- Graph compares MS and LC performance for 10 mM of each acid modifier to 10 mM FA
- Each acid was examined at varying concentrations (2-50 mM for fluorinated acids; 20-500 mM for FA), exhibiting progressive suppression of ESI signal with concentration: plateau at 50 mM for FA, 10-20 mM others
- Progressive improvement of LC performance: above 5-20 mM, except FA, requiring above 100 mM

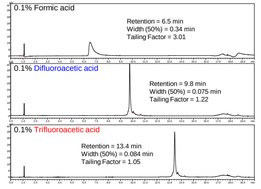
Application of Improved Materials and Conditions

- The use of DFA as an alternative to TFA and FA as the acidic modifier for RP-HPLC with and without MS detection has been explored in greater detail
- Separations of several intact IgGs (and other proteins) reveal that LC performance in FA is much poorer than in either TFA or DFA
- LC/MS comparisons of separations using DFA and FA, or mixtures thereof, indicate that DFA has useful LC properties, while allowing high resolution MS analysis, with moderate reduction in ionization
- Comparing the human hemoglobin subunit separation, improved LC performance in DFA largely compensates for lower ESI signal, due to improved band widths for eluting polypeptides, and concomitant resolution increase
- Fluorinated ion pairing acids decreased protein average charge state for all proteins examined

Effect of Modifier on IgG Separations

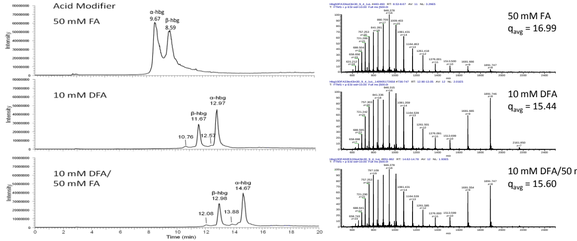
2.1 x 150 mm Halo Protein 400 C4

Gradient: 28-38% AcN/0.1% acid as indicated 30 min
Flow: 0.3 mL/min
Temp: 80ºC
Sample: 2 µL of Intact SILu™Lite SigmaMAb - 0.5 µg/µL (H_2O)



High Resolution Analysis of Human Hemoglobin Subunits by LC/MS

Halo Protein 400 C4, 0.3 mm ID x 100 mm PeekSil Capillary Column; 0.68 µL StemTrap; 33-45% AcN in 20 min; 8.0 µL/min, 50 ºC; Orbitrap Velos Pro (60,000 Res) 500-2500 m/z, +3.8 kV, 325 ºC desolvation capillary



Conclusions

- Superficially porous particles improve RP HPLC separations of many protein mixtures
- Alternative acidic mobile phase modifiers to the broadly used FA and TFA modifiers can be employed for useful protein separations, in some cases exhibiting a better compromise between separations performance and ESI-MS compatibility

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