Developing a New Separation Paradigm for Intact Protein LC/MS Analysis using Next Generation Wide-Pore Core-Shell Media

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Introduction

Analyzing intact proteins by reversed phase often pushes LC/MS to the limits of its capabilities. Reversed phase chromatography struggles to separate minor differences in post-translational modified proteins; electrospray mass spectrometers generate arrays of differentially charged forms requiring deconvolution software to identify minor differences. These limitations are especially evident when analyzing monoclonal IgG therapeutics. Identifying and quantitating by LC/MS minor glycoforms, glycations, as well as structural isoforms common with antibodies, are limited by complexity of the sample as well as poor recovery.

Recently introduced wide-pore core-shell media fundamentally changed the chromatography of large intact proteins. Analysis conditions are required to be dramatically altered versus the highly specialized isopropanol-based mobile phases previously reported with other intact antibody methods. Lower retentivity requires lower initial organic concentration, but also results in higher recovery of hydrophobic proteins.

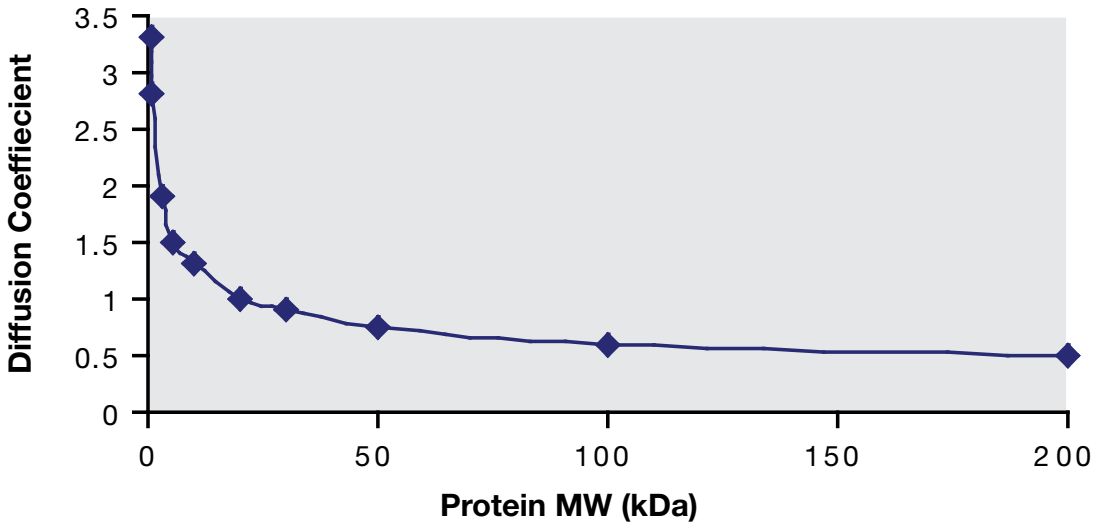
Efforts were undertaken to explain the rationale for particle design as well as present some parameters to consider when developing methods using such media.

Materials and Methods

Mouse IgG and EGF were purchased from ProSpec (New Brunswick, NJ); myoglobin, bovine IgG, and other chemicals were purchased from Sigma (St. Louis, MO). Solvents were purchased from EMD (San Diego, CA). Core-shell columns were packed at Phenomenex (Torrance, CA) and fully porous columns were purchased from column manufacturers (various sources).

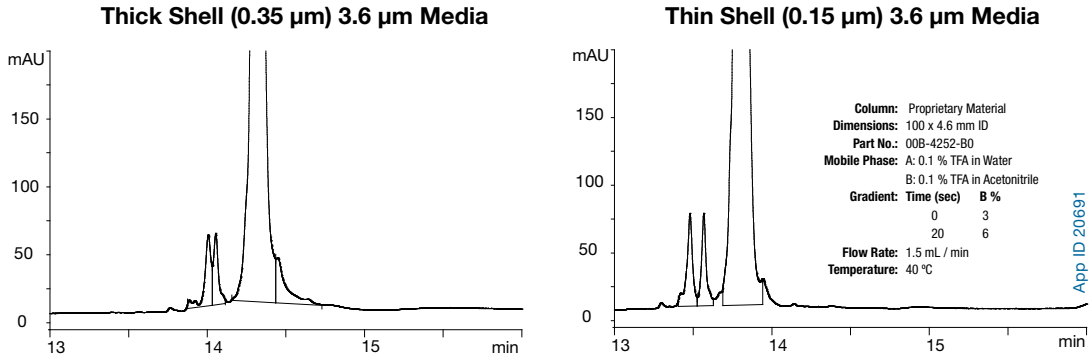
Myoglobin and other protein samples were partially degraded by incubation at room temperature for up to a week in dilute acid. For refolding analysis, samples were reduced with DTT; reduced/non-reduced mixtures were generated by spiking different ratios of the native to the reduced sample prior to injection on HPLC. The various protein samples were analyzed on an Agilent® 1200 HPLC system (Palo Alto, CA). Detection was monitored by either UV or MS (API 3000™ triple quadrapole MS, ABSCIEX™ Foster City, CA) in full-scan MS mode. Mass reconstruction was performed using ProMass software (Novatia, Monmouth Junction, NJ). Mobile phases used were either 0.1 % TFA in water (A) and 0.085 % TFA in acetonitrile (B) or formic acid in similar concentrations. Different gradients, flow rates, and column temperatures are listed with corresponding chromatograms.

Figure 1. Protein Diffusion vs. Size



Shown is a linear graph of protein diffusion rates through porous particles based on Gutenwick et al. (JCA 2004) and Davies et al. (1989).
While diffusion rates are a minor factor for small molecule chromatographic efficiency, for proteins larger than 25 kDa diffusion in and out of a porous column becomes the limiting factor in efficiency of reversed phase separations.

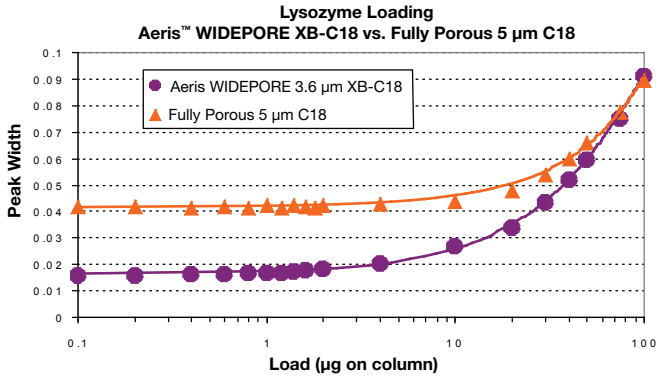
Figure 2. Resolution vs. Shell Thickness



Lysozyme is compared on two different core-shell prototypes with varying porous shell thickness. The thin shell media provided narrower peak widths and increased efficiency compared to the thicker shell particle. Optimal reversed phase protein separation is a balance between shell thickness, pore permeability, and loading.

Comparative separations may not be representative of all applications.

Figure 3. Loading vs. Peak Width



Peak width for Aeris WIDEPORE core-shell compared to a fully porous 300 Å high surface area media. Note the narrower peak width at analytical loads for lysozyme. As loading increases peak width for both columns increase, shell thickness was optimized to provide significantly better performance compared to fully porous particles of the same diameter at loadings up to 20 µg.

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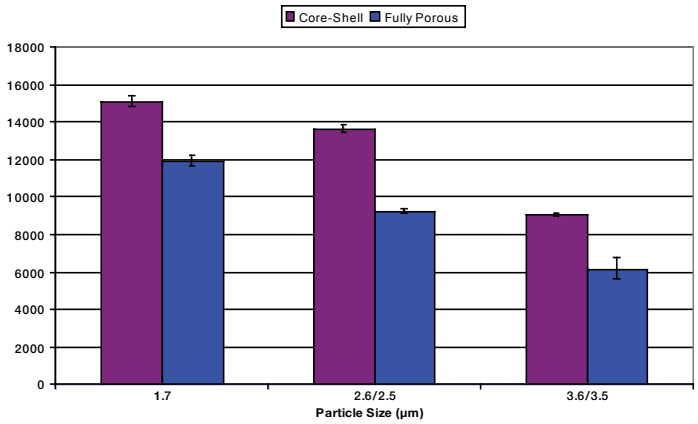
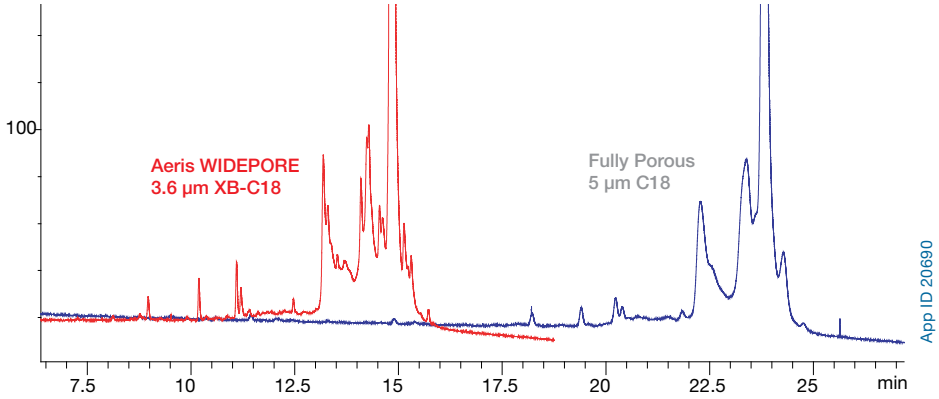
Figure 4. Core-Shell vs. Fully Porous Media



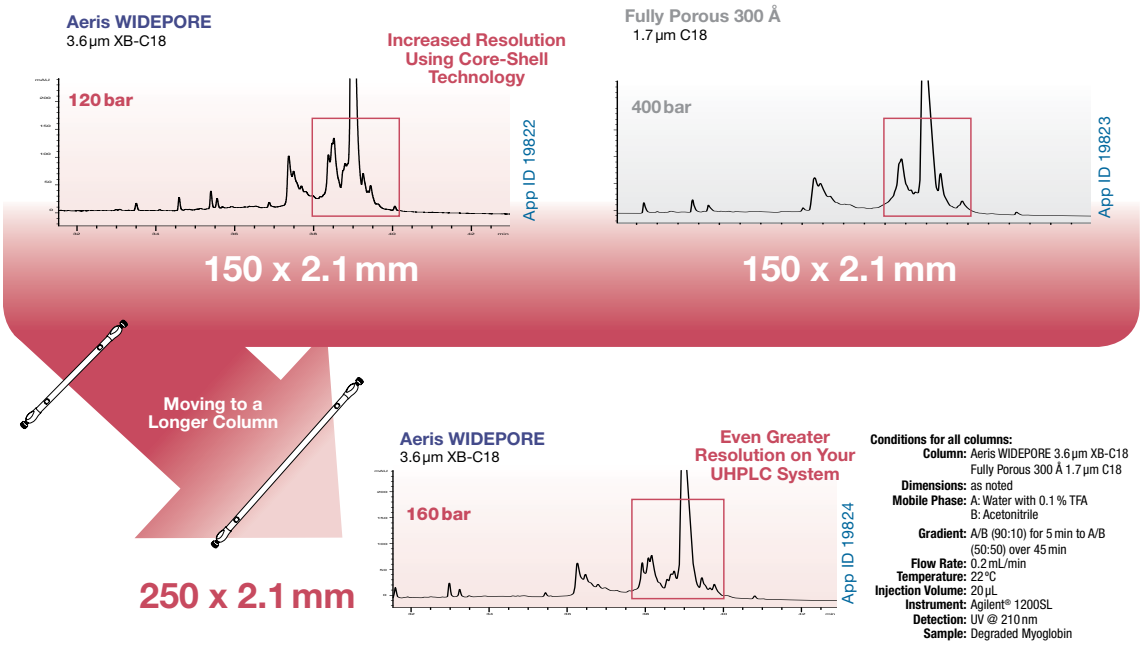
Plates per column efficiency of fully porous 100 Å particles compared to core-shell particles of a similar size. Across all particle sizes core-shell columns provided significantly higher efficiency over fully porous columns.
Running conditions:
50 x 2.1 mm, 0.5 mL/min, mobile phase 50:50 acetonitrile/water, sample naphthalene.

Figure 5. Retention Comparison



Retention of a core-shell Aeris WIDEPORE column and fully porous 5 µm 300 Å column for degraded myoglobin. The core-shell column was significantly less retentive than the fully porous column, yet provided dramatically improved resolution of closely related modified proteins. Optimizing methods for wide-pore core-shell column should use lower initial organic and shallower gradients to compensate for the reduced retention of the column. See **Figure 6** for column conditions.

Figure 6. Aeris WIDEPORE 3.6 µm Core-Shell vs. Fully Porous 1.7 µm

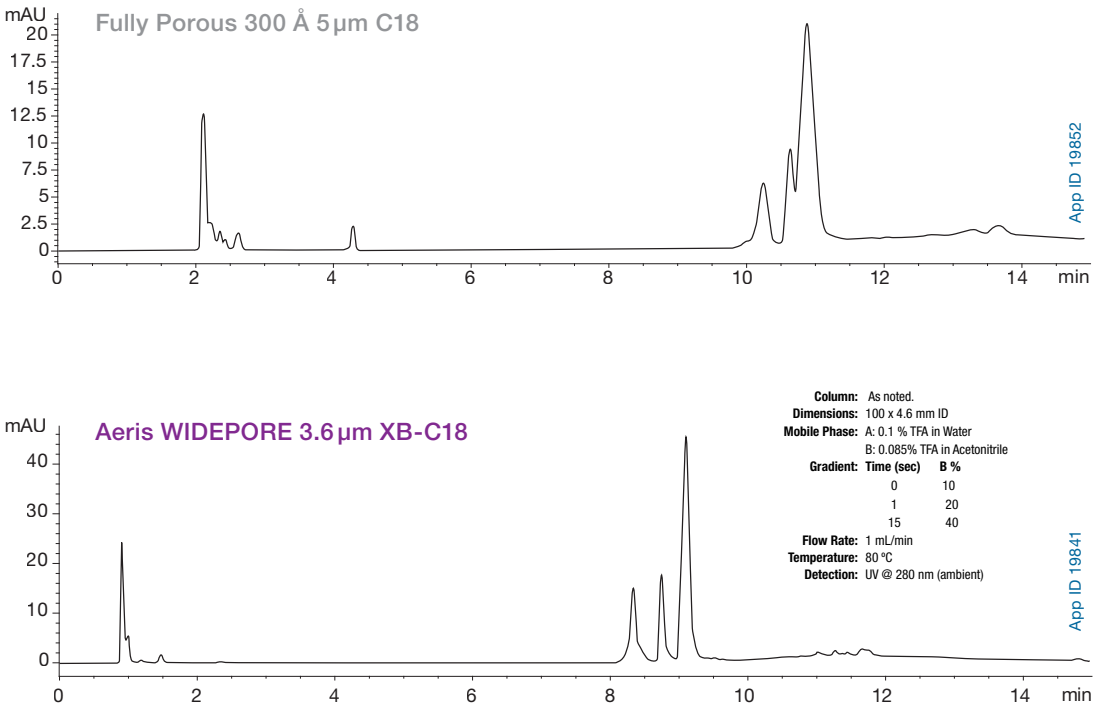


Conditions for all columns:
Column: Aeris WIDEPORE 3.6 µm XB-C18
Fully Porous 300 Å 1.7 µm C18
Dimensions: as noted
Mobile Phase: A: Water with 0.1 % TFA
B: Acetonitrile
Gradient: A/B (90:10) for 5 min to A/B (50:50) over 45 min
Flow Rate: 0.2 mL/min
Temperature: 22 °C
Injection Volume: 20 µL
Instrument: Agilent® 1200SL
Detection: UV @ 210 nm
Sample: Degraded Myoglobin

Substantially lower backpressure of the 3.6 µm core-shell column allow the use of longer columns (in this case 250 x 2.1 mm) to get increased resolution of closely eluting proteins.

Comparative separations may not be representative of all applications.

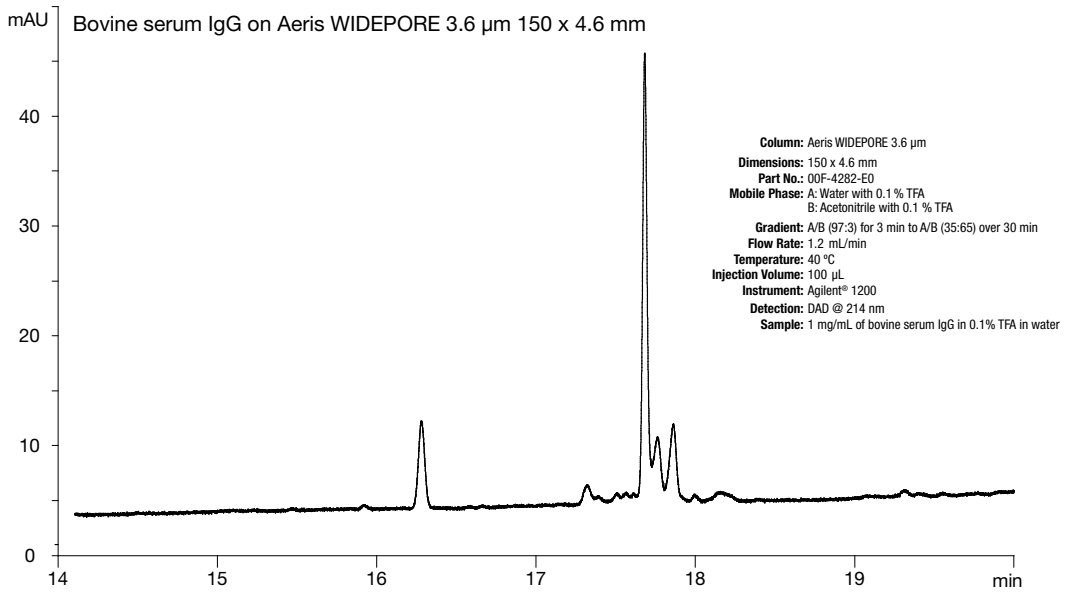
Figure 7. Intact IgG Comparison



Separation of intact mouse monoclonal IgG antibody. Note the increased resolution and recovery of IgG isomers for the core-shell column compared to a fully porous media.

Comparative separations may not be representative of all applications.

Figure 8. Intact IgG Separation of Core-Shell Media



Separation of bovine IgG on Aeris WIDEPORE 3.6 µm XB-C18. Good resolution and recovery of IgG isoforms are obtained using relatively low column temperature (40 °C) and acetonitrile-only organic mobile phase using the wide-pore core-shell column.

Results and Discussion

Previous reports have shown that diffusion rates of proteins are inversely proportional to the log of the molecular weight of a protein; data confirms that slow diffusion of large proteins in and out of a porous particle is the limiting factor in minimizing peak width of a protein in reversed phase chromatography similar to previous results (**Figure 1**). The newest generation of core-shell media utilizes thin porous layers to minimize peak dispersion of large proteins while maintaining strong shape selectivity; separation of closely related modified proteins demonstrate similar separation with a concomitant reduction in protein peak width based on the shell thickness of the core-shell particle (**Figure 2**).

Optimizing shell thickness is a balance between loading and performance; a thinner shell has a shorter diffusion path with lower surface area, loading, and retention. A thicker shell material, or in this case fully porous material, allows for greater loading and retention but reduces performance due to the longer diffusion path (**Figure 3**).

Setting a uniform thickness of 0.2 µm seems an acceptable balance in the development of a optimal column for "analytical" where one would typically load in the 1 to 20 µg range (for a 4.6 mm ID column).

However, even when a thicker shell is used as is typically used for small molecule separations, the performance of core-shell columns outperform fully porous media columns when equal particle sized columns are compared using small molecule probes (**Figure 4**).

Results show that the lower retentivity of the thin shell product requires significant reduction in organic content during gradient methods (resulting in subsequent reduction in electrospray efficiency); however, the increased resolution and efficiency of wide-pore core-shell columns far outweighs such losses with an increase in MS sensitivity for evaluated protein samples (**Figure 5**).

An additional example is show in **Figure 6** where the wide-pore core-shell column is compared against a fully porous 1.7 µm 300 Å column showing improved resolution. However, since the wide-pore core-shell column utilizes a 3.6 µm particle size, the significantly lower backpressure of the core shell column allows for the use of longer columns allowing for additional resolution of similar components when run time is not a factor.

Optimized methods for IgG antibodies demonstrate separation of major disulfide-related isoforms with significant increases in protein recovery (**Figures 7** and **8**). The reduced retention of these media allow the use of acetonitrile as the organic mobile phase (versus IPA mixtures for fully porous media) as well as lower column temperatures which allow for different selectivity and resolution of post-translational modifications.

Conclusions

The development of wide-pore core-shell media specifically designed to overcome the diffusion characteristics of large proteins allows for improved recovery and resolution of intact proteins. However, key considerations must be made in developing successful methods for intact protein analysis. Initial conditions and gradient slopes must be optimized to take into account the reduced hydrophobicity of wide-pore core-shell media. In addition, the media design necessitates its use for analytical applications specifically, as high loading adversely impacts performance of both core-shell and fully porous media.

High resolution and recovery of intact proteins using wide-pore core-shell media allow for enhanced structural information of large proteins, especially recombinant antibody therapeutics where peptide mapping application potentially do not fully address all folding and structural heterogeneity of IgG based therapeutic drugs.

References

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