Improving Intact Biogeneric Protein Separations with Aeris™ WIDEPORE Core-Shell Columns

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Aeris WIDEPORE is a recently introduced core-shell HPLC | UHPLC column specifically designed to provide improved resolution of intact proteins larger than 10 kilodaltons (KDa) in molecular weight. The improved resolution of proteins is accomplished by the use of a new core-shell particle morphology which minimizes protein band-spreading that occurs during diffusion in and out of the core-shell particle. The result is narrower peaks and better resolution of closely eluting proteins. This improved resolution is especially useful for refolding, impurity, and post-translational modification assays on intact biogeneric proteins where very slight differences between intact and modified proteins elute closely on reversed phase columns. Several examples are shown demonstrating the utility of Aeris WIDEPORE core-shell columns for such applications.

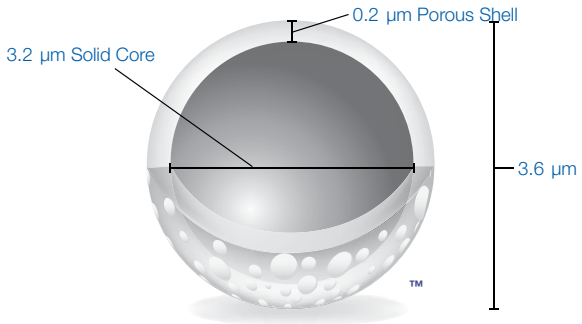
Introduction

Since their debut over two years ago, Kinetex® core-shell columns have introduced a new paradigm in ultra-high performance by decoupling column efficiency from high backpressures. This has resulted in small molecule applications with reduced run times and increased throughput without the need for expensive new UHPLC instrumentation.

While analysis speed has some value for protein separations, the principal focus is more on improving resolution of proteins from near-identical post-translationally modified impurities rather than reducing run times. A new wide pore core-shell column specifically designed to improve protein separations (Aeris WIDEPORE) has been introduced. Rather than utilize a similar morphology of small molecule core-shell columns with larger pores, a completely different particle that takes into account the slower diffusion of proteins in and out of porous particles was developed. A graphic representation of the Aeris particle is shown in **Figure 1**.

Figure 1.
Graphical representation of Aeris 3.6 µm WIDEPORE particle. A 0.2 µm porous shell surrounds a 3.2 µm solid core. This particle geometry is specifically designed to narrow the peak width and improve resolution of proteins and other large molecules.

3.6 µm Core-Shell Particle



By greatly reducing the path length of protein diffusion, protein peaks tend to be narrower allowing for better resolution between intact proteins and their post-translationally modified impurities. The larger particle size of Aeris WIDEPORE columns generates lower column backpressures than small particle fully porous wide pore columns. This lower column backpressure enables the use of longer columns for maximizing protein resolution.

In recent years several therapeutic proteins have gone off patent, allowing a large number of organizations to develop their own generic versions of these potent therapeutics. With bioequivalence being an important aspect in the regulatory success of any biotherapeutic it is of the utmost importance for researchers to develop analytical methods that fully characterize and quantitate all of the impurities present in a candidate molecule. While peptide mapping is the more common method for identifying low-level post-translational modifications (PTMs), mapping gives only minimal information about protein folding and can sometimes miss N-terminal and C-terminal modifications. Thus, intact protein analysis by reversed phase HPLC | UHPLC is usually part of the suite of testing performed on any protein therapeutic. The Aeris WIDEPORE core-shell column offers a new and improved solution for intact protein analysis by offering narrower peak widths and improved protein resolution when compared to fully porous wide pore media. This technical note will show several examples of using Aeris WIDEPORE for analyzing PTM's on small to moderate proteins similar in size and chemical characteristics to several protein therapeutics. In addition, separations on common biogenerics will also be shown to demonstrate the improved performance of the Aeris WIDEPORE core-shell columns.

Materials and Methods

All chemical and standard proteins were obtained from Sigma Chemical (St. Louis, MO, USA). Recombinant human EGF and alpha interferon were purchased from R&D Systems (Minneapolis, MN, USA). Solvents were purchased from EMD (San Diego, CA, USA). Fully porous 300 Å C18 columns were purchased from various HPLC column vendors. Core-shell Aeris WIDEPORE 3.6 µm XB-C18 columns were obtained from Phenomenex (Torrance, CA, USA).

Myoglobin samples were partially degraded by incubation at room temperature for up to a week in dilute acid. Ribonuclease samples were reduced with 100 mM DTT in 50 mM NH_4HCO_3 pH 8.0 for 20 minutes at 45 °C; reduced/non-reduced mixtures were generated by spiking different ratios of the native to the reduced sample prior to injection on HPLC. Different protein samples were analyzed on an Agilent® 1200 HPLC system with autosampler, column oven, solvent degasser, and UV detector set at 214 nm. Data was collected using ChemStation software (Agilent, Santa Clara, CA, USA). Mobile phases used were 0.1% TFA in water (A) and 0.085% TFA in acetonitrile (B). Different gradients, flow rates and column temperatures were listed with the corresponding chromatograms.

Results and Discussion

Intact protein analysis is performed on recombinant proteins to quantitate the purity of a protein and potentially identify any specific impurities in a sample. For most purified proteins the typical impurity is a post-translationally modified version of the protein or an improperly folded species of the protein. Since such PTM impurities are chemically similar to the intact therapeutic protein, achieving chromatographic separation by HPLC or UHPLC between the two species can be a challenge. Separation and quantitation of PTM proteins can be especially difficult for larger proteins since chemical differences induced by a single modification have a smaller net effect. For effective quantitation of impurities, a wide pore reversed phase media must maximize both efficiency and selectivity.

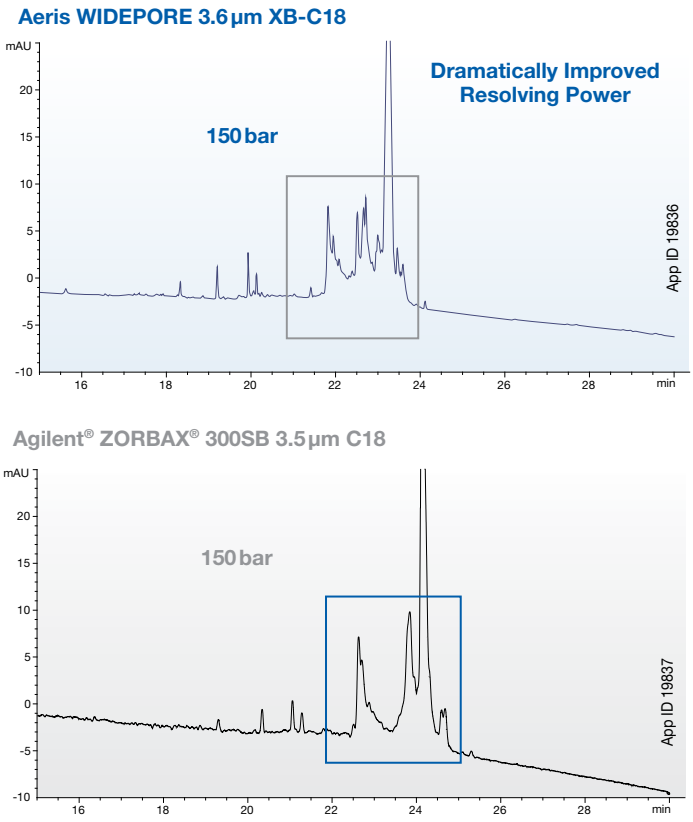
Aeris™ WIDEPORE, a recently introduced line of core-shell HPLC | UHPLC columns, utilizes a unique particle morphology specifically designed to reduce peak broadening resulting from slow protein diffusion in and out of the porous layer of the column (**Figure 1**). In addition, by using a large (3.2 µm) silica core, the resultant particle is 3.6 µm in diameter which allows for longer columns at lower backpressures. The overall result is a column that delivers a significant improvement in separation power for intact proteins and their PTM-based impurities. An example of this improvement is shown in **Figure 2,** where degraded myoglobin is compared between a fully porous 3.5 µm wide pore column and the Aeris WIDEPORE 3.6 µm column. Note the dramatically increased number of resolved impurities for the core-shell Aeris column compared to the fully porous column.

An additional example where improved resolution of the core-shell Aeris WIDEPORE column leads to more accurate quantitation of impurities for biogeneric proteins is shown in **Figure 3,** where an intact alpha interferon sample was compared between a fully porous wide pore column and the core-shell Aeris WIDEPORE column. While resolution is not complete between the interferon peak and modified components, one can see the additional component resolved by the Aeris WIDEPORE column leading to more accurate quantitation of the impurities present. One could potentially use a longer Aeris column and a shallower gradient to further resolve the components. Another characteristic of the Aeris WIDEPORE core-shell media compared to fully porous 300 Å media is that it is significantly less hydrophobic than most fully porous media, so proteins will tend to elute at a lower percentage organic. Thus, to improve resolution on existing protein methods, one should look to lower the initial percentage organic and potentially use shallower gradients when transferring a method to Aeris WIDEPORE core-shell columns.

An additional benefit of the low hydrophobicity of the column is better recovery of hydrophobic proteins. This, in combination with a well bonded inert surface and an optimized diffusion path, can lead to dramatic differences when compared to other fully porous 300 Å columns. An example of this behavior is shown in **Figure 4** where epidermal growth factor (EGF) is compared between Aeris WIDEPORE 3.6 µm C4 and a fully porous 3.5 µm 300 Å C3 column. In this example, the difference in peak heights between the chromatograms is mostly due to adsorption of the protein on the fully porous column. For most applications tested, Aeris WIDEPORE core-shell columns deliver higher protein recovery in addition to the improved resolution that the column demonstrates.

A final example to demonstrate the utility of Aeris WIDEPORE columns for intact biogeneric protein analysis is shown in **Figure 5**. In this example, RNase is reduced with DTT and different mixtures of the reduced and native protein are overlaid in the figure. Analyzing and quantitating the folding state of a recombinant protein is primarily done by reversed phase chromatography of the intact protein. This example shows how an Aeris WIDEPORE column can easily resolve folded and unfolded forms of the RNase protein making it an ideal solution for analyzing intact proteins.

Figure 2.
Comparison between the Aeris WIDEPORE 3.6 µm XB-C18 and a 300 Å fully porous 3.5 µm C18 column. A degraded myoglobin sample was used to compare performance between the two columns. Note the increase in resolution and narrow peak width of the multiple impurities partially resolved on the Aeris core-shell column. In both cases a 150 x 4.6 mm column was used. Flow rate was 1.5 mL/min and column temperature was 40 °C. Gradient was from 3 to 65 % B in 30 minutes after a 3 minute hold.

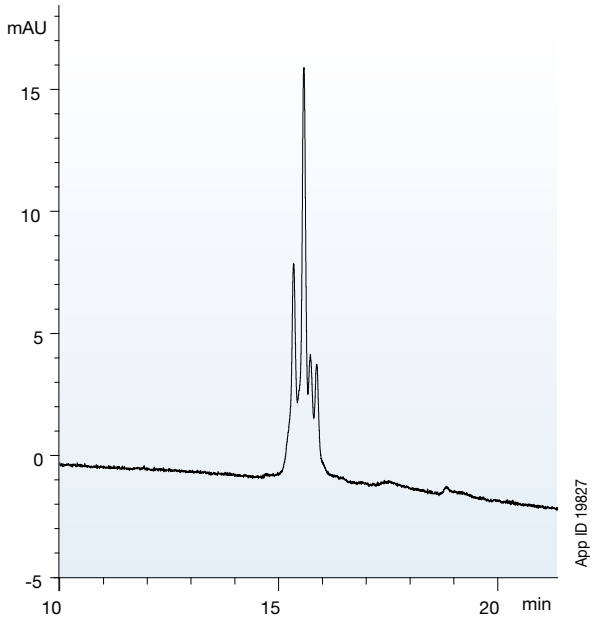


Conditions for both columns:

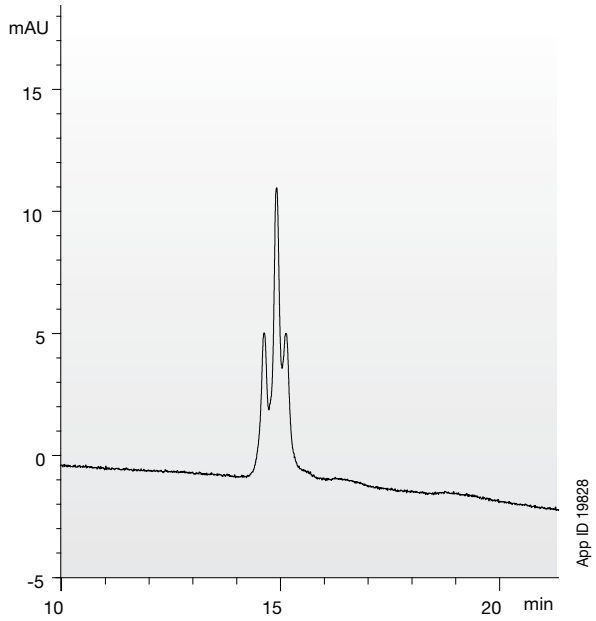
Column: Aeris WIDEPORE 3.6 µm XB-C18
ZORBAX® 300SB 3.5 µm C18
Dimensions: 150 x 4.6 mm
Mobile Phase: A: Water with 0.1 % TFA
B: Acetonitrile with 0.1 % TFA
Gradient: A/B (97:3) for 3 min to A/B (35:65) over 30 min
Flow Rate: 1.5 mL/min
Temperature: 40 °C
Injection Volume: 20 µL
Instrument: Agilent® 1200SL
Detection: UV @ 210 nm (ambient)
Sample: Degraded Myoglobin

Figure 3.
Comparison between the Aeris WIDEPORE 3.6 µm XB-C8 and a 300 Å fully porous 3.5 µm C8 column. Interferon alpha-2a was used to compare the two columns. Note the additional impurity partially resolved by the Aeris WIDEPORE core-shell column. In both cases a 150 x 4.6 mm column was used. Flow rate was 1 mL/min and the gradient was from 30 to 65 % B in 30 minutes. One could potentially use a longer Aeris column (250 x 4.6 mm) and a shallower gradient to improve resolution of the impurities.

Aeris WIDEPORE 3.6 µm XB-C8



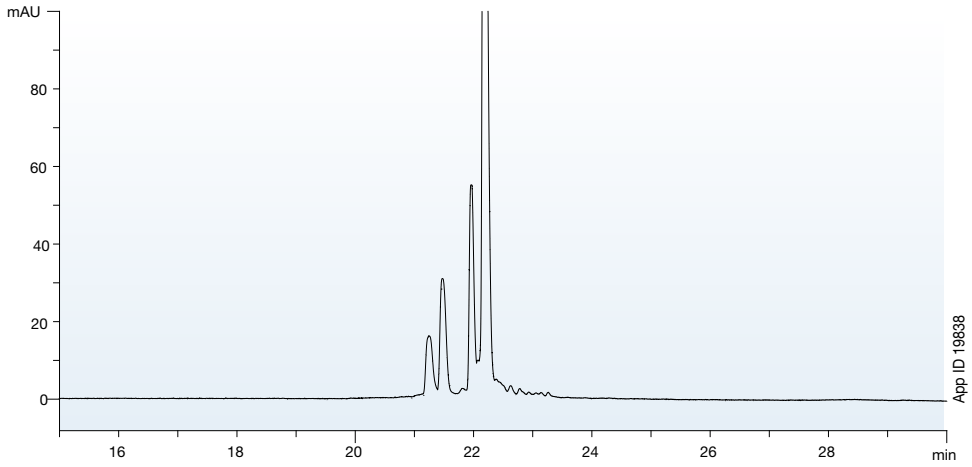
Fully Porous 300 Å 3.5 µm C8



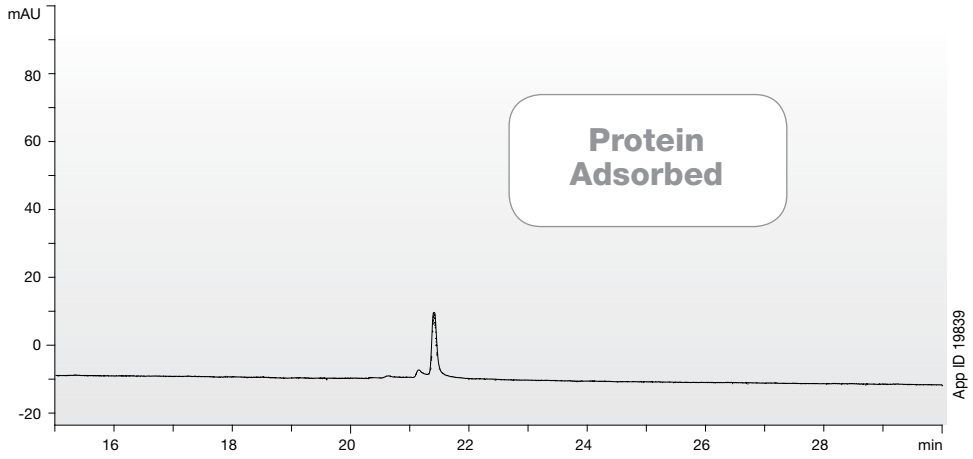
Column: Aeris™ WIDEPORE 3.6 µm XB-C8
Fully Porous 300 Å 3.5 µm C8
Dimensions: 150 x 4.6 mm
Mobile Phase: A: Water with 0.1 % TFA
B: Acetonitrile with 0.1 % TFA
Gradient: A/B (70:30) to A/B (35:65) over 30 min
Flow Rate: 1.0 mL/min
Temperature: 22 °C
Injection Volume: 5 µL
Instrument: Agilent® 1200
Detection: UV @ 214 nm (ambient)
Sample: Interferon alpha-2a

Figure 4.
Comparison between the Aeris WIDEPORE 3.6 µm C4 and a 300 Å fully porous 3.5 µm C3 column. Recombinant EGF was used to compare the two columns. Note the dramatic increase in recovery for the Aeris column and the multiple components present in the sample. In both cases a 150 x 2.1 mm column at a temperature of 40 °C was used. Flow rate was 0.3 mL/min and the gradient was from 3 to 65 % B in 45 minutes.

Aeris WIDEPORE 3.6 µm C4

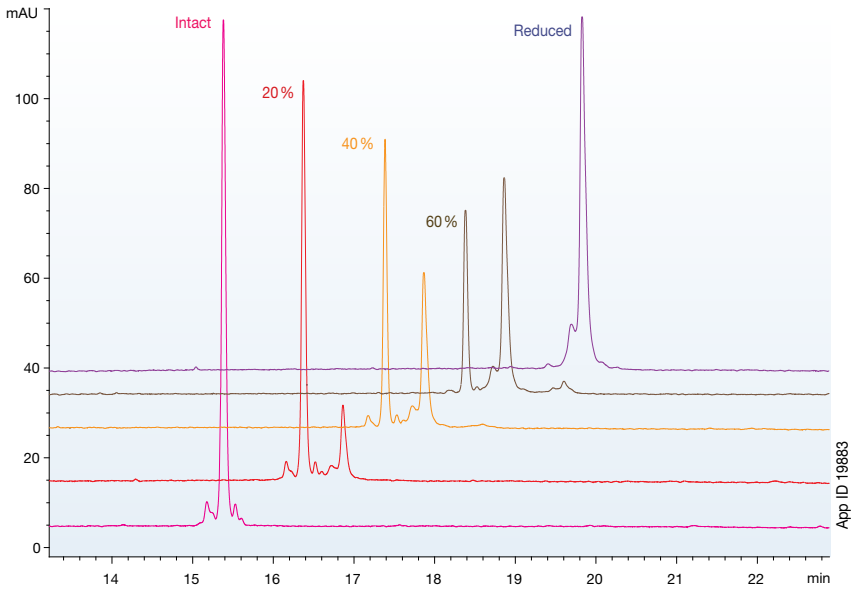


Agilent® ZORBAX® 300SB 3.5 µm C3



Column: Aeris WIDEPORE 3.6 µm C4
ZORBAX 300SB 3.5 µm C3
Dimensions: 150 x 2.1 mm
Mobile Phase: A: Water with 0.1 % TFA
B: Acetonitrile with 0.1 % TFA
Gradient: A/B (97:3) for 3 min to A/B (35:65) over 45 min
Flow Rate: 0.3 mL/min
Temperature: 40 °C
Injection Volume: 20 µL
Instrument: Agilent® 1200
Detection: UV @ 214 nm (ambient)
Sample: Human Epidermal Growth Factor (EGF)

Figure 5.
RNase refold assay on Aeris WIDEPORE 3.6 µm C4. Overlays of different mixtures of reduced and non-reduced RNase are shown on an Aeris 150 x 4.6 mm column. Flow rate was 1.2 mL/min and column was held at ambient temperature. The gradient was from 3 to 65 % B in 30 minutes after a 3 minute hold.



Column: Aeris WIDEPORE 3.6 µm C4
Dimensions: 150 x 4.6 mm
Part No.: 00F-4486-E0
Mobile Phase: A: Water with 0.1 % TFA
B: Acetonitrile with 0.1 % TFA
Gradient: A/B (97:3) for 3 min to A/B (35:65) over 30 min
Flow Rate: 1.2 mL/min
Temperature: 22 °C
Injection Volume: 20 µL
Instrument: Agilent® 1200
Detection: UV @ 214 nm (ambient)
Sample: RNase subject to reduction
100 % intact
20 % reduced
40 % reduced
60 % reduced
100 % reduced

Conclusion

Maximizing resolution between proteins and their modified impurities is critical in obtaining useful quantitation of post-translational modifications of biogeneric proteins. The different applications in this technical note show the utility of Aeris WIDEPORE columns for obtaining accurate data for intact protein applications. The optimized geometry of the core-shell Aeris WIDEPORE columns, as well as good selectivities of the three separate phases offered (XB-C18, XB-C8 and C4), deliver better resolution and recovery than existing fully porous 300 Å columns for intact protein analysis. Finally, the large particle size of the Aeris WIDEPORE column delivers a significantly lower backpressure than sub-2 µm 300 Å columns which allows for more flexibility in instrument used (HPLC or UHPLC) as well as column length in developing biogeneric protein applications.

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Ordering Information

Aeris™ WIDEPORE 3.6 µm Minibore Columns (mm)

	50 x 2.1	100 x 2.1	150 x 2.1	250 x 2.1
XB-C18	00B-4482-AN	00D-4482-AN	00F-4482-AN	00G-4482-AN
XB-C8	00B-4481-AN	00D-4481-AN	00F-4481-AN	00G-4481-AN
C4	00B-4486-AN	00D-4486-AN	00F-4486-AN	00G-4486-AN

Aeris WIDEPORE 3.6 µm Analytical Columns (mm)

	100 x 4.6	150 x 4.6	250 x 4.6
XB-C18	00D-4482-E0	00F-4482-E0	00G-4482-E0
XB-C8	00D-4481-E0	00F-4481-E0	00G-4481-E0
C4	00D-4486-E0	00F-4486-E0	00G-4486-E0

For more information on Aeris Core-Shell HPLC | UHPLC columns visit www.phenomenex.com/Aeris

guarantee

If Aeris core-shell technology does not provide at least an equivalent separation as compared to other products of the same phase and dimensions, return the product with comparative data within 45 days for a FULL REFUND.

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