Improved Peptide Maps Using Core-Shell Media: Explaining Better Biopharmaceutical Applications With Kinetex® C18 Columns

Michael McGinley, Jeff Layne, and Vita Knudson

Phenomenex, Inc., 411 Madrid Ave., Torrance, CA 90501 USA
Other posters presented at this meeting show many performance examples of how the Kinetex® core-shell media delivers “sub-2 µm” efficiency, better resolution, and reduced run times versus fully porous silica media, all while operating at backpressures amenable for operation using standard HPLC systems. While reduced run time and increased throughput are big motivators for using next generation LC columns in small molecule separations, bioanalytical separations require the increased resolution and sensitivity ultra-high performance LC columns offer for the analysis of closely related peptides in low concentrations.

Peptide mapping is the principal bioanalytical application where resolution is important; proteins are complex biopolymers that often have post translational modifications (PTM’s) that can affect the activity and immunogenicity of a protein therapeutic. As such modifications are only a slight chemical change in the overall protein; proteins must be enzymatically fragmented into dozens or hundreds of peptides that are then separated by reversed phase HPLC and positively identified by LC/MS. Good resolution of closely related peptides is critical for identifying PTM’s and next generation HPLC columns with higher efficiency have allowed for more accurate information from complex protein therapeutics like Ig-G proteins which represent a majority of candidates currently in clinical trials.

However, high efficiency is not the only important parameter in peptide mapping of complex proteins. HPLC medias must have high selectivity of minute chemical differences, have acceptable loading of analytical amounts of digested protein so that low level PTM’s can be detected, and media must maintain good performance in MS-compatible buffers used for such analyses. Kinetex®, a recently introduced core-shell media, was evaluated against popular fully porous media as well as other superficially porous media in regards to peptide mapping performance, peptide loading, performance in volatile buffers and with Ig-G peptide separations to demonstrate the advantages of such a media over existing chromatographic solutions.
Materials and Methods

Recombinant and natural proteins and peptides, dithiothreitol (DTT) and iodoacetic acid (IAA) were purchased from Sigma Chemicals (St. Louis, MO, USA). Recombinant human protein Interferon-alpha was obtained from Abcam, Inc. (Cambridge, MA, USA). Solvents were purchased from Fisher Scientific (Fairlawn, NJ, USA). Mobile phase modifiers and Asp-N endoproteinase were obtained from EMD (San Diego, CA). Lys-C endoproteinase was obtained from Wako Chemicals (San Diego, CA). Sequencing grade trypsin was purchased from Roche Applied Science (Indianapolis, IA, USA). Human Ig-G2 was obtained from Dako (Carpentaria, CA). Kinetex® 2.6 µm C18 columns (100 or 150 x 4.6 mm) and a wide-pore 3 µm C18 were used for HPLC analysis (Phenomenex, Torrance, CA). HALO® C18 columns were obtained from Mac Mod (Chadds Ford, PA) and XBridge™ C18 columns were purchased from Waters (Milford, MA).
For BSA digests, protein was digested with sequencing grade trypsin 1:50 E/S in 0.1 M NH₄HCO₃ pH 8.0 at 37 °C for 18 hours then quenched with TFA or formic acid before injection. Different gradients of TFA/water or formic acid/water from 5 to 45 % B (Acetonitrile) over 30 minutes were used based on the application shown. For peptide mixtures, fast gradients from 5 to 50 % in 5 minutes were used. For Human Ig-G, protein was digested with Lys-C for 18 hours at 37 °C (E/S 1:30) in 4 M guanidine/0.1 M ammonium bicarbonate. Sample was diluted to 1 M guanidine then digested with Asp-N for 12 hours (E/S 1:50). The reaction was quenched with TFA and 20 µg aliquots were injected on HPLC. HPLC analyses were performed on an Agilent 1100 HPLC equipped with an autosampler, column oven, and MWD using ChemStation software (Agilent, Santa Clara, CA). The mobile phases used were: A=0.1% TFA in water/2 % acetonitrile and B=0.085 % TFA in acetonitrile with a gradient from 2 to 45 % B in 30 minutes. A flow rate of 1 mL/min was used and peptide elution was monitored at 214 nm.
A direct comparison of a BSA tryptic digest run on the Kinetex® 2.6 µm C18 column versus a fully porous XBridge™ 3.5 µm C18 column using identical running conditions on a standard HPLC system is shown in Figure 1. The improved efficiency of the Kinetex® core-shell column is readily apparent with significantly narrower peak widths and increased peak height versus the fully porous media. Upon closer inspection one can also see the better selectivity that the Kinetex® media delivers over the gradient with over double the peak count (63 vs. 30) that the fully porous media delivers. Such results demonstrate the utility of the Kinetex® column for peptide mapping. Improved diffusion is one of the reasons why Kinetex® media far outperforms fully porous media of similar size; another relevant performance advantage that improved porosity of Kinetex® offers relates to the performance of large peptide/ small proteins on core-shell media as is shown in Figure 2. Small proteins above 10 Kilodaltons tend to perform poorly with badly tailing peaks on fully porous media. When proteins as large as 16 Kilodaltons are analyzed on Kinetex®, good peak shape is maintained probably due to the improved porosity that core-shell media delivers. This is especially useful for peptide mapping of large proteins where some glycosylated peptides can be especially large.

While core-shell media demonstrates better porosity than fully porous media, one area of concern relates to potential reduced loading capacity of such media due to their lower surface area. When different analytical loading levels of peptides on Kinetex® are evaluated against high surface area fully porous media, Kinetex® core-shell media still delivers narrower peak widths than fully porous media for peptide in excess of a 10 µg loads on a 4.6 mm ID column. Such high analytical loading demonstrates the superior performance of Kinetex® core-shell for peptide mapping applications where loading might be a concern. Another relevant concern for any high performance separation relates to the inertness of a media and its effect on the peak shape of basic peptides. Inertness of a media can be a significant concern when LC/MS compatible volatile (non-ion pairing) buffers are used. Figure 4 demonstrates the inertness of Kinetex® over other core-shell porous media (HALO®) when peptide maps of apomyoglobin are compared. Note that Kinetex® chromatograms maintain good peak shape in formic acid LC/MS buffer; HALO® demonstrates dramatic loss of selectivity and peak shape when volatile buffers are used. While phase bonding plays a likely role in media inertness, particle morphology may also be a factor. Figure 5 shows different electron micrographs of Kinetex® and HALO® particles.
Figure 1: BSA tryptic digests run on a 2.6 µm core-shell C18 column versus a fully porous 3.5 µm column. Note the dramatic improvement in efficiency and peak height for the Kinetex® core-shell column as well as a doubling in peak count versus the fully porous media.
Figure 2: Large peptides/ small proteins were run on a core-shell Kinetex® 2.6 µm C18 column. Note the reasonable peak width for myoglobin compared to smaller peptides. The good peak shape for myoglobin highlights an additional benefit of improved mass transfer; better performance for large peptides is much greater than expected for a fully porous 90 Å media.
Figure 3: Peptide loading studies on a core-shell Kinetex® 2.6 µm C18 column. The chromatogram shows increasing loads of met-enkephalin from 1 to 10 µg and the peak width at different loading is graphed against a high surface area fully porous 3 µm media and a competitor fused-core media. Note that even at high loads (>10 µg) the peak width is narrower than the fully porous media suggesting that analytical loading is not a concern with core-shell media like Kinetex®.
Figure 4: A comparison of peptide maps of apo-myoglobin with different core-shell media using LC/MS friendly buffers. Note that the Kinetex® column maintains good peak shape and resolution when using volatile buffers. This data suggests that the Kinetex® C18 media is more inert than other core-shell media and better suited for LC/MS applications.
Figure 5: Electron micrographs comparing Kinetex® core-shell media to HALO® Fused-Core® media. Kinetex® media is shown on the right. Note the spherical shape and improved surface smoothness of the Kinetex® media.
Figure 6: Ig-G Lys-C/ Asp-N peptide map on a 3 µm wide-pore C18 (red) and Kinetex® 2.6 µm C18 (blue). Note the large number of peaks generated from the 150 KDa Ig-G protein and the incomplete resolution of several peptide on the 3 µm C18 column (red). For the Kinetex® 2.6 µm C18 column note the dramatically improved resolution of many Ig-G peptide peaks compared to the 3 µm column. Kinetex® provides ultra-high resolution of complicated peptide maps run on a standard HPLC system.
Conclusion

Other presentations on core-shell Kinetex® media have demonstrated its utility for small molecule applications; however have not addressed its capabilities for peptide mapping and other bioanalytical applications. Results obtained in these studies clearly show how the better efficiency, selectivity, loading and inertness make core-shell Kinetex® a superior solution for peptide mapping and other bioanalytical applications. A final example of this is shown in Figure 6 where the improved performance of Kinetex® over a fully porous media for an Ig-G peptide map is readily apparent, even on a standard HPLC system.

Trademarks
Kinetex and Gemini are registered trademarks of Phenomenex, Inc. XBridge is a trademark of Waters Corporation. HALO and Fused-Core are registered trademarks of Advanced Materials Technology, Inc.

Disclaimer
Comparative separations may not be representative of all applications. Columns used for comparison were purchased directly from the original manufacturer. Phenomenex is not affiliated with Waters Corp. or Advanced Materials Technology.

© 2010 Phenomenex, Inc. All rights reserved.