Using Core-Shell UHPLC Columns for Improved Separation and Characterisation of Immunoglobulins and Other Large Intact Proteins

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The widespread popularity of developing monoclonal antibodies and other similar immunoglobulin-like proteins as human therapeutics has required new tools for reversed phase LC/MS analysis of proteins. Existing fully porous column technologies have struggled in resolving chemical and structural details of such large, hydrophobic proteins. Recently introduced UHPLC column technologies including wide-pore core-shell particle columns have addressed such issues by demonstrating increased peak capacity as well as improved recovery of hydrophobic proteins. Several examples of the utility of such materials are shown and important method development considerations are discussed which address the radically different chromatographic characteristics of such materials.

Introduction

Recombinant proteins are becoming evermore popular and are being employed in a wide area of natural science. Their uses are widespread and span many areas of science. In the detection of diseases they make up critical components in bio sensor kits and furthermore, recent advances in proteomic analyses are yielding a revolution in 'designer' treatments. In their own right, proteins and peptides are being used as treatments for illnesses such as diabetes and as anti-venoms. Moreover, biotechnology is being used to develop more effective treatments of diseases where small drug molecules have been up until now less efficacious.

Reversed phase HPLC analyses of peptides and especially intact proteins have always been more difficult to achieve than small molecules. There are a number of factors for this, the shear size of most proteins (>10KDa) have prevented columns packed with silica gel media with 100Å pores from being used. Quite simply the pores are too small and the proteins do not fit inside the pores, removing 99% of the available media surface from interacting with the protein. As a result, most protein separations larger than 10 KDa are typically performed on 300Å pore size media.[1] In addition, proteins tend to be more hydrophobic as they get larger is size (regardless of their chemical polarity) due to the ever-increasing volume of water displaced by folded protiens.[2] This resulted in a trend toward using columns with low carbon loads and short chain lengths especially when working with very large proteins like recombinant monoclonal Ig-G antibodies. Furthermore, due to the array of functional groups found on amino acid building blocks, unwanted interaction of the acidic silica backbone through electrostatic interactions and metal chelating effects has led historically to poor peak-shapes and adsorption. Finally, due to a higher resistance to mass transfer seen with large molecule separations, efficiencies are often lower than those of small molecule separations.

Major developments in chromatography (commonly called UHPLC) over the last decade have focused on improving speed and throughput of small molecule separations, but have also resulted in several new paradigms (sub-2µm porous particles, monoliths, and core-shell particle columns) that all have offered some positive contributions to improving separations of intact proteins. One of these recent innovations, wide-pore core-shell particle media (Aeris WIDEPORE), is of particular note because it was specifically designed to address the challenges of separating large intact proteins. As was previously discussed, overcoming the slower diffusion of large proteins in-and-out of porous particles is crucial in resolving any of the chemical complexity present in large glycoproteins.[3] For protein-specific core-shell columns, this is achieved by using a very thin porous layer surrounding a non-porous core to minimise diffusion distance as is show in Figure 1.

When one looks at different intact protein

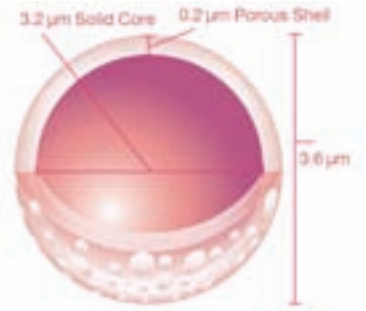

Figure 1: Graphical representation of Aeris 3.6µm WIDEPORE core-shell particle. A 0.2µm porous shell surrounds a 3.2µm solid core. This particle geometry is specifically designed to minimise the diffusion distance through the porous layer, resulting in narrow peak width and improved resolution and recovery especially for large proteins.

separations, the resolution of chemical and structural heterogeneity of therapeutic Ig-G antibodies probably stands out as one of the more difficult applications due to the large size and structure of Ig-G. Post-translational modifications are common; glycosylation is variable, somewhat random glycation can occur, and ragged c-terminal lysine residues can further complicate characterising Ig-G based therapeutics. [4] In this article critical parameters for analysing Ig-G are discussed, especially via reversed phase separation of intact Ig-G.

Materials and Methods

All chemical, protein standards and some antibodies were obtained from Sigma Chemical (St. Louis, MO, USA). Other antibodies were purchased from R+D systems (Minneapolis, MN, USA) and Dako (Carpenteria, CA, USA) Solvents were

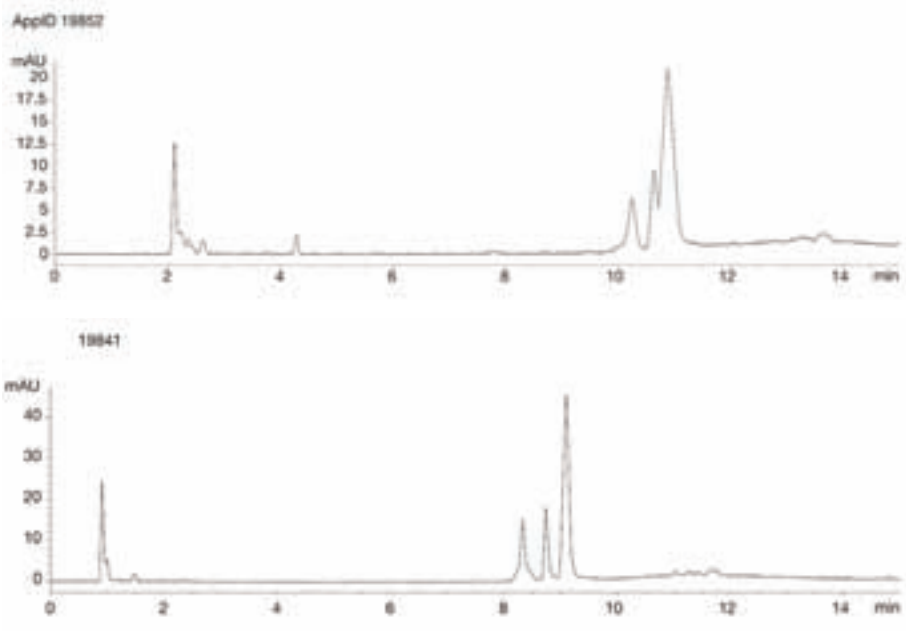

Figure 2: Comparison between a fully-porous 5µm 300Å C18 column (top chromatogram) to a 3.6µm wide pore core-shell C18 column (bottom chromatogram) for a mouse Ig-G sample. Note the significantly narrower peak width and greater protein recovery obtained on the core shell column. The thin shell minimises protein diffusion distance resulting in narrower peak widths and greater resolution of closely related species. The gradient was from 10 to 40%B in 15 minutes, column temperature was 80°C, and flow rate was 1mL/min.

purchased from EMD (San Diego, CA, USA). Fully porous 5µ 300Å C4 and C18 columns, core-shell Aeris 3.6µm WIDEPORE XB-C18 columns (100 x 4.6mm), and Gel Filtration Yarra SEC-2000 columns (300 x 7.8mm) were obtained from Phenomenex (Torrance, CA, USA).

All samples were analysed on an Agilent 1200 HPLC system with autosampler, column oven, solvent degasser, UV detector. Data was collected using Chemstation software (Agilent, Santa Clara, CA, USA). Mobile phase used was 0.1% TFA in water (A) and 0.085% TFA in acetonitrile for reversed phase gradient chromatography; for gel filtration chromatography the mobile phase used was 100 mM sodium phosphate pH 6.8 in water with 0.3 M sodium chloride. Flow rate, column temperature, and gradients used are listed with specific figure legends.

Results and Discussion

Of paramount importance in the use of any recombinant protein is identifying, characterising, and quantitating any natural or artificially generated post translational modifications that might be in a particular sample. As such, several different types of analyses are required to characterise a specific sample. While there are several different spectroscopic and immunologic methods used to characterise the bioactivity of a protein, analytical methods primarily focus on quantitative measurements of purity (both chemical and conformational). The main chromatographic methods for protein analysis are peptide mapping, reversed phase HPLC, ion exchange HPLC, and gel filtration chromatography. Peptide mapping (not discussed in this paper) is the primary method for sequence confirmation as well as identifying most post translational modifications that occur at a specific amino acid, but give only limited information about the global conformation of a protein. Gel filtration chromatography is primarily used for quantitating aggregation and non-covalent association in a protein sample. Reversed phase LC/MS analysis of the intact protein can be used to provide some quantitation of post translational modifications as well as resolve differences in protein folding, which is of great interest because it provides a rapid method for assessing the chemical and conformational purity of a protein. Key for the utility of any RP-HPLC method is having a protein column that provides high efficiency, selectivity, as well as good recovery.

As mentioned previously and shown in Figure 1, the core-shell particle is a significantly different morphology compared to small pore core-shell media (typically 2.6µm diameter, for example, uses a 0.35µm shell on a 1.9µm core). The wide-pore core-shell was designed to maximise resolution of proteins greater than 10 KDa molecular weight with specific emphasis on very large proteins where diffusion speed is greatly reduced and is the largest contributor to band broadening and reduced efficiency. The reduced diffusion path of core-shell particles results in two effects: increased efficiency by reduced path length, and reduced net hydrophobicity of the column. Reducing hydrophobicity (through lower surface area) allows the use of phases with greater methylene selectivity (like C8 and C18) resulting in greater selectivity for minor differences in hydrophobicity. This characteristic of wide-pore core-shell columns becomes important when analysing large proteins like Ig-G where any modification or conformational difference represents only a very minor change in hydrophobicity. Of near equal importance is the larger particle size or the wide-pore core-shell particle which reduces column backpressure allowing for the use of longer columns for increased resolution.

The practical advantage of this wide-pore core-shell particle is demonstrated in Figure 2 where an Aeris WIDEPORE XB-C18 column is compared to a 5µm fully porous widepore C18 column in the separation of a monoclonal mouse Ig-G. Despite being much less retentive than the fully porous C18 columns (note that all the components in the

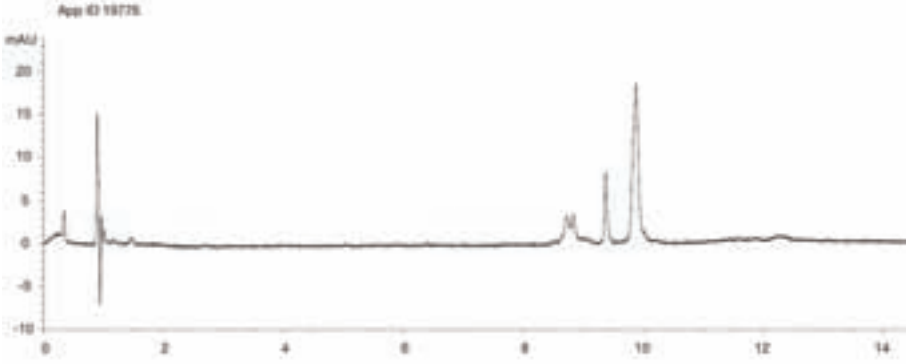

Figure 3: Separation of a mouse monoclonal antibody on a wide pore core-shell C8 column. Chromatographic conditions are the same as in Figure 2 except with a reduced sample amount. Note the different selectivity for the wide pore core-shell C8 column compared to the wide pore core-shell C18 shown in Figure 2. The overall resolution for three major peaks is less than the wide pore core-shell C18 column; however, the wide pore core-shell C8 column further resolves the first peak into at least two additional components while the wide pore core-shell C18 column cannot.

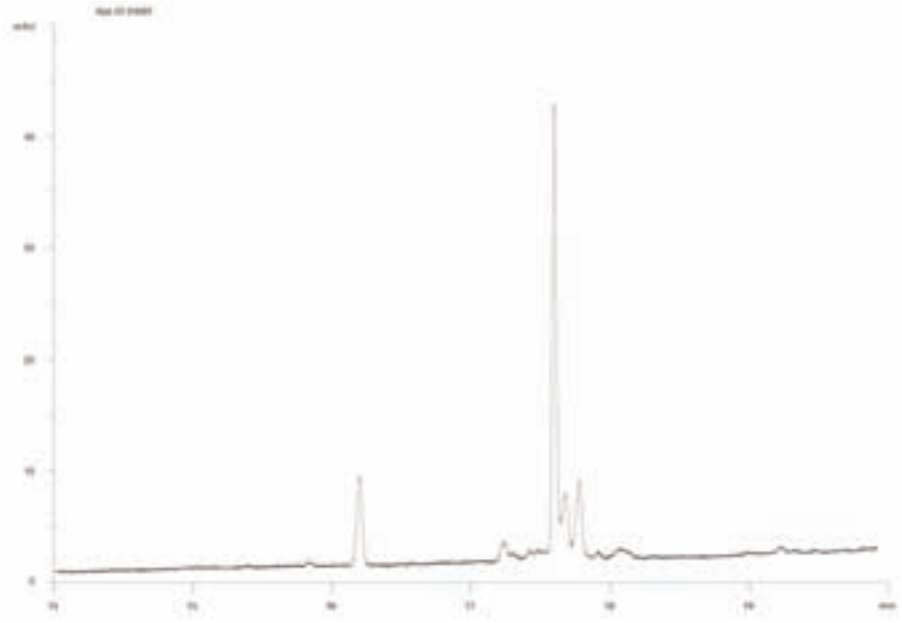

Figure 4: Bovine Ig-G run on a 150x4.6mm wide pore core-shell C18 column. Flow rate was 1.2 mL/min and column temperature was 40° C and monitored at UV 214 nm. An aliquot of 100µL of an approximately 0.1mg/mL concentration was injected. Note the unique elution profile compared to other Ig-G.

core-shell chromatogram elute almost two minutes earlier than on the fully porous column), greater selectivity and efficiency is seen for the core shell column. This greater selectivity (even under un-optimised conditions) allows for increased resolution of highly complex mixtures. In addition, the low hydrophobicity and superior inertness results in greater recovery for hydrophobic proteins which has also been a concern in previous methods for analysing large intact proteins by reversed phase.

The emphasis on both improved efficiency (or more properly peak capacity as protein separations are always run using gradient conditions) and selectivity is further supported in Figure 3 where the same mouse monoclonal Ig-G is run on a wide pore core-shell C8 column using similar running conditions. Close inspection of the chromatography reveals reduced overall resolution across the sample. However, the first peak in Figure 2B is actually further resolved into at least 2 peaks by the Aeris XB-C8 column. This supports a general observation that with large proteins additional fractionation and quantitation of these somewhat heterogeneous molecules is practical with improved chromatographic resolution, justifying the development of multiple column selectivities for protein analysis. The need for good selectivity does not just apply to mouse Ig-G antibodies, as is shown in Figure 4 where bovine Ig-G was separated on a wide pore core-shell C18 column. Note that different antibodies (often even in the same class) will tend to have significantly different profiles, emphasising the need for method development for each individual recombinant antibody.

While peptide mapping and reversed phase analysis of intact proteins can provide a large amount of information about the primary and secondary structure of recombinant proteins, one area where such analysis techniques are limited revolves around understanding the aggregation state of a protein. As many large proteins often have solubility limitations and tend to form multimers, it is typically required for any protein characterisation study to also analyse the aggregation state of a protein using gel filtration chromatography. An example chromatogram is shown in Figure 5 where a recombinant monoclonal antibody was run on a Yarra SEC-2000 gel filtration column (3000 series columns are also commonly used for Ig-G studies). Note in this example a significant amount of both dimer and aggregate were observed in this sample, which is usually not seen by reversed phase analysis of proteins (unless the dimer and aggregates are covalently attached). While not discussed in this article, ion exchange chromatography of intact protein could also potentially be used to further elucidate additional impurities in a protein sample.

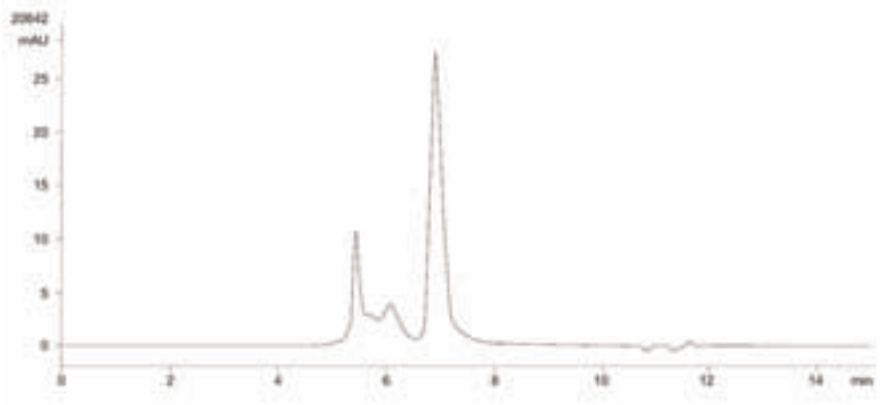

Figure 5: Gel filtration separation of an anti human IGF1 mouse Ig-G1 antibody on a Yarra SEC-2000 column. 5µL of a 0.2mg/mL solution was injected and the HPLC flow rate was 1mL/min, column temperature was 20°C, and monitored at UV 220nm. Note the presence of high molecular weight aggregate (peak 1) as well as Ig-G dimer (peak 2) present along with monomer Ig-G (peak 3). A major consideration with any characterisation of any large recombinant proteins is quantitating aggregate present in the sample

Conclusions

In a previous paper the utility and advantages of using UHPLC (core-shell columns or otherwise) for improved characterisation of peptides and peptide maps has been discussed.[3] However, for the analysis of large intact proteins the separation requirements are vastly different than for peptides and small proteins. In the past, to overcome resolution and recovery limitations of fully porous reversed phase columns, exotic mobile phase systems (iso-propanol or iso-propanol/ acetonitrile as the organic mobile phase) and elevated column temperatures (up to 90°C) have been used to overcome column limitations for large hydrophobic proteins.[4] With protein specific adaptations to core-shell technology new columns have been introduced that have been specifically designed to overcome diffusion limitations of large proteins as well as provide reversed phase solutions where good resolution and recovery can be obtained with traditional acetonitrile organic mobile phases and at lower column temperatures (even though offered Aeris core shell phases are stable to 90°C) where additional protein folding information might be elucidated. Various phase selectivities (C4, XB-C8, and XB-C18) have been introduced that provide alternate selectivities that can potentially each resolve unique

differences in a heterogeneous protein sample.

These new wide-pore core-shell columns provide enhanced tools for identifying some of the complex post-translational modifications that are often seen with antibodies including different glycoforms, disulfide isoforms, C and N-terminal modifications, and other site specific modifications like deamidations and glycations. Because these materials are significantly less hydrophobic than existing fully porous columns, method modifications are required which include using lower initial organic concentrations and shallower gradients. Although not shown here, protein loading limits for such columns are actually very similar to fully porous media for analytical applications (preparative loading is not applicable for such materials). When peak widths are compared, core shell columns demonstrate narrower peak widths than fully porous columns out to 100µg loads (for a 4.6mm ID column) which allow for improved resolution even at increased loadings where a low-level impurity is trying to be detected.

In summary, wide-pore core-shell columns offer new application-specific solutions for the analysis of large intact proteins like monoclonal antibodies that should supply improved structural information for the many therapeutic candidates in development. This technology along with improved "normal pore" (100Å) core shell UHPLC columns and next generation GFC columns (Yarra) provide better solutions for the analysis of large proteins.

References

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