Column Technologies for Protein/Peptide Analysis and Purification

Introduction
Ion-exchange and size exclusion chromatography have long been used in the biochemistry laboratory for the purification of proteins, primarily using compressible gels. The use of reversed-phase HPLC dates from the late 1970s and early 1980s when silica-based separation materials with 300 Å pores were developed. The introduction of large-pore silica materials coincided with the rise of the biotechnology industry and these columns became the standard columns for the analysis of synthetic peptides and recombinant-derived protein therapeutics. They were also implemented in the purification processes of several protein therapeutics. For many years large-pore reversed-phase columns were available from a limited number of companies. In recent years, with the advent of high-purity silica particles made synthetically from silane starting materials, many companies have developed large-pore silica particles that are now used in the separation and purification of proteins and peptides.

The emphasis on column development in recent years has been on higher resolution and faster separation times. A number of technologies have appeared with these ends in mind. The rapid rise in mass spectrometry coupled to HPLC and the expanding field of proteomics have both significantly affected the development of new HPLC columns for protein/peptide separations.

Ion-Exchange Columns for Protein Separations
Although ion-exchange chromatography has long been used for protein separations, it has generally not had the resolution capabilities of reversed-phase HPLC. Ion exchange has traditionally been used for general-purpose purification of proteins. Dionex (Sunnyvale, California, USA) has developed several new ion-exchange materials based on pellicular, hydrophilic polymer-based materials that offer much better resolution than previous columns. With these newer columns the use of ion-exchange chromatography has expanded and it is now widely used to monitor charge variants in biotechnology-derived and natural proteins. With these columns carboxy-terminal processing of monoclonal antibodies can easily be determined (Figure 1), and determining deamidation and monitoring the sialic acid content of glycoproteins is possible.

Most recently, the use of capillary- and nano-LC cation-exchange columns have found widespread use as selective columns for the multidimensional chromatographic fractionation of complex protein digests in proteomics studies.

Figure 1: Hydrophilic pellicular supports for ion-exchange separations of proteins provide rapid, high-resolution separation of charge variants such as C-terminal lysine processing in monoclonal antibodies and glycoprotein separations based on the sialic acid content of the glycan. (a) Control humanized IgG1 MAb; (b) humanized IgG1 MAb treated with carboxypeptidase B for 2 hr at 37 °C. Column: ProPac® WCX-10, 4 x 250 mm; eluent A: 10 mM sodium phosphate, pH 7; eluent B: 1 M sodium chloride, 10 mM sodium phosphate, pH 7; gradient: 4–15% B in 30 min; flow-rate: 1 mL/min; injection volume: 10 µL; detection: UV at 220 nm. (Data courtesy of Dionex Corp.)
Small-Particle Reversed-Phase Columns
Small-particle (<5 µm) separation materials have been available for several years. 3 µm particles packed in shorter columns (less than 10 cm in length) are now being increasingly used for protein/peptide separations when faster analysis times are desired. Separation times for protein digests have been shown in the range of 10–15 min, which is much faster than the analysis times of 45–90 min normally achieved with conventional 5 µm particles packed in longer columns (15–25 cm in length). Whether these improvements in analysis time hold true for real samples in the laboratory, however, is uncertain.

Superficially Porous Reversed-Phase Columns
Some of the earliest columns used in HPLC were made of a solid glass core of 30–40 µm diameter with a thin coating of silica. These “pellicular” or “superficially porous” particles were fairly dense and packed well into columns. The thin surface coating of silica provided good mass transfer resulting in relatively good efficiency. Column efficiency, however, was well below what was theoretically possible and these pellicular materials gave way to smaller diameter, more porous silica particles and, eventually, the spherical particles that are used in HPLC columns today. Zorbax (Agilent Technologies, Wilmington, Delaware, USA) has revived the pellicular concept with a high-performance twist by introducing Poroshell HPLC columns for protein/peptide separations. Poroshell particles are 5 µm in diameter with an impervious core and a surface coating of microspherical particles akin to Zorbax itself (Figure 2(a)). As shown in Figure 2(b) rapid separations of protein digests (6 min) can be accomplished on these superficially porous materials, the advantage of which is to offer high resolution in short time frames at relatively high flow-rates, up to 4 mL/min.

Monolithic Columns
In the quest for new column technologies to give faster and/or higher resolution separations, many researchers have reported on work with “monolithic” columns. These columns are not packed with conventional small particles, but rather are formed as a single rod of very porous material that is encased in a column “package.” The materials used have been either silica gel or a variety of synthetic organic polymer materials. The advantage of the monolithic columns is high permeability, enabling their use at relatively high flow-rates with reasonable back-pressures. The final result is generally much faster separations with resolution comparable to that obtained with conventional particle columns. Several monolithic columns are now commercially available from Isco (Lincoln, Nebraska, USA), Dionex and Merck (Darmstadt, Germany), and a few of these, mainly the polymeric monoliths, have large porous channels suitable for proteins. These columns have the potential to significantly speed up protein and peptide separations. For more information on monolithic columns, see the article by Svec in this issue (p 24-28).

Low TFA Columns
The 2002 Nobel Prize in Chemistry recognized the development of mass spectrometry techniques in the analysis of large biological molecules, particularly proteins and peptides. The electrospray ion source, developed in the 1980s by John Fenn, 2002 Nobel Laureate, has been responsible for a tremendous proliferation of LC–MS in the protein laboratory. A limitation of LC–MS in protein/peptide analysis is that the commonly used HPLC reversed-phase ion-pairing reagent for polypeptide separations, trifluoracetic acid (TFA), binds so strongly to the polypeptide molecules that they became neutral complexes and, as such, cannot pass through the mass spectrometer and be measured. In affect TFA reduces the ion signal obtained via the electrospray ion source. Several solutions to the problem with TFA have been investigated by the mass spectrometry community including changing to a volatile and weaker reagent such as formic acid, and using very low concentrations of TFA (~0.01%), which do not affect the polypeptide electrospray signal very much. The problem with the latter solution is that many HPLC columns give inferior peak shape and separations at low TFA concentrations. In response to this several companies have developed special silica reversed-phase columns that separate proteins and peptides effectively at these low concentrations of TFA. These companies remain silent on the methods they use to achieve this result, however it is believed that modification of the silica surface is at the heart of these so-called “MS compatible” columns.

Capillary Columns
The use of columns with small internal diameters (<4 mm) has increased in recent years, in part because small-diameter columns use much less solvent and provide higher sensitivity than traditional analytical columns and, in part, because they are more compatible with coupling to MS. LC–MS with electrospray mass spectrometry today largely uses “narrow-bore” columns of about 2 mm diameter because the flow-rates...
of these columns are compatible with pneumatic-assisted electrospray ion sources. Capillary columns (<1 mm i.d.) are also used with conventional electrospray operating at flow rates of a few microlitres per minute. Proteomic analysis, consisting of large-scale separation and identification of proteins, has moved in the direction of using “nanoelectrospray” which requires sub-microlitre per minute flow-rates and employs exclusively capillary columns. This has greatly expanded the use of capillary columns and these are available from a variety of suppliers, packed with the same materials as used in larger diameter columns. The drawback is that specially adapted instruments must be used because of the very low flow-rates.

The primary benefits of using capillary columns in proteomic analysis is sensitivity – the ability to detect extremely low levels of proteins. Because of the complexity of proteomic samples, often containing thousands of peptides, high resolution is required together with very high sensitivity. Capillary reversed-phase columns coupled to electrospray mass spectrometry enable separation and identification of very large numbers of peptides in a matter of 2–3 hours (Figure 3).

To chromatographically resolve such complex peptide mixtures as noted in Figure 3 and to identify individual peptides as needed by mass spectrometry, researchers have resorted to multiple chromatography modes. Multidimensional protein identification technology (MudPIT) uses two chromatography steps interfaced back-to-back with various configurations. Separation is achieved on the first column and fractions are collected which are then injected onto the second column. It is often more convenient to perform this experiment on-line using column switching valves. A popular configuration is depicted in Figure 4. Here the first stage is a cation-exchange capillary column on which many peptides from a tryptic digest are retained and selectively eluted by increasing the salt concentration, usually in a stepwise manner. The second stage is a reversed-phase capillary column on which the eluted fractions are separated based on their hydrophobicity. Because the mass spectrometer inlet does not permit the direct injection of salt used to elute fractions from column 1 to column 2, sometimes an intermediate desalting capillary column is used as depicted in Figure 4. Salts are flushed to waste and the peptides backflushed into the reversed-phase capillary.

Size Exclusion Columns
Size exclusion columns are different from other chromatographic columns in that the goal is for the molecules to have no interactions with the surface of the particle. In this way the separation is based exclusively on the hydrodynamic size of the molecule determining the degree of penetration into the particle pores. Consequently, size exclusion columns, often called gel filtration when used with aqueous mobile phases, have been developed with highly hydrophilic surfaces to

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**Figure 3:** Proteomic analysis involves separating a very large number of peptides by reversed-phase HPLC while obtaining MS and MS/MS data on as many peptides as possible. Sample sizes are small and sensitivity is critical in order to identify very minor protein species. Capillary columns such as this 300 µm i.d. × 150 cm long column packed with 5 µm, silica-based C18 particles are the basis for proteomic chromatography wherein large numbers of peptides are identified. The chromatograms aren’t as “pretty” as we normally see because of the large numbers of peptides eluting. The mass spectrometry dimension is able to further separate and subsequently identify many of the peptides. (Data courtesy of GraceVydac, Baltimore, Maryland, USA.)
eliminate interactions with proteins. The few advances in size exclusion columns that have been made in recent years rely on a decrease in the particle size of packings and surface modifications for their uniqueness (biocompatibility). Zorbax (Agilent) GF-250 and GF-450 size exclusion columns employing a zirconia-modified surface coupled with a diol bonded phase have been used for many years. Thermo Hypersil-Keystone BioBasic size exclusion columns are made with silica particles that are coated with a hydrophilic polymer. Particles are generally around 5 µm in diameter.

**Conclusion**

HPLC has become a standard in the separation, analysis and purification of proteins and peptides. These uses now range from isolation of natural peptides and proteins, to characterization of therapeutic, biotechnology-derived proteins, to purification of large quantities of protein therapeutics, to separation of complex proteomic samples. Although conventional columns packed with 5 µm spherical silica-based particles continue to dominate the field, several recent technical innovations offer faster and/or high-resolution separations, often in less time than conventional columns. Greater compatibility with mass spectrometry is also an important part of the benefits of much new column technology. To resolve complex peptide mixtures, multidimensional chromatography is quickly becoming an alternative to 2D gel electrophoresis.

**References**


**David Carr** was formerly the marketing/technical manager at Vydac, a leading supplier of columns for protein/peptide separations. He is currently the Director of Training at Bioanalytical Technologies and specializes in developing and teaching seminars and training courses in HPLC, protein therapeutic analysis and proteomics. Comments regarding this article can be directed to David at carr@bioanalyticaltech.com