Chromatography Solutions

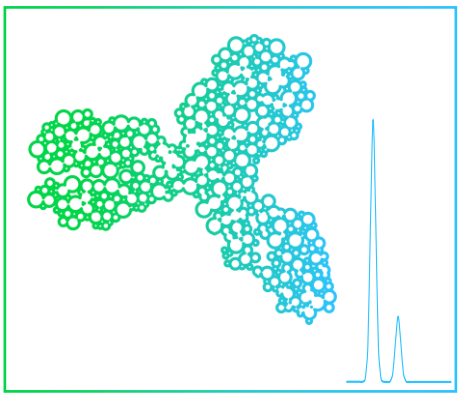
Technical note #040

Column selection for peptide and protein RPLC separations in biopharmaceutical analysis

INTRODUCTION

Biopharmaceuticals are a class of therapeutic compounds that include oligonucleotides, monoclonal antibodies, vaccines and fusion proteins. These compounds are extracted or semi-synthesised from a biological source, although in the case of oligonucleotides, may also be produced using fully synthetic approaches. Each of these areas is seeing growth in terms of the development of new chemical entities, and this growth is resulting in changes to the types of drugs that are now routinely taken by patients, with improvements in efficacy and a reduction in toxicity.

One group of biopharmaceuticals that is receiving a lot of interest is monoclonal antibodies (mAbs). These compounds have been successfully applied for a range of therapeutic uses, including oncology and cardiovascular diseases. Monoclonal antibodies are derived from a biological source and are typically synthesised within cell cultures or *in vivo* in a mammal. The process of manufacture is based on several ground-breaking research activities in the 1970's [1,2], and involves



the generation of antibodies in the host, followed by a series of downstream purification steps, which often involve process scale chromatography.

It should also be stated that there are biotherapeutics on the market that are not mAbs. One of the most

important types of therapies, where liquid chromatography is having an important impact in the determination of the quality of the drug is oligonucleotides. The synthetic manufacturing process for oligonucleotides is very different to that associated with the manufacture of protein-based drugs. The same regulations still apply to the analysis of these types of therapies, however the types of impurities that can be observed is quite different and consequently, a different approach is required for their characterization.

One aspect that all such biomolecules share is their structural complexity. This means that there is great scope for small structural variations to arise in the biomolecule that could influence the drug's safety and efficacy. This complexity can also result in the presence of many potential impurities. The structure and impurity profile must therefore be comprehensively characterized using a variety of analytical techniques. Chromatographic techniques are widely utilized within analytical workflows, with a variety of different chromatographic approaches being used to provide a comprehensive characterization. Figure 1 gives an overview of some of the common techniques used for the characterization of protein based biotherapeutics.

The use of such a variety of chromatographic approaches means that column selection is vital and can be confusing. The aim of this article is to summarise some of the key considerations for column selection in approaches that utilize reversed-phase liquid chromatography (RPLC), highlighted in Figure 1.

PARTICLES

In reversed-phase applications, proteins and peptides are separated by interacting with the hydrophobic surface of particles packed in columns. These particles are typically manufactured from fully porous silica, due to the material's physical robustness, high surface area, stability under most solvent conditions and the ability for silica to be made into spherical particles of various sizes and morphologies, with pores of different diameters. The latter physical characteristics ensure optimal chromatographic performance.

The choice of particle size is dictated by the application and the specifications of the (U)HPLC systems available, with smaller particles providing higher chromatographic efficiency, at the expense of higher back pressure. For many reversed-phase HPLC applications, silica particles of 3 – 5 µm diameter are widely utilized. These can be successful used on standard HPLC systems with operating pressures below 400 bar (5,800 psi). More recently, the availability of highly optimised UHPLC systems has enabled the use of smaller, sub-2 µm particles to achieve faster analysis and/or more efficient separations at higher operating pressures up to 1,400 bar (20,000 psi).

Another recent development is solid core particles, which feature a different morphology outlined in Figure 2. These particles offer several compelling advantages over fully porous equivalents and can provide higher resolution separations without the need for higher

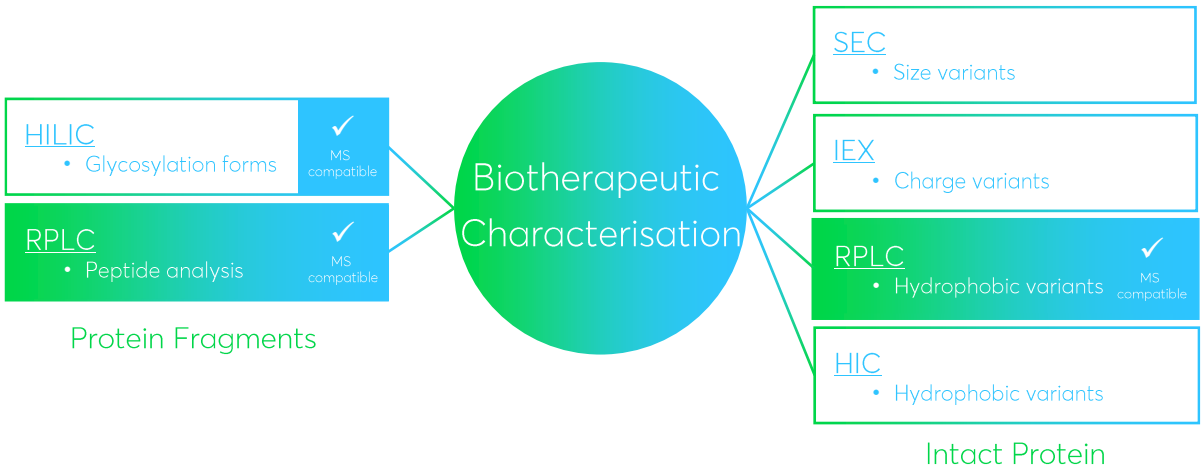


Figure 1: Summary of the key chromatographic techniques used for the analysis of critical quality attributes in protein biotherapeutics.

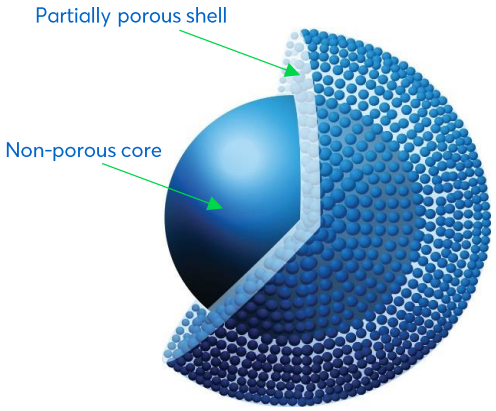


Figure 2: Schematic representation of a solid core particle.

pressure UHPLC systems. For large biomolecules, solid core particles can reduce detrimental mass transfer effects that result in less efficient chromatographic peaks and are becoming more commonly utilized. For more details on the advantages of solid core particles, please refer to references 3 and 4.

SILICA PURITY

The purity of the silica used in HPLC columns is critical to obtain good separation performance. Metal ion impurities within the base silica particle result in undesirable secondary interactions with the target analytes and increases the acidity of residual silanols at the silica surface. This can cause peak tailing and loss of resolution of proteins and peptides, as shown in Figure 3. Low-purity silica with metal impurities requires the use of high concentrations of an ion-pair reagent, trifluoroacetic acid (TFA), to maintain good peak shape. The use of low concentrations of TFA results in poor peak shape and loss of resolution. With high-purity silica, TFA concentrations as low as 0.005% result in good peptide peak shape. This is especially important for LC-MS applications as the use of TFA causes a signal reduction due to ion suppression when using the electrospray interface. Lower concentrations of TFA therefore reduce the impact of ion suppression, resulting in enhanced sensitivity in LC-MS. Modern columns based on type B ultra-pure silica, such as Avantor® ACE® columns, therefore offer distinct advantages in terms of efficiency and peak shape for biomolecule analysis.

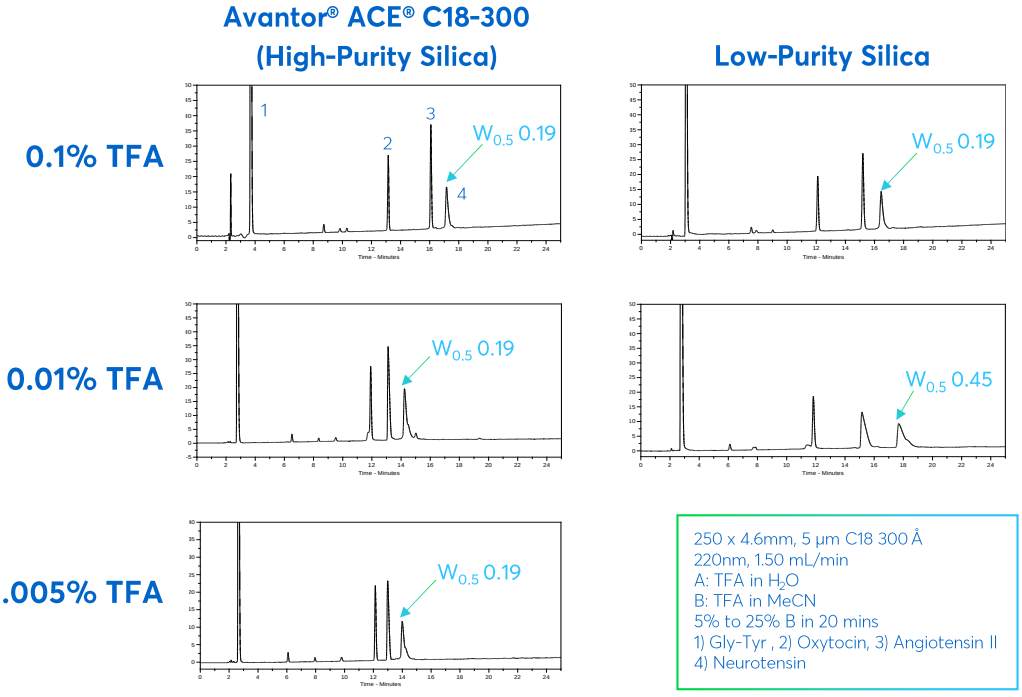


Figure 3: The effect of lowering mobile phase modifier (TFA) concentration with low- and high-purity silica base columns.

PORE DIAMETER

High-resolution separations of peptides and complex protein digests can be readily achieved using columns packed with high-purity, high-performance silica-based particles with a pore size of approximately 80-100 Å (e.g. Avantor® ACE® Excel® C18). However, intact proteins require a modified approach. Proteins have considerably higher molecular weights and are therefore physically much larger. For example, a mAb has a molecular weight of ~150,000 Da compared to ~150-1,000 Da for typical small molecule drugs. Using the small pore (~100 Å) silica normally used in reversed-phase HPLC applications will result in inferior separations for proteins (Figure 4A). Due to their size, proteins are unable to enter the stationary phase pores, leaving only the very small exterior surface for separations to occur. This poor accessibility of the analyte into the stationary phase pores, restricts diffusion and increasing peak broadening and potentially tailing.

Wide pore silica materials have therefore been developed, which overcome these accessibility issues, allowing proteins and even larger peptides to enter the pore and fully interact with the surface, thus resulting in better peak shape and resolution (Figure 4B). The use of wider pore silica (with pore sizes greater than 300 Å) is therefore mandatory for protein separations and are universally used today.

CHOOSING THE SEPARATION SURFACE

The silica is modified with a hydrocarbon based ligand to create a hydrophobic surface. In the case of a C18 stationary phase, a chlorosilane with an attached C18 hydrocarbon chain (i.e. octadecylchlorosilane) is reacted with surface silanol groups to attach the C18 chain to the silica surface. The organosilane molecules do not react with every silanol on the silica surface because of steric hindrance and a significant number of polar silanols remain on the surface. These silanol groups can interact with polar moieties present in the peptides and proteins and, since this interaction is typically stronger than the hydrophobic interactions present between the C18 chain and the analyte molecules, it will cause the peaks to elute later, or can cause tailing. A process called "end-capping" is used to react a small organosilane with the silica surface, to reduce the number of polar silanol groups present.

The chemistry used to modify the silica surface allows various organic groups to be attached to the silica. The most common modification is attachment of a linear, aliphatic eighteen carbon chain resulting in a "C18" or ODS type column. This creates a fairly thick layer of hydrocarbon on the surface. It is to this thick hydrocarbon layer that proteins and peptides adsorb. The C18 column is particularly useful for the separation

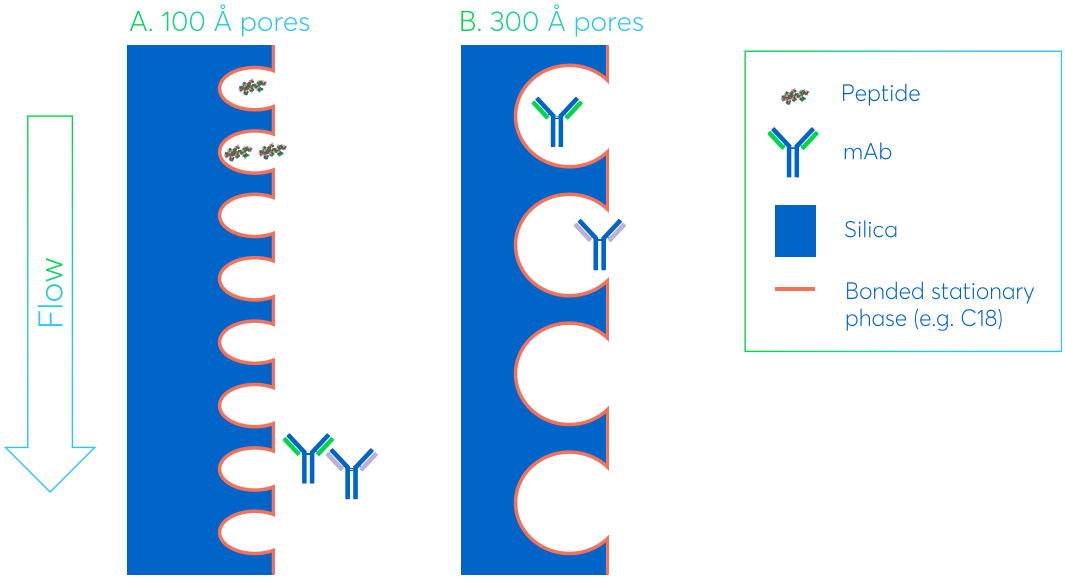


Figure 4: Schematic representation of A. 100 Å and B. 300 Å pore size material for the reversed-phase separation of small peptides and mAbs. In A, the smaller peptides can fully access the stationary phase pores and fully interact with the stationary phase surface, resulting in good separation. The larger mAbs are unable to enter the pores and show poor separation. In B, the mAbs can completely access the pores, resulting in improved retention and separation.

of peptides less than 3,000 Da and is usually the column of choice for the separation of peptides resulting from protease digestion of proteins as well as the separation of natural and synthetic peptides. Figure 5 shows an example of a complex tryptic digest of BSA using an Avantor® ACE® UltraCore 2.5 SuperC18 column. The thickness and space between the C18 groups allow the smaller peptides to partition between the bulk mobile phase and the stationary phase, and this partitioning results in different retention times, which are dependent on the amount of organic solvent present. With the larger proteins, these are not able to fully partition into the stationary phase due to their size and the resulting interaction in then based on an adsorption process. This results in a great deal of sensitivity to the amount of organic solvent, and typically the protein will either be retained or not be retained, with the amount of organic having a digital effect on the retention mechanism.

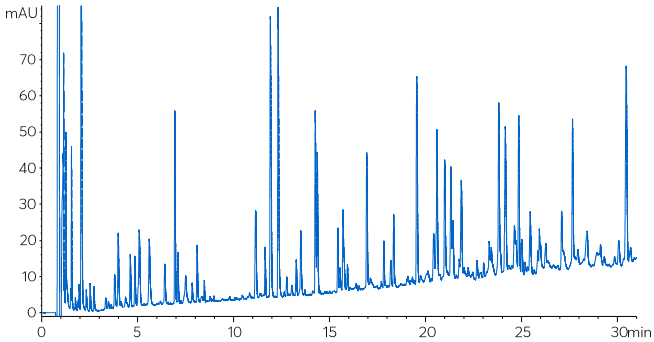


Figure 5: Gradient analysis of a bovine serum albumen peptide digest sample on a C18 solid core column. Column: Avantor® ACE® UltraCore 2.5 SuperC18, 150 x 2.1 mm; Mobile phase A: 0.05% TFA in H_2O, B: 0.05% TFA in MeCN; Gradient: hold at 5% B for 1.5 minutes then 5-35 %B in 30 minutes, then 35-96 %B in 5 minutes; Temperature: 60°C; Injection volume: 20 µl; Detection: UV, 214 nm.

A less hydrophobic phase results from the attachment of butyl (C4) groups to the silica surface. The butyl phase is most suitable for protein separations, such as mAbs (Figure 6) but can also be used to separate large or hydrophobic peptides. Proteins can be separated with a C18 column, however some proteins have poor peak shape or tailing peaks on C18 columns and C4 columns are recommended for protein separations. Phenyl phases, which offer similar hydrophobicity to C4 phases, but may provide different selectivity, are also widely available for available for protein separations.

Column selectivity for peptides is affected by the nature and characteristics of the bonded stationary phase, as

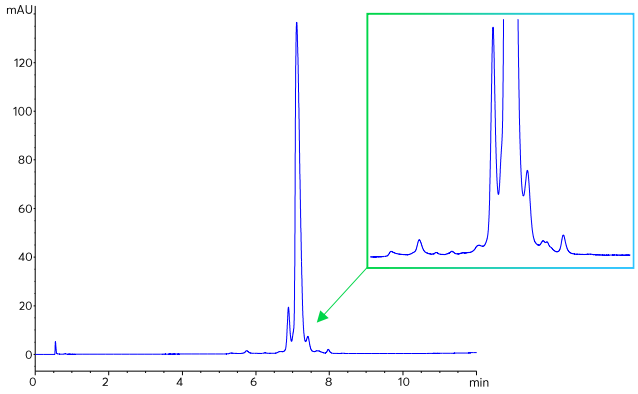


Figure 6: Analysis of NIST mAb on an Avantor® ACE® UltraCore BIO C4-500, 100 x 3.0 mm column. Mobile phases: A: 0.1% TFA in H_2O, B: 0.1% TFA in MeCN/H_2O 90:10 v/v; Gradient: 36 to 45% B in 10 minutes, then 45 to 80% B in 2 minutes, hold at 80% B for 2 minutes; Flow rate: 0.8 ml/min; Injection volume: 1 µl; Temperature: 80 °C; Detection: UV, 280 nm.

well as the underlying silica surface. Different reversed-phase stationary phases may offer substantially different selectivity for peptides, for example, figure 7 shows a mixture of peptides separated on six different Avantor® ACE® 100 Å stationary phases under identical gradient separation conditions. The different stationary phases offer different retention, peak spacing and in some cases different elution orders for the six peptides. Varying the stationary phase can therefore be useful for resolving coeluting peptides and can be exploited to provide optimal peptide coverage for more complex applications such as peptide mapping.

Additionally, certain applications may require use of a specifically designed stationary phase for optimal separations, for example, the Avantor® ACE® Excel® Oligo column has been developed specifically for the analysis of oligonucleotides (Figure 8), whilst the Avantor® ACE® Excel® Glycan column can be used for the analysis of glycans using a HILIC approach in the characterisation of mAbs. [5]

COLUMN LENGTH

For small molecule separations, including lower molecular weight peptides, the more interaction the analytes have with the particle surface, the better the resolution and long columns give higher resolution than short columns. Larger proteins, however, adsorb near the top of the column, are subsequently desorbed and do not

interact appreciably with the particle surface after being desorbed. Column length is therefore not important in protein separations and short columns separate proteins as well as long columns.

Since peptides interact less strongly with the hydrophobic reversed-phase surface than proteins, length appears to play a greater role in peptide and protein digest separations. Longer columns usually result

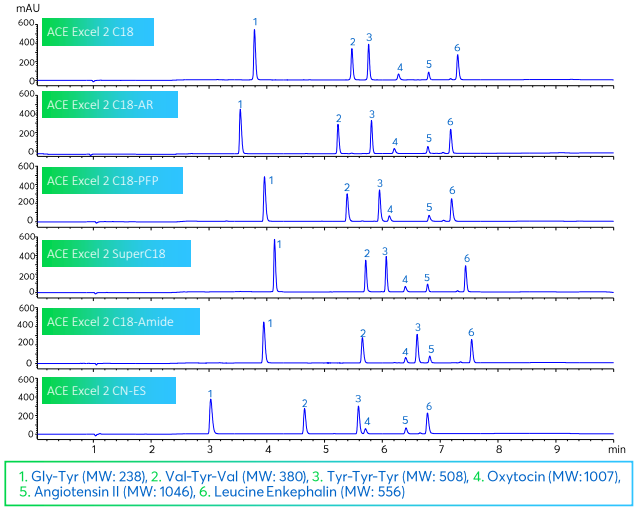


Figure 7: Gradient separation of a mixture of six peptides on six different Avantor® ACE® 100 Å columns under identical conditions. Column dimensions: 100 x 3.0 mm; Particle size: 2 µm; Mobile phases: A: 0.05% TFA in H_2O, B: 0.05% TFA in MeCN; hold at 5% B for 1 minute then 5-40 %B in 8 minutes, hold at 40 %B for 1 minute; Flow rate: 0.6 ml/min, Temperature: 22 °C; Injection volume: 2 µl; Detection: UV, 220 nm.

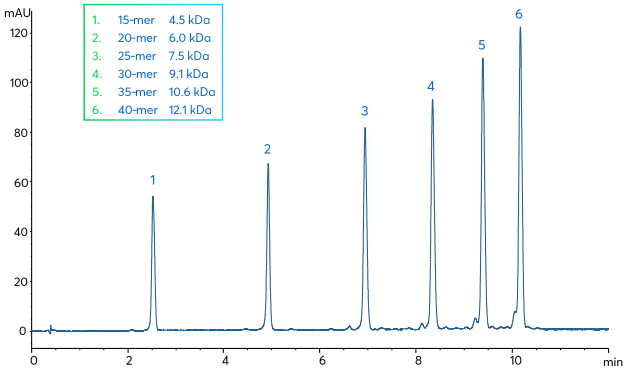


Figure 8: Gradient separation of an oligonucleotide ladder standard using a 100 x 2.1 mm Avantor® ACE® Excel® 1.7 Oligo column. Mobile phases: A: 15 mM Dibutylamine + 25 mM 1,1,1,3,3,3-Hexafluoropropan-2-ol in H_2O, B: 15 mM Dibutylamine + 25 mM 1,1,1,3,3,3-Hexafluoropropan-2-ol in MeOH; Gradient: 30-50 %B in 15 minutes, then hold at 50 %B for 2 minutes; Flow rate: 0.6 ml/min, Temperature: 60 °C; Injection volume: 10 µl; Detection: UV, 260 nm.

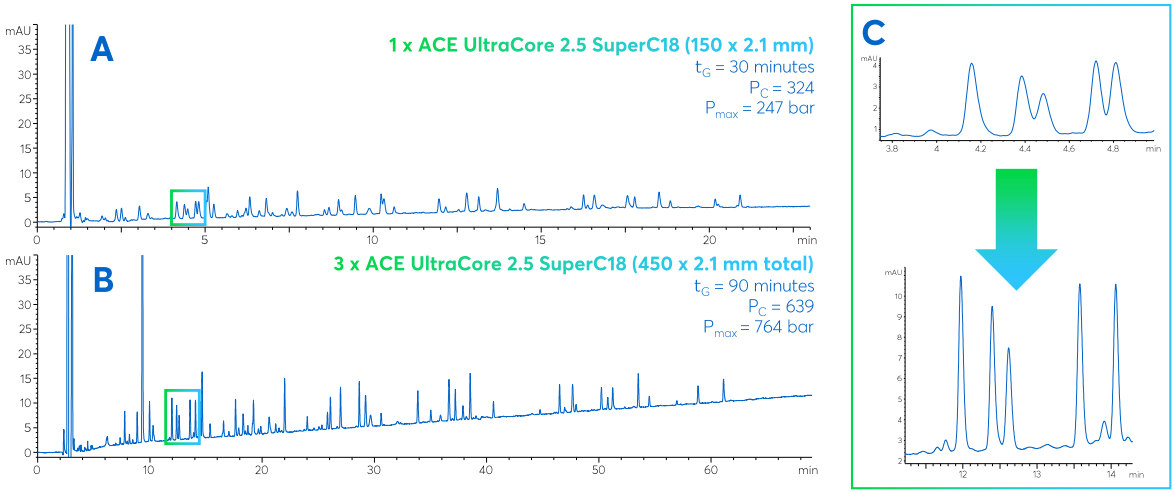


Figure 9: Gradient analysis of a protein digest sample on (A) one single Avantor® ACE® UltraCore 2.5 SuperC18 150 x 2.1 mm column packed with 2.5 µm solid-core particles and (B) three 150 x 2.1 mm columns connected in series. (C) Zoomed-in partial chromatogram comparing the regions highlighted in (A) and (B). Mobile phase: A = 0.05 % TFA in H_2O, B = 0.05 % TFA in MeCN; Gradient 10-40% B; Flow rate: 0.35 ml/min; Temperature: 60 °C; Detection: UV, 214 nm; Injection volume: 20 µl.

in better resolution for peptides than shorter columns. Fifteen or twenty-five centimetre columns are recommended for peptide separations when 3 or 5 µm particles are used, whilst if 2.5 µm solid core particles, or sub-2 µm UHPLC particles are used, 10 cm columns often provide good performance. The use of UHPLC does provide some additional possibilities for the use of even longer columns. For example, Figure 9 shows an ultra-high resolution separation of a peptide digest sample using three coupled solid core columns with a total column length of 450 mm.

COLUMN INTERNAL DIAMETER

Figure 10 summarises the characteristics of different column IDs. The standard internal diameter of analytical HPLC columns is 4.6 mm. These columns are typically run at flow rates of around 1 ml/min. Narrow bore columns (2.1 and 3.0 mm ID) are more widely used for UHPLC and LC-MS applications. These are run at lower flow rates (0.2 to 0.6 ml/min for 2.1 mm ID and 0.4 to 1.0 ml/min for 3.0 mm ID) and therefore use substantially less solvent than 4.6 mm ID analytical columns. Narrow bore columns can also be used to increase sensitivity compared to standard analytical columns. This is because the amount of solvent passing through the detector per minute is lower, resulting in a higher concentration of protein or peptide in the peak. Concentration dependent detectors, such as the UV detector and electrospray mass spectrometer, show greater sensitivity with smaller bore columns.

Microbore columns operate at flow rates of approximately 50 µl/min and therefore their use results in even better sensitivity, about 50 times that of an analytical column. Finally, capillary columns operating at 1 - 50 µl/min flow rates exhibit even higher relative sensitivity, about 200 times the sensitivity of an analytical column. Microbore and capillary columns, however, require specialized, highly optimized instruments because of the flow rates being used and the greater significance of extra-column dead volume.

CONCLUSIONS

The wide range of chromatographic techniques that are utilized for biopharmaceutical characterisation means that selecting an appropriate column for a given analysis can be complex. This article has focussed on the important column selection decisions that are required for reversed-phase separations involving large biomolecules such as proteins. The selection of column dimensions and particle size are typically driven by the specific application, LC system and detection mode used. Careful consideration of the pore size is required to ensure adequate interaction between the target analyte and the stationary phase. Smaller pores are suited to the analysis of protein fragments such as peptides, whereas

COLUMN TYPE	Analytical	Narrow Bore	Microbore	Capillary
Diameter	4.6 mm	2.1 mm	1.0 mm	<1 mm
Flow rate	~ 1 mL/min	0.2 – 0.6 mL/min	~ 50 µL/min	5 - 50 µL/min
Relative sensitivity	1	5	50	200

Figure 10: Characteristics of columns of different internal diameters.

larger pore sizes are required for intact protein analysis. A wide variety of stationary phase chemistries are available that can be tailored to specific applications. The use of different stationary phases provides different analyte selectivity and can be used to provide adequate retention and tune the relative peak spacing and resolve co-eluting peaks.

ACKNOWLEDGEMENTS

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