Understanding and Overcoming Separation Challenges in the Biological Drug Development Process

Overview of the Drug Development Stages

Biotherapeutics go through multiple development stages before they are ready to be sold commercially. The list below details the stages:

1. Selection of a new or matching protein sequence
2. Identification of lead clone(s) using the DNA sequence for proposed protein
3. Cell culture development
4. Process development and final process "lock" – no more changes after this stage
5. Clinical trials and pharmacological assessment
6. Scale-up to commercial quantities
7. Obtain data for regulatory filings
8. Obtain approval and prepare for product launch

Within this process there are different primary analytical needs at each development stage. The identification of lead clones requires titer (protein concentration/run) and protein sequence verification. At this stage, there is also generation of a Master Cell Bank (MCB) and Working Cell Bank (WCB). For cell culture development, titer is still required along with ion exchange (IEX) chromatography to identify charge states, and size exclusion chromatography (SEC) to monitor monomer and aggregates. During process development multiple batches are made which require complete analysis to define the biotherapeutic drug. Included in this is an assessment of the diversity of the protein that is produced. For example, a monoclonal antibody (mAb) has a primary sequence with 150 kDa molecular weight and then there are a variety of post translational modifications (PTMs) and glycosylation modifications that are possible. See Figure 1 for a representative structure of a mAb. These variations create different forms of the same mAb --- theoretically millions of possibilities! Thankfully, nature does not support all of the variations. Separation techniques and mass spectrometry are used to identify these different forms of the mAb. Additionally, stability studies are conducted at this stage.

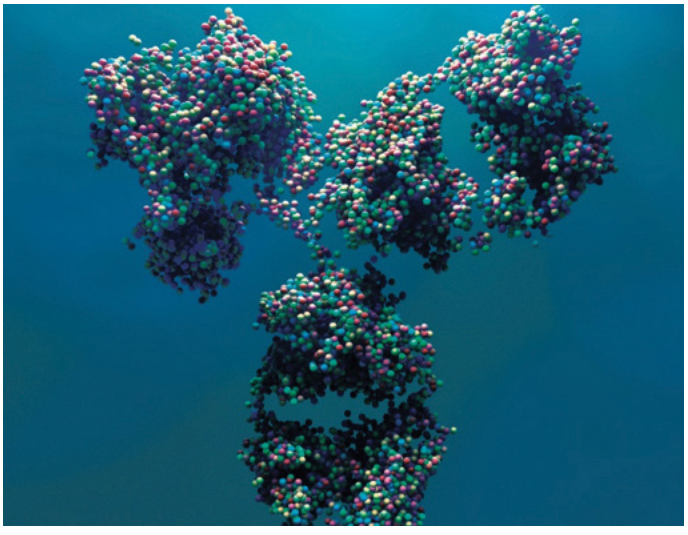


Figure 1. Representative Structure of a mAb

Table 1 shows an overview of some properties

CHARACTERISTIC	PROPERTY MEASURED
Protein Backbone	Amino acid sequence Molecular Weight Amino acid composition Charge profile distribution
PTMs Glycosylation	Galactosylation Galactose-a-1,3 galatose Sialylation N-glycolylneurominic acid Core fucosylation High mannose structure Low abundance glycan species Aglycosylation
Protein Backbone Modifications	N-terminal variation C-terminal variation Deamidation Oxidation C-terminal amidation Glycation
Higher Order Structure	Protein Folding Disulfide connectivity Free cysteine Enthalpy of unfolding Tertiary structure Spectroscopic properties
Aggregation	Percent Monomer Aggregates Fragmentation Sub-visible particles Hydrodynamic radius
Formulation and Drug Product Properties	Protein extinction coefficient Protein concentration Solution properties Formulation Components Container Closure Components Process Impurities Leachables and Extractables
Stability Profile	Comparative stress stability Stress stability Long term stability studies
Host Derived Impurities	Host Cell Protein Host Cell DNA

Table 1. Properties Measured for Physicochemical Characterization of Biological Drugs

measured for physicochemical characterization. They are grouped by which aspect of characterization is being addressed. The methods listed in red utilize HPLC separations.

HALO® BioClass offers columns that fit the separation needs for several of the methods used for physicochemical characterization. Whether intact mAb analysis, reduced and alkylated heavy and light chain fragment analysis, protein digests, or glycan analysis, Advanced Materials Technology offers tailored particle solutions to meet the most challenging separation requirements. Figure 2 shows the various particle sizes and bonded phases that comprise HALO® BioClass products. There are two particle designs for protein analysis: a 1000 Å 2.7 µm particle and a 400 Å 3.4 µm particle. The 1000 Å particle is used for the ultimate resolution of mAbs and other large proteins, while the 400 Å particle is designed for fast analysis of these compounds at lower back pressures. For peptide analysis, Advanced Materials Technology offers three particle sizes from which to choose: 2 µm columns which offer the highest efficiency and are desirable for UHPLC systems, 2.7 µm columns for a good compromise between efficiency and back pressure, and 5 µm columns where longer columns can be utilized and also placed in series for high resolution while maintaining moderate back pressure. For glycan analysis, an application specific column is available which utilizes a proprietary bonded HILIC phase. All of the HALO® BioClass products are designed for high temperature operation and long column lifetimes.

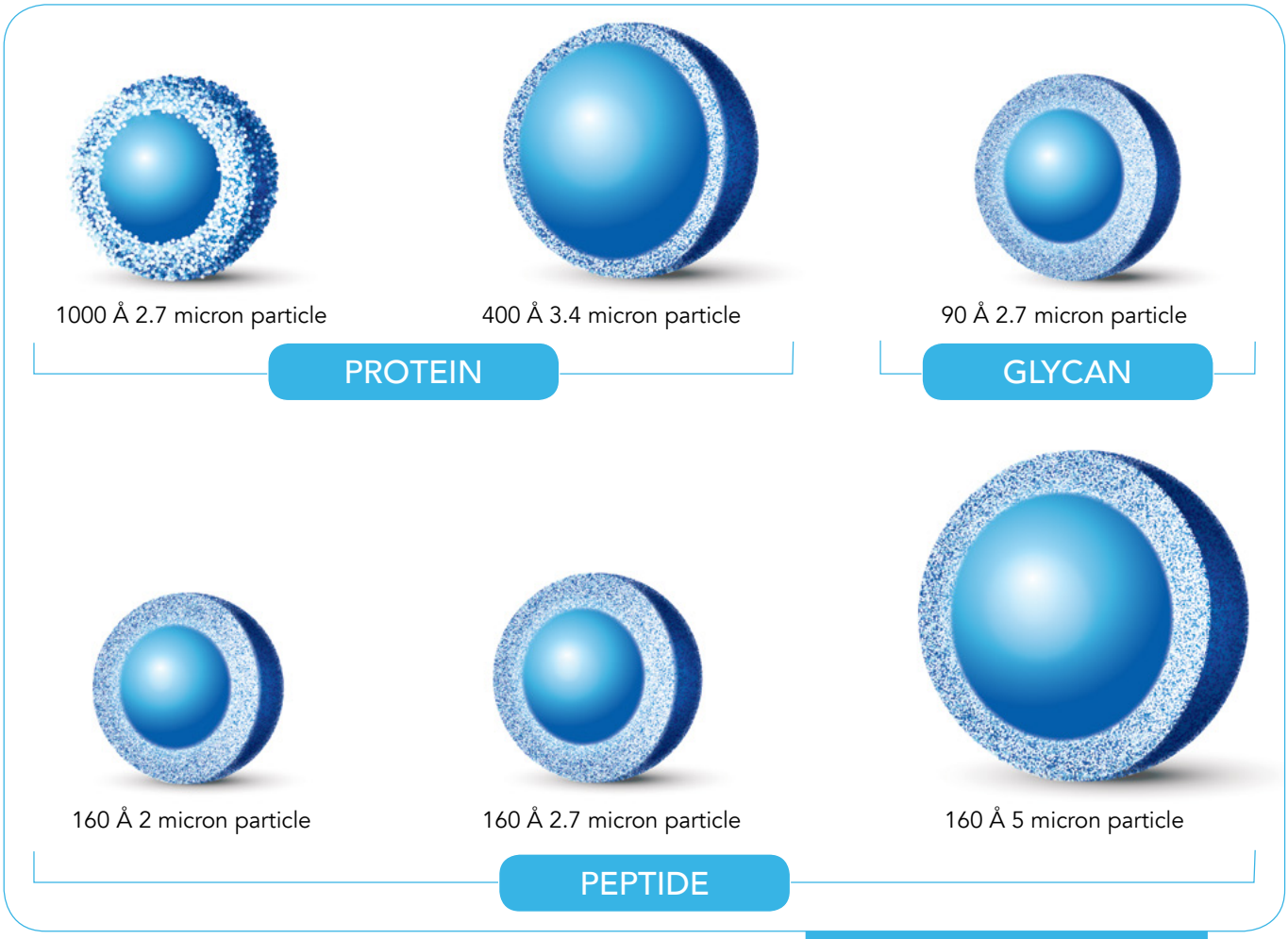


Figure 2. Particles available from AMT for Biomolecule Analysis

BONDED PHASES

C4
ES-C18
DIPHENYL
PHENYL-HEXYL
ES-CN
PROPRIETARY POLY-HYDROXY

Why Fused-Core® columns are especially useful for biomolecule separations

While many separations can be done with multiple types of modern columns, one of the major advantages of Fused-Core® columns is their ability to generate high efficiency separations with reasonable back pressure. Furthermore, multiple columns may be run in series since the back pressure is sufficiently low. In contrast with sub-2 µm fully porous particle (FPP) columns, high pressures would prevent one from coupling several columns together. Figure 3 shows several different particle types and their back pressures relative to a 5 µm FPP. The relative back pressure is highest for the 1.7 µm FPP column with a value of 8.7. However, the 2 µm Fused-Core® particle has a relative back pressure of 6.2, the 2.7 µm Fused-Core® particle has a relative back pressure of 3.4, and the 5 µm Fused-Core® particle has a relative back pressure of 1.2. Low back pressure not only enables Fused-Core® columns to be run in series, but it also means longer lifetimes of pump seals since they are not running at such high pressures.

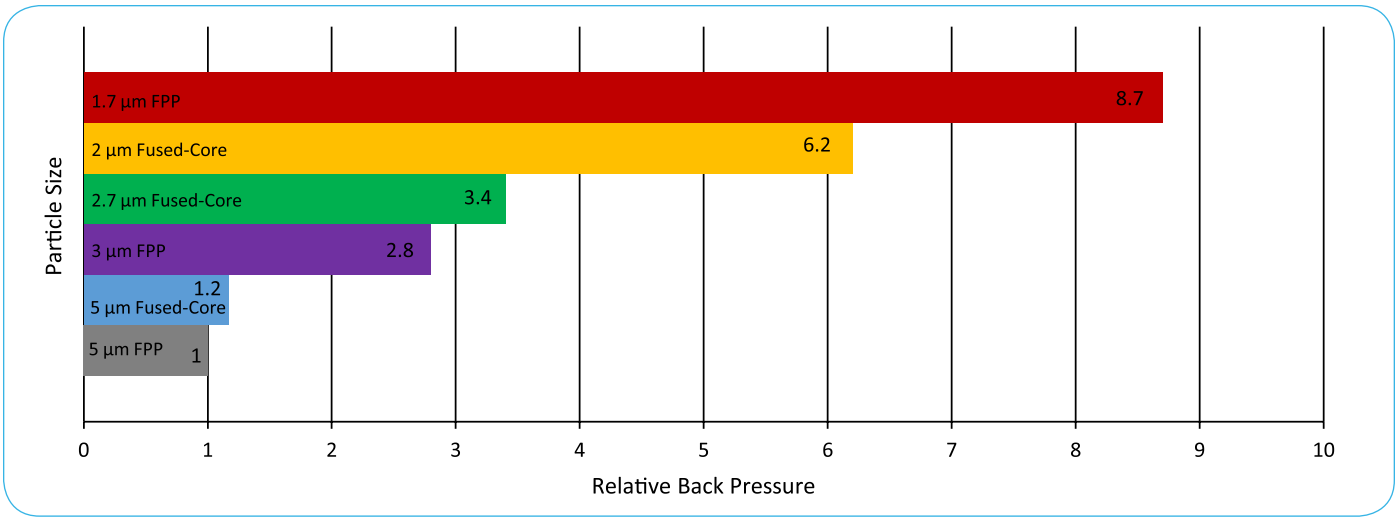


Figure 3. Back Pressure of HALO® and FFP Particles of Various Sizes Relative to 5 µm FPP Back Pressure

Peptide Mapping

Peptide mapping is widely used and needed for both characterization and release of biological molecules. Typically, a protein is digested using an enzyme (Trypsin, Chymotrypsin, Lys-C, etc.) and then the peptides generated are separated using reversed phase chromatography. This process generates a sample with many peaks (50-100 or more), which requires a high efficiency separation. Figure 4 shows the comparison of a trastuzumab tryptic digest run on a HALO 160 Å ES-C18, 2.7 µm column compared to a 1.7 µm FPP C18 column. While slightly more peaks are observed on the FPP column, it is at a cost of twice the back pressure of the HALO 160 Å ES-C18 column.

Columns: as indicated
Flow Rate: 0.6 mL/min
Temperature: 50 °C
Injection Volume: 10 µL of 1 mg/mL trastuzumab, trypsin digest, reduced and alkylated, 50 TRIS pH= 7.8
Instrument: Shimadzu Nexera
Detection: PDA at 210 nm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/ Water/0.08% TFA
Gradient: 3-45% B in 30 min

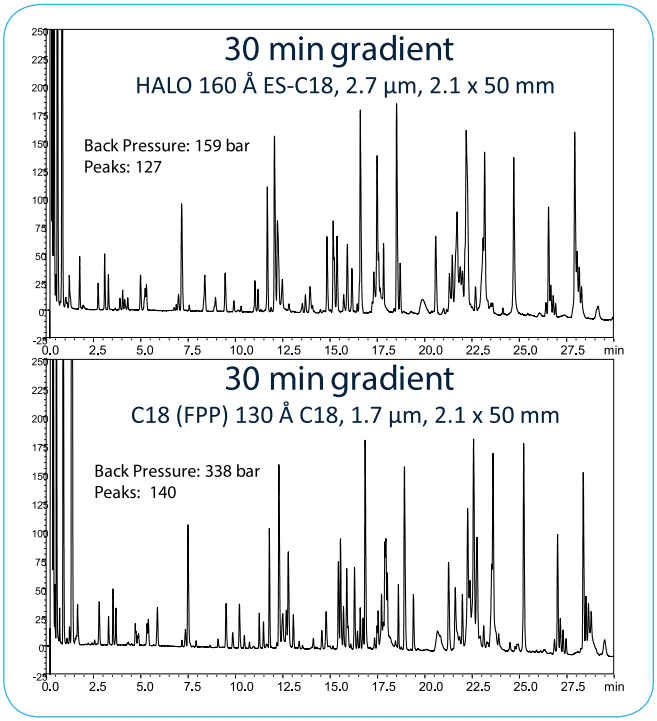
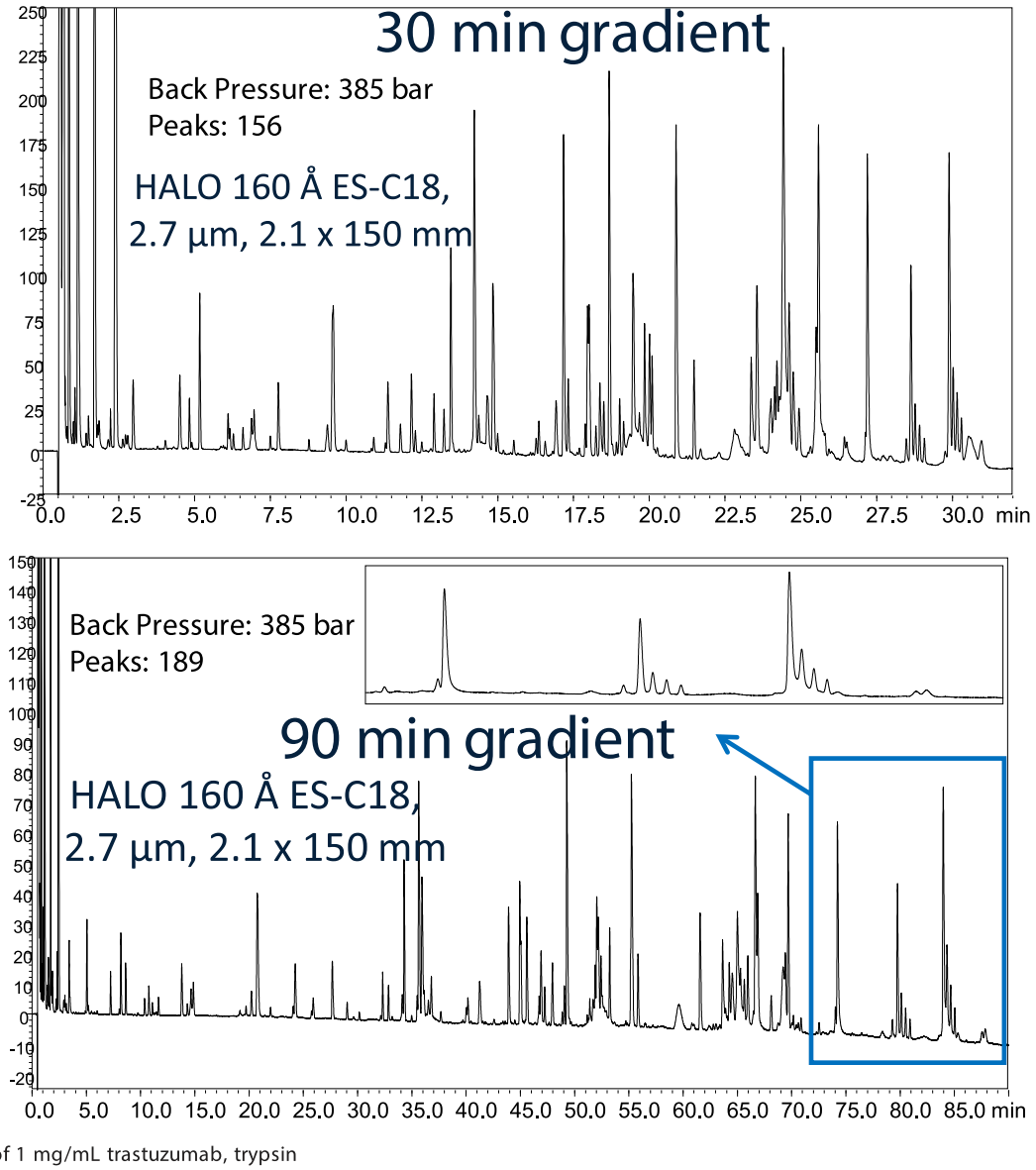


Figure 4. Comparison of HALO 160 Å ES-C18 to FPP 130 Å C18 for a Tryptic Digest

TEST CONDITIONS

Since the back pressure is low with the HALO 160 Å ES-C18 column, a 150 mm length column can be used to increase the efficiency of the separation. See Figure 5. The back pressure is now similar to the FPP column, and more peaks are observed (156 vs. 140). Ultimate performance is achieved when the gradient time is extended to 90 minutes, yielding 189 peaks. This is 35% more peaks than observed with the FPP column!

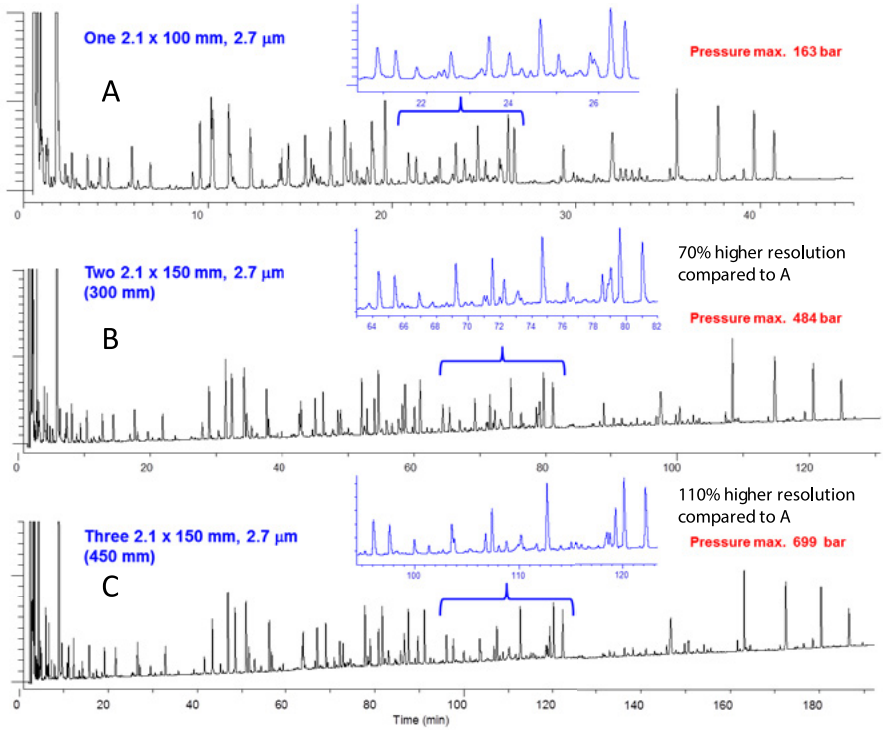


TEST CONDITIONS

Columns: as indicated
Flow Rate: 0.6 mL/min
Temperature: 50 °C
Injection Volume: 10 µL of 1 mg/mL trastuzumab, trypsin digest, reduced and alkylated, 50 TRIS pH= 7.8
Instrument: Shimadzu Nexera
Detection: PDA at 210 nm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/ Water/0.08% TFA
Gradient: 3-45% B in 30 or 90 min

Figure 5. Comparison of 30 minute Gradient to 90 minute Gradient using HALO 160 Å ES-C18

To utilize the higher performance at lower pressure benefit of SPP columns, an example of columns run in series is shown in Figure 6. Figure 6B demonstrates the gains in resolution that can be attained by coupling two 2.1x150 mm HALO 160 Å ES-C18 columns for a 70% increase in resolution compared to using a single 2.1 x 100 mm column Figure 6C takes the example one step further and couples three 2.1x150 mm HALO 160 Å ES-C18 columns for a 110% gain in resolution compared to when one 2.1x100 mm HALO 160 Å ES-C18 column is used!



TEST CONDITIONS

Columns: HALO 160 Å ES-C18, 2.7 µm, lengths as indicated
Flow Rate: 0.4 mL/min
Temperature: 60 °C
Injection Volume: 10 µL of 1 mg/mL apotransferrin tryptic digest
Instrument: Shimadzu Nexera
Detection: PDA at 215 nm
Mobile Phase A: water/0.1% TFA
Mobile Phase B: 80/20 ACN/ Water/0.1% TFA
Gradient: 5-60% B in 60, 180, or 270 min

Figure 6. Effect of Column Length on Resolution with HALO 160 Å ES-C18 Columns

Another advantage of Fused-Core® columns for biomolecule separations is their particle morphology. The thin shell with large pores around a solid silica core provides proteins unrestricted access to the bonded phase and shorter overall diffusion paths compared to sub-2 µm FPP particle columns. In Figure 7, a separation of an IgG2 is compared across several different protein columns. Figure 7A shows limited resolution of the multiple isoforms of the IgG2 using a 1.8 µm 300 Å FPP column. Figure 7B shows a 5 µm 300 Å SPP column beginning to resolve some of the IgG2 isoforms. In contrast, maximum resolution is obtained by using the HALO 1000 Å C4 column as shown in Figure 7C. For intact protein analysis, it is imperative to use columns with pores that are wide enough to accommodate large biomolecules so the bonded phase accessibility to the protein will lead to better resolution and ultimately the best characterization details.

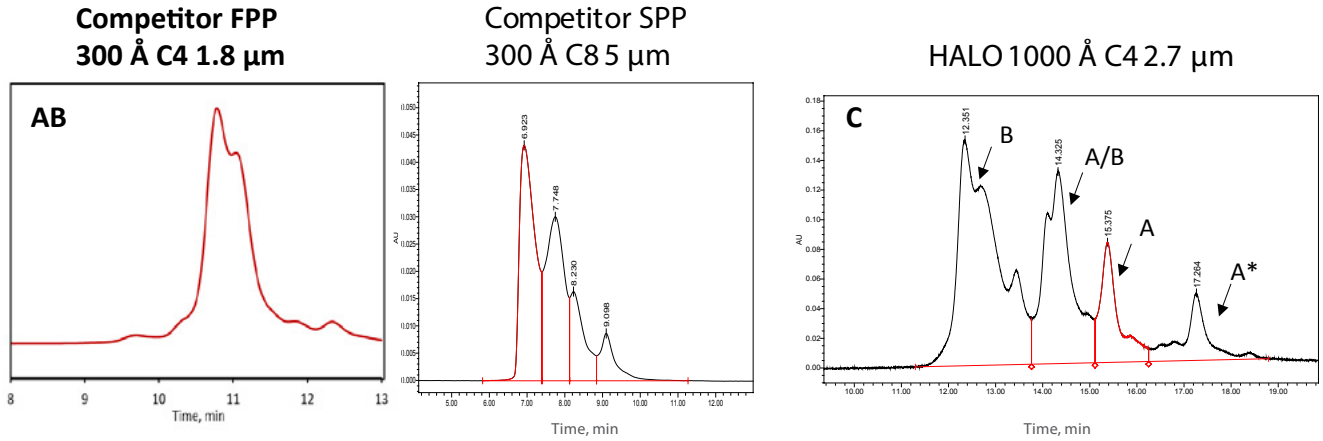


Figure 7. Comparison of HALO 1000 Å C4 to Different Pore Size and Particle Size Columns for the Separation of Intact IgG2

Proposed strategies for Developing versatile platform methods that can be used or easily modified for all stages and needs in drug development

Within biotherapeutics drug development the three main areas for separations are process support, characterization, and final release/QC. Process support can be rapid and low resolution, but more efficient separations can be developed and then "detuned" using the same column and mobile phase, thus not requiring a complete new qualification or validation. Characterization methods can be designed to be very highly efficient, focusing on the highest resolution, usually with longer times. This is often acceptable since these full characterizations are only done on a limited basis. Similar to the process work, high efficiency methods can be "detuned" for final release/QC and stability to be faster when the highest resolution is secondary to time.

For instance, one could develop high resolution methods using long SPP columns (150 mm or longer) with either 2.7 µm or 5 µm particles and wide pore, 1000 Å. These methods will be used for the characterization of the biotherapeutic drug. The methods can then be modified by shortening the column length and/or increasing flow rate for process support, QC work and release assays. The advantage of this approach is that the same column is used for multiple purposes and translation from one method to another is easy.

To illustrate the above concept, the development of a characterization method and QC method for glycan analysis will now be discussed. Glycan analysis consists of several steps:

1. De-glycosylation of protein
2. Labeling of released glycans
3. Separation by HILIC
4. MS used to identify glycans during development
5. Final quanitification done by fluorescence

First, the protein is deglycosylated. Then the released glycans are labeled with a UV or fluorescent small molecule tag. The labeled glycans are separated using the HALO® Glycan in HILIC mode. Mass spectrometry is used to identify the glycans during development while final quantitation is performed using fluorescence. Figure 8 shows a 90 minute HILIC separation of the released and labeled glycans from a highly sialylated protein.

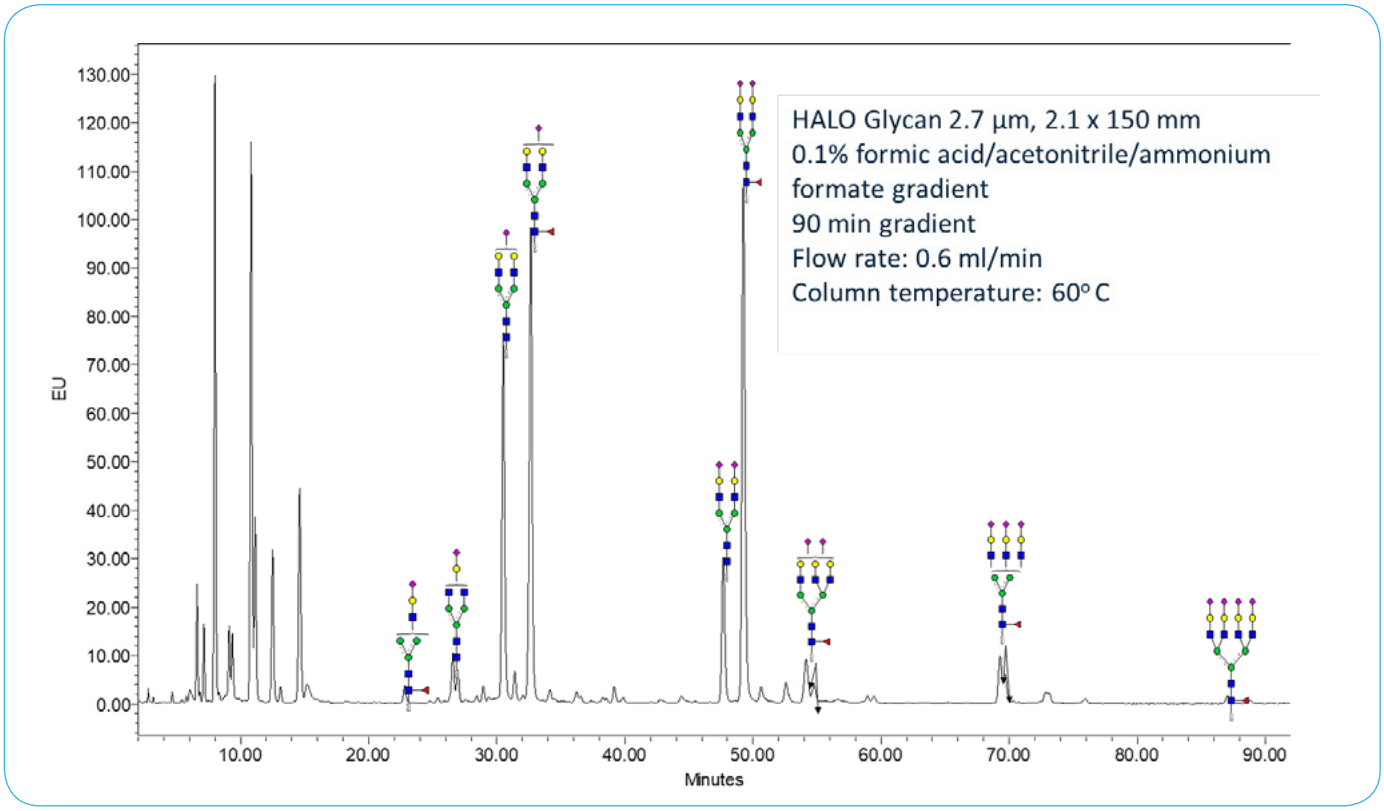


Figure 8. HILIC Analysis using HALO Glycan for a Highly Sialylated Protein

The HILIC method using a HALO® Glycan column was able to separate > 70 glycan species with a peak capacity of ~200. A limitation of the method was that only 1 µL of sample could be injected due to sample solvent interactions while running in HILIC. Additionally, the k* value was 178, which is much higher than the recommended range of 5-10. See Equation 1 for the k* formula:

Equation 1 $$k^* = \frac{0.87 t_G F}{V_m \Delta\%B * 4}$$

(where t_G is the time of the gradient, F is the flow rate, Vm is the column volume and $\Delta\%B$ is the difference in starting and ending mobile phase composition).

In order to reduce k*, the time of the gradient was reduced (shorter time) and the flow rate was also reduced. Figure 9 shows the comparison of the 90 minute gradient to the 45 minute gradient.

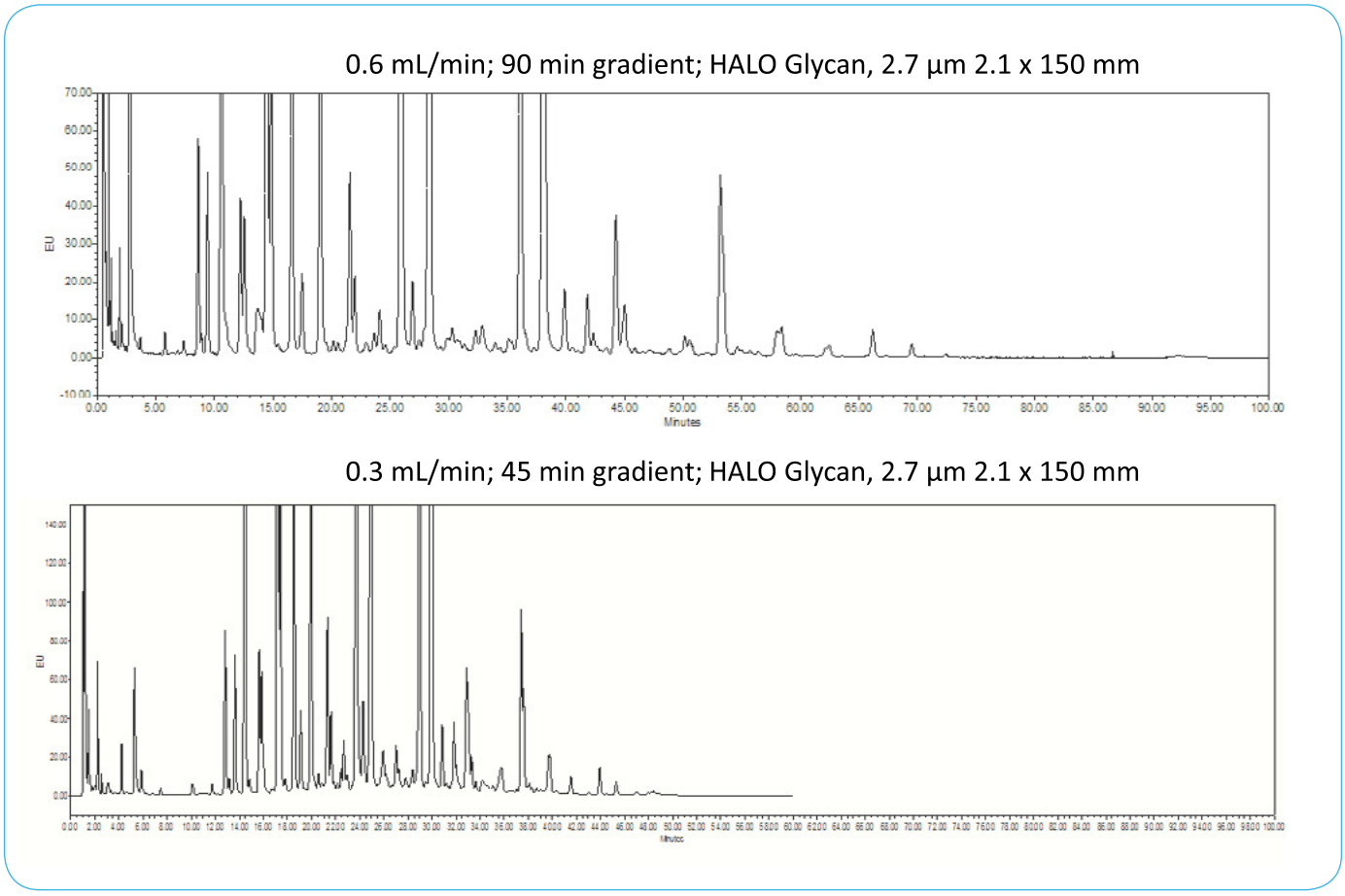
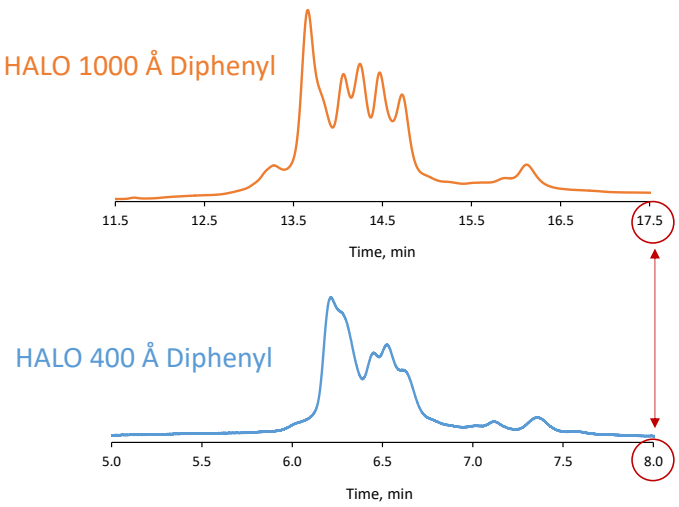


Figure 9. Comparison of 90 min and 45 min gradients for Glycan Analysis

Not only is the method (**twice as fast)**, but also the peak heights are higher which (**increased the sensitivity)** of the method. In addition the k* has been reduced to 44 from 178. This can be counterintuitive, but is an example of how proper use of theory can lead to better separations.

Another strategy implementing methods for both characterization of intact mAbs and a quick release assay is shown in Figure 10. A wide pore, 2.7 µm HALO 1000 Å Diphenyl column could be used for the detailed characterization workup. Then for the release assay, the 3.4 µm 400 Å Diphenyl column could be used for a higher speed analysis due to its thin shell. In this case, complete resolution may not be required rather, only the critical quality attributes need to be identified since the full characterization was completed at an earlier stage of the biotherapeutic drug development process.



TEST CONDITIONS

Columns: HALO 1000 Å Diphenyl, 2.7 µm, 2.1 x 150 mm
HALO 400 Å Diphenyl, 3.4 µm, 2.1 x 150 mm
Flow Rate: 0.2 mL/min (HALO 1000 Å Diphenyl)
0.4 mL/min (HALO 400 Å Diphenyl)
Temperature: 60 °C
Injection Volume: 2 µL of 2 mg/mL denosumab
Instrument: Shimadzu Nexera
Detection: PDA at 220 nm
Mobile Phase A: 88/10/2 water/ACN/n-propanol + 0.1% DFA
Mobile Phase B: 70/20/10 n-propanol/ACN/water + 0.1% DFA
Gradient: 18-28% B in 20 min (HALO 1000 Å Diphenyl)
18-28% B in 10 min (HALO 400 Å Diphenyl)

Figure 10. Intact Denosumab Separation Using HALO 1000 Å and HALO 400 Å Diphenyl Columns

Conclusion

Biological drug development requires testing at many stages during and after the process. Separations, especially HPLC, are very important in this testing. Process, Characterization, and Final Release/QC all have specific requirements. Use of the HALO® Fused-Core® columns can be beneficial in all developmental stages to maximize analytical efficiency.