An Executive Summary

Applying High-Resolution and High-Efficiency Size Exclusion Chromatography Technology to Biotherapeutic Characterization

More efficient and accurate analysis of potential biotherapeutics at an early stage of development can speed up successes and reduce failures.

Overview

Scientists use size-exclusion chromatography (SEC) to study many biomolecules. SEC columns with sub-2 µm particles can afford high resolution and efficiency separations for characterizing size variants of biotherapeutics. SEC can also be used in two-dimensional (2D) liquid chromatography–mass spectrometry (LC–MS) separation and with MS detection to support structural characterization of biotherapeutics. This summary discusses some of the challenges and solutions for getting the most accurate data out of an SEC separation.

Column Features

Over time, particle sizes in SEC columns have become smaller, thus offering columns with higher efficiencies. Additionally, optimized surface coatings helped to improve the separation characteristics.

It is critical to choose an SEC column with the correct pore size that would allow the molecule of interest to permeate the pores while having the maximum amount of pore volume to help resolve other size variants. SEC separation is based on the size of the molecules rather than their molecular weight. However, since most proteins are globular in nature, resolving ranges is often expressed in terms of molecular weight.

A column with sub-2 µm particles, such as the Agilent AdvanceBio SEC 200 Å 1.9-µm columns, increases the resolution of monomers and other fragments with low molecular weights. In this case, the 200 Å pores are designed to analyze molecules with a hydrodynamic radius of 6.7 nm or less, which works well with monoclonal antibody (mAb) monomers and dimers as well as separating low molecular weight (LMW) molecules. It can also reduce non-specific interaction and increase the life of the column. For example, AdvanceBio SEC 200 Å 1.9-µm columns provide better resolution at a relatively low pressure and a reduced risk of clogging in the column, which allow for faster separations.
Sub-2 μm Applications

Using smaller particles, especially ones less than 2 μm in diameter, provides many benefits. For example, the high resolution makes quantification possible. In characterizing biotherapeutics, this technology can be used in four ways:

1. LC with ultraviolet (UV) detection can be used to quantify high molecular weight (HMW) dimers and LMW fragments;
2. LC–MS in native or denaturing modes can be used in various ways, including analyzing protein-ligand and protein-protein interactions;
3. With 2D-LC, scientists can analyze aggregate and free drugs, plus other forms of analysis; and
4. Bioprocessing, such as monitoring antibody aggregation.

With antibodies, as shown in Figure 1, analysis can involve separations of various components, including mAb dimers, monomers, and fragments, including antigen-binding (Fab)2 and fragment crystallizable (Fc) fragments. When separating these mAb components, the resolution depends on particle size, in addition to other factors that affect the column performance.

The surface of the particles, for instance, impacts nonspecific interactions. As an example, AdvanceBio SEC 200 Å 1.9 μm particles are inert due to unique phase chemistry, a hydrophilic coating, and optimized bonding density. Also, using a highly crosslinked phase makes the column chemically stable. Together, this results in a sharp monomer peak and well-resolved fragment peak. Under similar conditions, a sample performed on other vendor columns showed poor performance with split peaks. This might be because of high hydrophobicity of sample causing secondary interaction with column media.

Looking for a Long Life

In addition to achieving desirable separations, scientists want a column that can be used as long as possible with consistent peak areas and stable column backpressure over hundreds or even thousands of injections.

Agilent’s R&D team has designed a stop-and-run method to understand the mechanical strength of the particle and probe for column lifetime stability. In this instance, flow is started, completely stopped, and then started again. This represents the use of a column that is not constantly running. Some columns show reduced efficiency after 200 injections by this method. With an AdvanceBio SEC 200 Å 1.9 μm column, the performance stayed the same for at least 400 injections in one comparison.

The length of the column also plays a role in performance (Figure 2). For example, reducing the length of a column by half cuts the runtime in half and doubles the throughput. The reduced length also produces less back pressure, which allows for higher flow rates.

In one example, the run time was reduced to three minutes—compared to previous SEC columns taking 20 minutes to run. As Figure 2 shows, this increases the throughput from 72 samples in 24 hours into 480.

Characterizing Biologics

High-efficiency SEC makes an effective tool for characterizing biology-based therapies, which are often called biotherapeutics or biologics. An example application is stability characterization.

For product quality, pharmaceutical scientists assess a biologic’s chemical and physical properties. Running these studies early in the drug-development process reduces late-stage failures. In short, early protein characterization reduces risk in chemistry, manufacturing, and controls development to bring the final pharmaceutical product to the market.

Native Analysis

Scientists at Frontage Laboratory analyze large molecules for many features: charge variants, size variants, as well as the glycan profile, binding potency, and host-cell proteins. Some of these scientists also use native MS on...
proteins in their native condition, which means that the folding is intact. To maintain a protein’s natural structure, no denaturing solvent is used during LC and only soft ionization is used in the MS stage.

The pharmaceutical industry uses native MS for many reasons, such as analyzing protein–protein and protein–ligand interactions. This method can also be used to assess how much of a large-molecule sample is oligomer versus monomer.

Despite many technological advances, Figure 3 shows why native MS remains challenging. One challenge is caused by normal salt buffers used in SEC being incompatible with MS. Another challenge includes potential denaturation of proteins caused by ionization.

In a study using an MS-compatible mobile phase, 100 millimolar ammonium acetate at pH 7.5, in the AdvanceBio SEC 200 Å 1.9 µm columns, UV detection showed good separation. Then, using this mobile phase and column, scientists at Frontage Laboratory characterized a mAb with native MS, as shown in Figure 4. The UV spectra (top) shows two peaks: the major species and a LMW species. The zoomed-in MS spectra (lower left) shows charge states from 24 to 30, and deconvolution (lower right) shows six major glycoforms of the intact mAb, around 150 kDa.

The scientists were able to identify different glycoforms of the intact mAb. For the LMW species, its mass is about 64 kiloDaltons, as revealed after deconvolution (not shown here), which matches the molecular weight of the transition from the hinge region of the mAb. The key here is that one method can be used for relative quantification and identification of the components.

For looking at oligomerization, the company’s scientists analyzed bovine serum albumin (BSA). The results of SEC and a UV spectra showed two major peaks—presumably one being the monomer and the other the dimer. The MS spectra and deconvoluted spectra for the major and minor peaks showed molecular weights that matched the calculated weights for the monomer and dimer, respectively.

Figure 3: Challenge of native analysis.

1. Separation condition may be incompatible with MS condition.
   Mobile Phase, pH, Salt concentration, Flow rate etc.

2. Ionization could also lead to denature.

3. High m/z range and low sensitivity
Such studies of oligomerization give an idea of how SEC-MS can be used to study binding between proteins or proteins and ligands. This technology can also be used to analyze cysteine-conjugated antibody–drug conjugates (ADCs).

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In-Practice Pointers

In all applications of a sub-2 µm SEC column, several elements of the system should be considered. One is to use tubing with a 0.12 millimeter internal diameter, which reduces the extra-column volume and minimizes band broadening.

To extend a column’s lifetime, the flow of the mobile phase should be increased gradually. As an example, start at 0.1 mL/min and slowly increase the flow in increments of 0.1 mL/min. Continue the slow increase to the desired flow rate.

Bad connections can also reduce the quality of data. High data collection rates are also essential to accommodate the sharp peaks that sub-2µm columns can provide.

Conclusion

Scientists can use SEC to characterize biotherapeutics for a wide range of applications. This technology can also be combined with UV or MS detection to quickly analyze a range of samples—from protein mixtures or mAb and fragments, to more complex structures such as a mAb bound to an antigen. These techniques can provide pharmaceutical scientists with the information from early stages of drug development that can prevent costly late-stage failures.