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CHROMATOGRAPHY FUNDAMENTALS FOR THE ANALYSIS AND PURIFICATION OF BIOLOGICS

In 1982, the first biologic drug—a recombinant human insulin made by cloning human DNA in *Escherichia coli*—hit the shelves. Nearly 40 years later, 8 of the 10 top-selling drugs globally are biological molecules.[1]

Biopharmaceuticals now account for over a third of the new drugs approved annually by the US Food and Drug Administration. In 2019, for example, the FDA gave the thumbs-up to five new monoclonal antibodies, three antibody-drug conjugates, three peptides, one oligonucleotide, one small interfering RNA, and one fusion protein.[2]

The majority of biologics are still produced by genetically engineering cells. These cells—including bacteria, yeast, mammalian, and plant—are grown in bioreactors. Once they are harvested, "you need to do a purification in order to remove everything that you don't want from the biological soup inside the bioreactor," says Jeffrey A. Kakaley, Marketing and Applications Manager for high-performance liquid chromatography (HPLC) and ultra-HPLC (UHPLC) columns at YMC America.

Harvested biologics are purified about 1,000,000-fold before they are given to patients.[3] Purification methods vary between biomolecules, but, as with small-molecule drugs, chromatography dominates these processes. Purification of biomolecules is often more challenging than that of small molecules. Reasons include their larger size—biologics can be 1,000 times as large as small-molecule drugs—and greater structural complexity.

For example, bulk monoclonal antibody purification typically starts with affinity chromatography, followed by ion-exchange (IEX) chromatography. A subsequent hydrophobic interaction chromatography (HIC) step is often used as well.

HPLC and UHPLC are also widely used throughout biologic manufacture for quality-control purposes. Popular modes for biomolecule analysis are size-exclusion chromatography (SEC), IEX chromatography, HIC, and reversed-phase (RP) chromatography. The same techniques are also used to ensure biologics are not degrading during storage.

AFFINITY CHROMATOGRAPHY

Affinity chromatography uses the specific and reversible binding interactions between a chromatography resin and the biomolecules being separated. Interactions typically used include antibody-antigen, enzyme-substrate, and enzyme-inhibitor.

For antibody purification, resins containing immobilized protein A are a popular choice. Protein A, which is found in the cell wall of *Staphylococcus aureus*, binds strongly and reversibly to a broad range of monoclonal antibodies. As a crude sample travels through the column, protein A will bind the target antibody. Impurities can then be washed off the resin. The pH of the buffer is lowered, to trigger the unbinding of the antibody and its elution from the column.

Affinity chromatography has been used for antibody purification since the 1950s, and products on the market have been evolving ever since to meet consumer needs. Most recently, chromatography manufacturers have needed to keep pace with the pharmaceutical industry's shift toward continuous manufacturing for biologics, says Gerard Gach, Chief Marketing Officer at YMC Process Technologies. The aim of continuous manufacturing is to produce biologics from their starting materials in a single multistep flow process. In contrast, traditional batch biologic manufacturing comprises a series of distinct steps, often with a break in time or change in location between stages.

Estimates vary, but it is widely believed that continuous manufacturing will at least halve the cost of producing biologics.[4] "The FDA is encouraging biologic manufacturers to use continuous production to reduce cost, increase availability of the drug, and increase consistency of product quality," Gach says.

There are, however, many technical and logistical challenges posed by such a significant switch in manufacturing approach. Still, continuous processing is slowly being embraced for the upstream stages of biologic manufacture, including cell growth and harvest.[5] Progress for downstream processes such as purification has been slower. Affinity chromatography proved a particularly challenging step to adapt to a continuous process, according to Gach. Suitable systems are coming on line, such as the dual affinity column technology YMC Contichrom Twin CaptureSMB. This system is suitable for antibody purification at bench through production scale.

The YMC Contichrom Twin CaptureSMB (shown here) is a dual affinity column suitable for antibody purification at bench through production scale.

Image credit: YMC Co, Ltd.

Another common problem for biologics manufacturers is affinity columns that are only 40–60% efficient in binding to the target biomolecules. When columns are pushed to achieve higher loading, target molecules tend to pass through the columns uncaptured. To counter this, the YMC Contichrom Twin CaptureSMB has two affinity columns in series, meaning the second column can capture any target molecule that has passed through the first column, effectively making the first column 95% or more efficient.

The YMC system allows the first column to be taken off-line and the product eluted while the second column is still loading, Gach says. When the first column is empty, it is reconnected and the second removed for product elution. "That just goes on continuously," he adds. This twin column setup has been shown to improve productivity by up to three times, while using up to half the buffer and protein A resin used in conventional processes.

Pharmaceutical companies that have embraced the YMC technology include Bristol-Myers Squibb, which has introduced the YMC Contichrom Twin CaptureSMB into the clinical production for some of its antibody therapies.[6] A European Union–based biopharma company has also purchased a twin unit for good manufacturing practice production, and multiple other end users in Europe and the US have used it on the pilot scale.[7]

YMC also has a sister technology, Contichrom Twin MCSGP HPLC, geared toward continuous purification of other biomolecules such as peptides, antibody-drug conjugates, and oligonucleotides.

SIZE-EXCLUSION CHROMATOGRAPHY

SEC, which sorts biomolecules by size, is a popular quality-control tool for detecting and monitoring aggregate formation. Biomolecules have a tendency to clump, forming dimers and trimers or even larger aggregates, during both production and storage. "The aggregates can cause immunogenicity," says Cinzia Stella, Senior Scientist in the protein analytical chemistry department at Genentech. "We use a size-exclusion method to monitor the level of size variants to ensure consistency and product quality of the released batches."

The resin used for SEC is highly porous. A biomolecule's size determines whether it can penetrate the pores and, if so, how deeply. This in turn determines how rapidly it is eluted off the resin. "Larger molecules cannot enter the pore and just pass through the column, while smaller molecules go into the pores deeply and are eluted later," says Takashi Sato, Sales and Marketing Manager at YMC in Japan.

YMC has two SEC phases suitable for biomolecules: YMC-Pack Diol and YMC-SEC MAB. The first phase comes in four pore sizes. The 60 Å one is for separating small biomolecules. The 120, 200, and 300 Å columns are for separating proteins. The second phase has 250 Å pores and is specifically designed for detecting aggregates and fragments of monoclonal antibodies and antibody-drug conjugates.

Manufacturing platforms are a set of well-established processes suitable for an array of products. These platform technologies are increasingly popular for most stages of biopharmaceutical manufacture, including SEC. For example, a platform SEC method that uses the same column and mobile phase can be routinely implemented for all applicable monoclonal antibodies. "We've been working with [immunoglobulin G antibodies] for a long time, and we try to use platform methods—that fit most of our molecules—as much as possible," Stella says. "It makes things much easier in the quality control labs if the same method can be used for as many immunoglobulin G antibody products as possible instead of having to develop and validate a new one every time."

Platform technologies are not static, however. "We stay up to date with the latest analytical technologies, and if we see that there is another column that is much better than what we've been using so far, then we do try to implement the change when possible," Stella says.

ION-EXCHANGE CHROMATOGRAPHY

"Ion-exchange chromatography is useful for both analytical separations and preparative purification of most biologics," Kakaley says. This chromatography mode is used frequently for the bulk purification of monoclonal antibodies as a second step, after affinity chromatography.

IEX chromatography separates biomolecules according to differences in their overall surface charges. The resin contains charged groups that form reversible interactions with the charged amino acid side chains on a biomolecule's surface. These are then eluted from the column using gradients, either salt concentration or pH.

Salt ions compete with biomolecules for the charged groups on the resins. This means that as the salt concentration in the mobile phase increases, the biomolecules will start to be knocked off the resin and eluted from the column. The proteins with the lowest net charge will be eluted first, and those with the highest net charge come off last.

With a pH gradient, the order of elution depends on the isoelectric points of the biomolecules being separated. The isoelectric point is the pH at which the charged groups on a biomolecule cancel one another out, making the overall charge for that biomolecule zero. With cationic resin, for example, while the pH of the mobile phase is below the isoelectric point of a biomolecule, its overall charge will be positive and it will bind to the resin. When the pH of the mobile phase rises above the isoelectric point, the overall surface charge of the biomolecule will be negative; it will be repelled by the resin and will elute from the column.

YMC produces hydrophilic polymer beads tailored for the IEX chromatography of proteins, peptides, and nucleic acids: the YMC BioPro IEX. This is available as both a cationic and anionic resin. Both types are produced in porous and nonporous forms. Porous resins offer the possibility of better separation with the trade-off of slower flow rates, according to Sato. “The nonporous resin is particularly good for fast analytical applications,” he says.

All YMC BioPro IEX resins are available as analytical chromatography columns and bulk materials for preparative-scale separations.

YMC BioPro IEX resins are hydrophilic polymer beads tailored for the IEX chromatography of proteins, peptides, and nucleic acids.

Image credit: YMC Co, Ltd.

HYDROPHOBIC INTERACTION CHROMATOGRAPHY

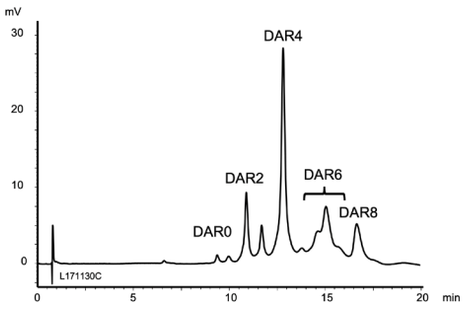
HIC separates biomolecules using differences in surface hydrophobicity. This mode is frequently used during the bulk production of biomolecules as a third step, after affinity and IEX chromatography.

The resin for HIC contains hydrophobic groups that form reversible interactions with hydrophobic amino acid side chains on the surface of biomolecules. A salt gradient is used during the separation. High salt concentrations strengthen the interactions between the biomolecules and the column, while lower salt concentrations weaken them. The more hydrophobic the overall biomolecule, the less salt is needed to promote its binding. The salt concentration is run from high to low during the separation, meaning the least hydrophobic biomolecule comes off first and the most hydrophobic biomolecule elutes last.

This chromatography mode is also gaining traction at the analytical scale for the quality control of antibody-drug conjugates. Antibody-drug conjugates are composed of small-molecule drugs attached to monoclonal antibodies by chemical linkers. The desired drug-to-antibody ratio varies between therapeutics, but an average of three or four molecules of a given drug attached to a single antibody is a typical ratio.[8] If the average ratio is too low, efficacy can be reduced, but a ratio that is too high can pose the possibility of overdose. Measurement of this ratio is therefore a critical quality-control step during the production of all antibody-drug conjugates.

"We routinely use HIC to measure the drug-to-antibody ratio of antibody-drug conjugates," Stella says. Genentech manufactures Kadcyla, a HER2-positive metastatic breast cancer treatment that gained FDA approval in February 2013 and became one of the first antibody-drug conjugates to market.

HIC works well for antibody-drug conjugates because most small-molecule drugs are hydrophobic. An antibody without a drug attached will come off the column first, while the antibody with the most drug molecules attached elutes last. The ratio can be determined by comparing peak areas.



Column	: BioPro HIC BF 4 µm, 100 X 4.6 mmI.D.
Eluent	: A) 50 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0) containing 1.5 M $(NH_4)_2SO_4$/2-propanol (95/5)
	B) 50 mM NaH_2PO_4-Na_2HPO_4 (pH 7.0)/2-propanol (80/20)
	0%B (0-1 min), 0-100%B (1-15 min), 100%B (15-20 min)
Temperature	: 25°C
Flow rate	: 1.0 mL/min
Injection volume	: 5 µL
Detection	: UV at 280 nm
Sample	: Cysteine-conjugated ADC mimic (5 mg/mL)

The BioPro HIC BF column shows superior resolution compared to competitors' columns and is highly effective for determining drug-to-antibody ratios of Antibody Drug Conjugates.

Image credit: YMC Co, Ltd.

In 2018, YMC launched the YMC BioPro HIC BF phase, optimized for the analysis and lab-scale purification of antibody-drug conjugates, antibodies, and other proteins. "We are currently aggressively developing a new HIC column for faster separation," Sato says.

REVERSED-PHASE CHROMATOGRAPHY

The fundamentals of RP chromatography are the same as those for HIC. Hydrophobic groups immobilized on a resin bind reversibly to hydrophobic groups on biomolecule surfaces. A concentration gradient of organic solvent is used to elute biomolecules from a column by order of their hydrophobicity.

However, the resins for RP chromatography are typically more hydrophobic than those developed for HIC. This leads to stronger interactions between the biomolecules and the resin, requiring nonpolar organic solvents, such as acetonitrile, to break the bonds and elute the biomolecules.

Historically, RP chromatography was not commonly used for the separation of large biomolecules. Eluting these requires harsh conditions that classical (pure silica) RP columns cannot tolerate. The introduction of hybrid resin materials with higher chemical durability made these separations possible. For example, the recently released YMC-Triart Bio C4, a hybrid 300 Å porous resin, is specifically designed for the RP separation of intact monoclonal antibodies and other large proteins.

RP chromatography with hybrid resins is also a popular tool for separating small biomolecules. The YMC-Triart Bio C18 is designed specifically for this purpose. The YMC-Triart Bio C4 and YMC-Triart Bio C18 range are produced with 1.9, 3, and 5 µm particle sizes and as analytical- and semipreparative-scale columns. These columns are also well suited for use during peptide mapping, an important protein structure confirmation method.

CONCLUSION

Biologics are here to stay. The monoclonal antibody Humira—an autoimmune disease treatment produced by AbbVie—has been the top-selling drug globally for a number of years. It had global sales of $19.9 billion in 2018, and it is expected to retain its top spot until 2023, when its US patent expires.[9] Sales of another monoclonal antibody—Merck & Co.'s cancer immunotherapy Keytruda—are then expected to overtake it.

The types of biologics gaining FDA approval in recent years have extended beyond monoclonal antibodies to include more complex designs, such as antibody-drug conjugates. At the same time, biomolecule manufacture is shifting away from batch production to continuous processing. Chromatography manufacturers are keeping pace with industry changes, evolving resins and releasing novel chromatography setups suitable for the wave of effective biologic treatments on the horizon.

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