The Basics of Chromatography

Chromatography can be described simply as a separation method in which different components of a mixture migrate through a column at different rates. The mechanisms by which it works involve intricate molecular binding chemistries and delicate operating specifications.

Because biotechnology-derived therapeutic proteins are produced and formulated in liquid solutions, biopharmaceutical manufacturers use liquid — as opposed to gas — chromatography to analyze and purify their products. To work, it relies on diffusion or interactions between molecules in the sample mixture (the fluid phase) and a matrix or gel medium (the solid or stationary phase), which is packed into a column made of glass, plastic, or stainless steel (see Figure 1). An eluent (elution buffer) is used to recover (elute) the separate components from the matrix.

In characterization, chromatography is used — along with other analytical techniques — to demonstrate the identity, purity, potency, and stability of the biopharmaceutical. In production, however, chromatography is used as part of the manufacturing process to purify the biopharmaceutical.

Virtually all protein purification processes rely on at least one column chromatography step. The number and sequence of such steps are usually tailored to the protein, the feed material, and the application of the product.

Step by step. Chromatographic separations are a stepwise process by nature. Although the science behind the technique is complex, the actual steps involved are relatively simple: column equilibration, sample application (and adsorption), column washing, elution of bound molecules, column regeneration, and reequilibration. Those steps are repeated as long as the column resolution is good, as long as it can be reused.

Elution of sample fractions can be done several ways. The mode of elution usually depends on the scale of the process. Isoelectric elution makes use of an elution buffer solution to move the solute through the column. Gradient elution changes their relative affinity by changing the conditions (such as pH or salinity), either stepwise or continuously. Displacement elution uses a substance with greater affinity for the stationary phase to displace the molecule already bound.

Column chromatography: Separation method in which different components of a mixture migrate down through a column at different rates of speed based on their various chemical properties.

Chromatography column made from acrylic.

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There are three stages when chromatography is applied in a downstream process. The capture phase focuses on the “capture” (stabilization and concentration) of the target molecule. During intermediate purification, specific impurities such as host cell proteins are removed. Finally, in the polishing phase, final trace impurities are removed and product safety is assured.

“In capture, step-wise washing and elution are used and throughput is maximized,” says Eric Grund, director of bioprocess applications for Amersham Biosciences. “In intermediate purification, finer beaded media and gradient techniques might be used to increase resolution,” he adds. “In polishing, high resolution is a must and the media used are usually the finest for any given process.”

**Separation technologies.** Chromatographic techniques can be classified by their respective modes of separation: In nonadsorptive chromatography, the rate at which substances pass through the column is controlled by basic physical, kinetic characteristics such as the size and shape of molecules and particles. Gel filtration chromatography falls into this category.

In adsorptive chromatography, molecules in the sample mixture adsorb (adhere) onto matrix particles through chemical interaction, so the rate at which they move is determined by the chemical and thermodynamic properties of both the mixture components and matrix material. Adsorptive chromatography works by ionic interactions (electrostatic binding), by hydrophobic interactions (less soluble materials attracting each other), or by the myriad specific chemical interactions used in affinity chromatography.

Adsorptive chromatography relies on the distribution of various molecules between the two phases with which they are in contact — that is, between the solid phase in the column and the sample mixture itself. Molecules with greater affinity for the solid phase will concentrate there. Because different component molecules in a mixture have different physical and chemical properties, they can be separated based on their relative attraction to the medium.

Ion-exchange (IEC), hydrophobic-interaction (HIC), size-exclusion (SEC) or gel-filtration, and the various affinity chromatographies are most commonly used in large-scale separations of biotechnology products.

**Gel Filtration**

Although it is neither the most powerful nor most commonly used type of liquid chromatography, we first look at the gel filtration technique because it has the simplest mechanism of action. Also known as size-exclusion chromatography (SEC), it operates on the principle that impurities differ in size. Smaller components of the mixture enter pores in the gel matrix as larger ones pass them by. When the holes are large, this method can be used to separate cells and cellular debris from soluble proteins, slowing the protein and other molecules while the larger material washes through. When the holes are smaller, the technique is good for trapping and thus removing salt, buffers, and small-molecule impurities from the protein solution.

The quality of an SEC separation depends on the gel medium used (based on how it packs into the column and on the size and distribution of pores), on the volume and concentration of the mixture passed through the column, and on the flow rate of the mixture to be separated.

SEC media most often used include agarose, dextran, polyvinyl, silica, and acrylamide gels. The choice of medium is based on such parameters as the exclusion limit and fractionation ranges of the particles (that is, what size molecules they can fractionate or separate out) compared with known properties of the impurities. The technique works best when the component to be separated out (such as the protein of interest) is in the first half of the fractionation range.
Only for very large protein molecules does total size exclusion (using particles that will trap everything but the product itself) work best, and then only if no components present are bigger than the product.

**Ion Exchange**
The ion-exchange technique is so common in biopharmaceutical purification that any given separations process can be assumed to have at least one IEC step. One example of its use is in the preparative purification of “core” proteins from *Escherichia coli*. Ion-exchange is an electrical phenomenon. Ions are positively or negatively charged atoms or molecules because they have gained or lost protons. It is the amphoteric nature of proteins that makes this method useful; their net charge depends on the pH of their environment. We speak of a protein’s “net” charge because areas of positive and negative electric charge are scattered across the surface of each molecule according to the location and orientation of its amino acid residues. How those varying charges balance out to an overall positive or negative charge is expressed as the protein’s net charge.

The pH value at which a given protein’s net charge is neutral is called its isoelectric point (pI). At pH values above the pI, the protein is negatively charged; at pH values below pI, it is positively charged. Based on that, there are two types of IEC: cation-exchange chromatography, which binds positively charged proteins, and anion-exchange chromatography, which binds negatively charged proteins.

The media used in ion-exchange chromatography are also made of porous particles. However, their pores are not intended simply to trap molecules that are small enough to fit inside. They are locations for ionic interactions to take place. Commonly used ion-exchange media include agarose, cellulose, and dextran. IEC resins pack into columns commonly as tiny beads. Their capacity to bind proteins, expressed as mg/mL, depends on the size and distribution of pores and the electrically charged molecular groups across the surface of each bead.

**Hydrophobic Interaction**
When biopharmaceutical proteins are hydrophilic, meaning they are soluble in water, hydrophobic-interaction chromatography (HIC) takes advantage of the fact that they have pockets of hydrophobic amino acids that can react with immobilized hydrophobic groups on a matrix. Because hydrophobic interactions are strongest at high ionic strengths, HIC is a good choice to follow salt precipitation in a purification process. A mixture is applied at high salt concentrations. Inside the column, the hydrophobic proteins bind reversibly to hydrophobic resins. The medium is not strongly hydrophobic so that the bound proteins will be released under washing conditions (low salt concentration).

**Reverse Phase**
If your matrix is extremely hydrophobic, then you are using “reversed-phase chromatography” (RPC). (The “reverse” in the name derives from its being the opposite of another type of chromatography. “Normal phase chromatography” uses a hydrophilic matrix as opposed to the hydrophobic matrix used in RPC.) RPC uses organic solvents for separation and elution of a protein. Because the conditions are harsh, and because the proteins are bound tightly, this method works best with smaller, more stable proteins. RPC is powerful, but it is also limited in its application. The organic solvents used (butanol, isopropanol, ethanol, methanol, and acetonitrile, for example) are sometimes flammable and require special equipment for their storage and use to prevent fires or explosions. They may denature proteins, and they can be toxic as well, necessitating the use of special ventilation systems. Solvents that provide the best resolutions can also be too difficult
to separate from the product after the fact, adding another purification step that can negate the advantage of the RPC separation power in the long run.

Both HIC and RPC are used sometimes as concentrating steps in the purification process. RPC is less common in protein purification because of the harsher conditions it employs, but it is used quite often in preparative separation of peptides.

Affinity
By far the most varied (and often the most expensive) chromatographic technique used in biopharmaceutical production is affinity chromatography. The term covers a wide range of methods, some of which are customized and all of which are based on the specific binding of one molecule to another. In each case, a ligand with a high specificity for the molecule of interest (or a particular impurity) must be found. For example, immunoaffinity chromatography uses antibodies to bind antigens, metal affinity chromatography uses chelation, and dye affinity chromatography uses the tendency of some dyes to bind to proteins. Ligands must be immobilized on a resin support so that binding reduces the mobility through the affinity column of the material to be separated out. Thus, the medium used depends entirely on the molecule(s) present.

Affinity chromatography is the most powerful chromatographic technique, offering 100- to 1,000-fold purification (or better) in a single step. It also can be the most expensive method, particularly because of the custom nature of the media used. Selecting a good affinity ligand often requires a trial-and-error experimentatation. Too strong a molecular bond cannot be broken to free the protein of interest without denaturing it, but a bond that is too weak will allow it to be washed away by eluent. The better the specific bond is understood chemically, the better conditions can be predicted that will allow separations to go smoothly.

“The power of affinity chromatography encourages its use early in a purification scheme, although the greater expense and sensitivity of the ligand means that cleaning-in-place and lifetime studies are critical,” says Amersham’s Grund. “Several industrial examples, such as monoclonal antibodies, use affinity chromatography in the capture stage, reducing the number of purification steps needed further downstream.”

There are many different affinity chromatography techniques, but the types used for biotherapeutics can be grouped into three categories: dye-ligand, immobilized-metal, and biospecific-affinity chromatography.

Dye-ligand chromatography can be the least expensive of those to use, and it is based on the tendency of some dyes (like those used in clothing manufacture) to bind fairly specifically and quite solidly to certain types of proteins. Some types of dye-ligand affinity adsorbents can exceed the power of biospecific adsorbents. Dye-ligand chromatography is often used in the purification of diagnostic enzymes. However, says Nandu Deorkar, senior research chemist at Mallinckrodt Baker, dye-ligand chromatography is “usually only used in diagnostics because dyes are considered toxic.”

Immobilized-metal (or metal-chelate) chromatography is a newer technique that is gaining popularity because of its powerful resolution. The protein product is retained by coordinating electron-donating groups of atoms on the protein’s surface with the immobilized transition metal ions such as copper, nickel, or zinc. Some companies use this form of affinity chromatography to purify immunoglobulin G.

Biospecific-affinity chromatography refers to the use of biological ligands (often proteins such as enzymes or antibodies) to grab hold of very specific molecules in a mixture. The ligand may have an affinity for a group of molecules (such as all immunoglobulins) or it can be very specific for one product. When an antibody is bound
to a matrix, the resulting medium is called an immunoadsorbent. As a mixture flows through the column, the antigens bind to the antibody and stay inside while the rest of the mixture continues through. The antigens are then flushed out of the column and collected separately. This technique has been used to purify interferon.

“The most widely used example of affinity chromatography is Protein A affinity chromatography, which is frequently used for the separation of monoclonal antibodies,” says Deorkar.

Affinity tails (or affinity tags) are an important development in biotechnology. A so-called fusion protein is produced by fermentation, cell culture, or other means with a tiny protein fragment genetically engineered onto one end. The purpose of that tag or tail is to enable very specific affinity purification, and it is removed after separation is complete. Smaller-molecule ligands such as synthetic peptides also can be used in affinity chromatography; they’re more stable and sometimes enable more stringent cleaning methods to be used.

Chromatography Works
An elution profile (see Figure 2), or chromatogram, records how much material is carried out of the column by the eluent over time. This line graph will show a number of different peaks; each peak represents a different separated material from the original mixed substance. Practiced chromatographers can often recognize the peak of their protein of interest (along with any product variants and impurities) on a chromatogram. Chromatography has been used in analysis for many years, and it is a popular choice in biopharmaceutical quality control and development laboratories to show the relative purity of various materials.

Two different animals. Large-scale biopharmaceutical chromatography is vastly different from traditional analytical chromatography done at the laboratory scale. However, all the modes of chromatography already mentioned play key roles in characterizing biopharmaceuticals. “SEC is the current method of choice for the analysis of aggregates, although other techniques such as field flow fractionation (FFF) are emerging as alternatives to SEC,” says Jeffrey Mazzeo, applied technology director of Waters Corporation. “Protein variants, or modified forms, can be separated by ion exchange, reversed phase, and HIC,” he says. “Ion exchange is particularly useful for separating C-terminal lysine variants of antibodies.”

The internal diameters (IDs) of analytical-scale columns are usually 1–7 mm; using high-pressure liquid chromatography (HPLC), they can operate under pressures up to 8,000 psi using beds of resin with particles as small as 3 μm in diameter. By contrast, production (or “preparative”) scale operations can use beads that are more than 70 times that size in columns whose IDs range from a few centimeters up to two meters. Two-meter columns are currently the largest used, but some manufacturers are even envisioning three-meter columns as production demands increase on some higher-volume
products. Operating pressures are lower on the large scale, though, from 5 to 50 psi.

At such large scales, problems that arise can involve pressure limitations, media packing irregularities, and flow distribution across wide columns. Sample overloading, unpredictable adsorption rates, and low separation resolution are concerns at all scales (see Figure 3). The economics are such that a perfectly viable, efficacious, and useful molecular entity may not make it as a drug simply because it cannot be produced and purified efficiently enough to fall even within the wide realm of “affordability” as it is understood in the pharmaceutical market today.

Reproducibility means consistent, predictable performance. It depends most on the characteristics of the feed stream and the chromatography medium. The surrounding equipment must support realistic conditions for both the medium and the protein product.

**Bioprocess design.** Because chromatography is required to achieve the very high product purity standards set by regulators, biochemists are indispensable in biopharmaceutical purification process design. Their intimate understanding of the molecular interactions that characterize protein chemistry is essential to developing efficient purification processes. Computer modeling may make their job easier, but very often decisions come down to the trial and error of laboratory bench experiments.

The choice of chromatographic methods and the stationary phases to be used are important to the design of a purification system. Equipment is designed and optimized for the necessary conditions to provide robust, reproducible performance. Process design takes into account the economics and efficiency of purification based on scientific parameters such as hydrodynamics and kinetics, mass transfer rates, molecular interactions, and surface chemistries.

In general, a chromatographic matrix should be reusable and composed of spherically shaped particles with a definite size range (usually between 20 and 100 µm) and sufficient binding capacities, even at high pressures and flow rates. Leakage profiles for the base matrix and ligands must be determined during the conditions of washing, elution, and storage.

**Continuous Improvement**

In response to industry demands, vendor companies put a great deal of their research and development dollars into improving chromatographic systems. Sometimes they focus on creating better media for existing techniques; sometimes they look for ways to improve the techniques themselves; and sometimes they seek to invent whole new methods.