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IMPROVING THE PURIFICATION OF MODERN BIOTHERAPEUTICS WITH ANTIBODY-BASED AFFINITY CHROMATOGRAPHY

INTRODUCTION

Biotherapeutics are a powerful and widely researched class of drugs in the modern pharmaceutical industry. Since the inauguration of the field in 1982 with the development of recombinant human insulin, biotherapeutics have become far more diverse, encompassing therapeutic antibodies, other large biomolecules, and even adeno-associated virus vectors (AAVs) that deliver gene therapies to address a disease at its root cause.[1,2]

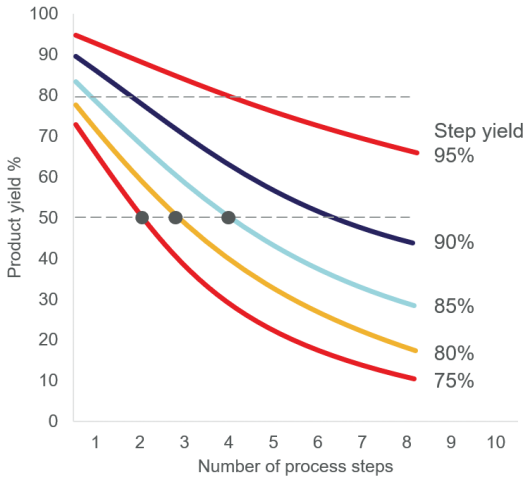
By 2014, more than 300 drugs in this class had been approved for use, representing a market of more than $100 billion. The market has only continued to grow, but so have the associated challenges.[1] Biotherapeutics are produced in genetically engineered cell lines rather than by the traditional methods of synthetic organic chemistry. As a result, the drug molecule is accompanied by a complex stew of host cell proteins and cell debris. Biotherapeutics themselves are also getting more complex, and this complexity can become a source of impurities. To obtain high-purity biotherapeutics from these mixtures and impurities, scientists are adopting separation methods that offer better selectivity.

CHROMATOGRAPHY IN BIOLOGICS MANUFACTURING: OVERVIEW AND LIMITATIONS

Chromatography is an indispensable step in producing biotherapeutics, as it allows scientists to isolate a single molecule from among the chaff of by-products and impurities. Many chromatographic methods are available and most of them take advantage of one or more physical properties that differ between the target molecule and associated impurities. These properties include the molecule's size, surface charge, or hydrophobicity. In cases involving impurities formed by degradation of the target molecule, the oxidation of one or several functional groups can alter a molecule's overall hydrophobicity and enable separation on that basis. In other cases, impurities may be drastically larger or smaller than the

target molecule, which allows them to be separated by size-exclusion chromatography.

These methods are usually too general to remove all impurities in a single pass, however. Affinity chromatography is a more advanced purification technique that can be tailored to the target molecule. This separation technique makes use of the highly specific but also reversible binding that occurs between some proteins in nature—specifically, between antibodies and antigens. This binding works on the basis of a number of factors, including protein shape, secondary structure, local regions of hydrophobicity, and the presence of solvent interactions. As such, affinity chromatography can be far more selective for a single molecule than other chromatographic methods and can often be used to achieve precise separations and high yields in a single purification step.[3]



Each step in the purification process results in a yield loss. Even at higher step yields the overall product yield decreases rapidly with the increase in process steps. Optimizing the purification process to minimize the total number of steps is crucial to increase efficiency in the total process.

Source: Thermo Fisher Scientific

The purification of biotherapeutics is an increasingly important field of study, as these molecules continue to be promising targets for pharmaceutical research. The number of them entering clinical trials each year is rising, particularly for the larger and more complex biotherapeutics that make up the cutting edge of drug research. Viral particles, recombinant proteins, Fc-fusion proteins, and other emerging therapies have great potential but also require new developments in manufacturing technology. They must often be purified by multistep processes that include size-exclusion chromatography, ion-exchange chromatography, and specialized filtrations. Though multiple chromatography steps are necessary to achieve the desired purity, they can severely reduce a process's yield while increasing manufacturing costs.[4]

Manufacturers (or process developers) are thus faced with the challenge of shortening purification processes while purifying compounds to the pharmaceutical industry's exacting standards. As it turns out, affinity chromatography is a good solution.

"Typically, nothing can compete with affinity chromatography in terms of the degree of purification that you can get in a single step," says Andrew Zydney, professor of chemical engineering and director of the Center of Excellence in Industrial Biotechnology at Pennsylvania State University.

In most forms of affinity chromatography, a protein ligand selected for its high affinity for the target molecule is immobilized on a porous support. When the crude sample is passed through the column, the target molecule binds to the ligand, effectively remaining stuck on the column while impurities and other undesired compounds are washed away. When this process is complete, the eluent can be changed to one in which the target molecule and the ligand do not have high affinity for one another, allowing the target molecule to disengage from the ligand and exit the column.[3,5]

PROTEIN A AFFINITY CHROMATOGRAPHY

Though the biotherapeutic market is quickly diversifying, it was previously dominated by monoclonal antibodies. These lab-grown versions of our immune system's defense mechanism are powerful drugs and can be used to treat a number of cancers as well as other disorders. Affinity chromatography to purify monoclonal antibodies can be accomplished using a bacteria-derived affinity ligand called protein A, which binds to the Fc region of monoclonal antibodies. This region remains the same in all antibodies, while the variable region can be engineered to recognize a wide variety of antigens.

For this reason, "the same protein A can bind to basically every monoclonal antibody that is produced," Zydney says. "There are some differences in degree of affinity and exactly the optimal buffer conditions, but in general it's an incredibly robust technology."

But he warns that protein A ceases to be an all-powerful affinity ligand when researchers leave the harbor of monoclonal antibody purification. Newer types of antibody-derived therapies, like antigen-binding fragments, often fail to be effectively purified with protein A affinity chromatography. Protein A will also be of little use to scientists developing chromatographic methods to purify newer products, such as gene therapies, clotting factors, and hormones.

Of course, looking at those other classes of biomolecules is often exactly what scientists want to do—and they often have to find new chromatographic techniques to address those challenging purifications.

ANTIBODY-BASED AFFINITY CHROMATOGRAPHY

Antibody-based affinity chromatography, a relatively recent development in the separation sciences, has arisen partly because of a swiftly growing assortment of biotherapeutics. One way chromatographic techniques are keeping pace with the demands of biologic research is by replacing protein A with an antibody as the affinity ligand. Using an antibody as a ligand transforms a general approach into a highly optimized one, as a new ligand can be identified for each target molecule. The key to this method is the amazing variability of antibodies. With minor modifications to the variable regions, antibodies can be optimized for binding to almost any target protein while keeping the same overall structure. Thus, antibody-based affinity chromatography does not share protein A's narrow applicability. It can be used to purify gene therapies and other viral vectors, cell

therapies, recombinant proteins, and a diverse range of antibody-related products beyond the human monoclonal variety.[5]

One powerful tool in affinity chromatography comes from the camelid family. The immune systems of animals in this clade, which includes camels, alpacas, and llamas, take a unique approach to antibody design.

By comparison, a human antibody, like the antibodies of most other organisms, is not a single protein chain but rather a complex of several protein subunits. Some of these subunits are constant—they are the same in every antibody—while others are variable. The variable regions can support nearly endless diversity to bind with almost any protein. In human antibodies, the actual binding occurs at the interface between two subunits of different sizes: the variable light chain and the variable heavy chain.

HOW AN ANTIBODY-BASED AFFINITY RESIN IS DEVELOPED

With Thermo Fisher Scientific's platform, an antibody-based affinity chromatography resin is developed in five steps, according to Laurens Sierkstra, Thermo Fisher Scientific's senior director of business leader purification. The company, which looks to industry trends for problems that can be addressed with off-the-shelf affinity chromatography, currently has products in all five stages of the development pipeline. But Thermo Fisher Scientific can use the same ligand development techniques to provide customized solutions. Some customers will be interested in purifying multiple proteins from their own research program. In those cases, Thermo Fisher Scientific can tailor a library of camelid antibodies for up to 10 targets from the pipeline to save time later if any of those candidate compounds advance to the manufacturing stage. "You can then pick out from this library every time you actually have a need for a specific affinity ligand and resin," Sierkstra says. The company also maintains libraries for common proteins of interest.

The resulting ligands undergo a high-throughput screening step, as researchers work to optimize the specificity, on- and off-rates of binding, and stability of the ligand itself. During this step, ligands are also evaluated for mild elution conditions. The optimal case is that the target can be washed off the affinity resin with only a mild change in pH.

Next, a few prototype resins are manufactured using promising ligands in combination with one or two resin backbones (even with the same ligand, different resin supports can offer varying degrees of performance). These are used in a one-step test purification of the mixture to see how the ligand performs when actually immobilized on the resin and to give researchers further opportunities to optimize the chromatography.[7]

"We will not only design the ligands against the target, but we will as well check the backbone for the specific product and process," Sierkstra says. For applications in which the chromatography will be run at high flow rates, the ligand can be immobilized on a proprietary poly(styrene-divinylbenzene)-based resin with relatively large pores. If a lower flow rate and higher capacity are desired, an agarose-based resin can be used instead.[8]

In the fourth development step, two promising lead resins are tested at larger scale and under actual process conditions to identify which gives the best separation. Finally, once the resin and ligand have been identified, Thermo Fisher Scientific validates the process and produces ancillary tools to complement the use of the resin. One of these is an enzyme-linked immunosorbent assay, which is capable of detecting the affinity ligand in solution if it were stripped from the resin backbone during chromatography. Many users of the technology, particularly manufacturers of biologic drugs, will need to demonstrate that the affinity chromatography step is not inadvertently adding unwelcome proteins into the mixture.[5]

Camelids also possess these antibodies, but they have an additional type of antibody, which does not contain light-chain subunits. In these molecules, binding occurs at a single, highly variable heavy-chain subunit.[6] Weighing 15 kDa, the subunit is still far smaller than comparable antibodies. At the same time, it doesn't require a light-chain complement in order to bind target molecules, and so it is much more stable and easy to manufacture.

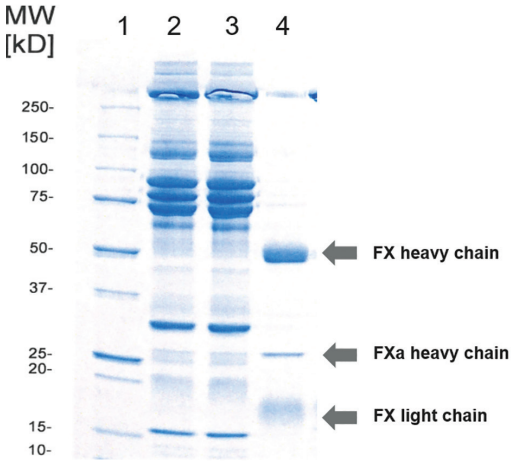
This subunit appears in the CaptureSelect™ antibody-based affinity chromatography technology developed by Thermo Fisher Scientific. The ligands are manufactured in *Saccharomyces cerevisiae*, so although the structure of the ligand comes from camelids, the product is not derived from an animal. Besides making the manufacturing process more scalable and flexible, this means the affinity ligands can be used in good manufacturing practices (GMP) purification processes, according to a webinar presented by Sierkstra.[5]

The versatility of this process makes it easy for Thermo Fisher Scientific to develop affinity ligands for commonly used proteins, and the company maintains a suite of affinity resins for popular classes of biomolecules, including AAV vectors, human proteins, and a large assortment of therapeutic antibodies.

CASE STUDY: HUMAN FACTOR X WITH EDTA FOR ELUTION

When screening possible ligands for an affinity chromatography process, researchers remain alert for any opportunities to make the target protein's elution conditions as mild as possible. This is especially true if the target protein is found to elute under acidic conditions. Researchers are often able to find that a small pH adjustment changes the protein's conformation enough that the protein will detach from the ligand and wash away from the support. But at other times, more elegant solutions appear.[7]

"Some proteins have an altered conformation in the presence or absence of calcium," Sierkstra says, so the ligand screening step includes a test with this ion as a matter of course. In his webinar presentation he explained that when Thermo Fisher Scientific was developing a CaptureSelect resin for human factor X protein, researchers discovered that it was possible to engineer the ligand so that the protein would bind only in the presence of calcium. When calcium was removed, the protein was easily eluted from the column. The process was therefore optimized to include calcium in the mixture.



Reduced SDS page gel showing affinity purification of Factor X. The affinity resin binds Factor X and Factor Xa in the presence of calcium, which allows mild elution with the addition of EDTA. 1: Molecular weight marker. 2: Load. 3: Flow through fraction. 4: Elution fraction.

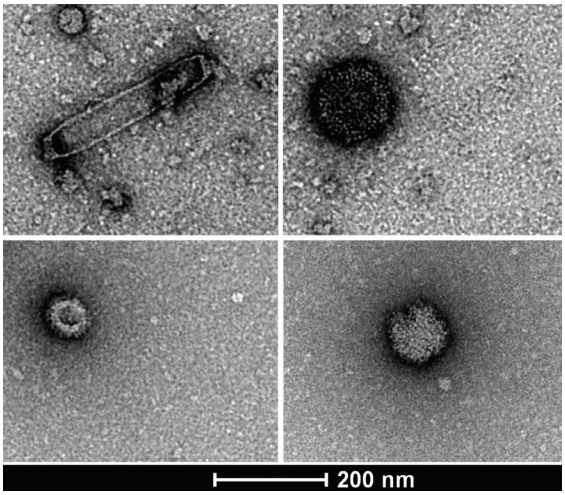
Source: Thermo Fisher Scientific

Once the impurities were washed away, elution of the target protein could be achieved with the addition of ethylenediaminetetraacetic acid (EDTA), a chelating reagent known for its ability to sequester calcium. The entire process could thus be run at neutral pH, protecting the valuable factor X protein from degrading during elution.[5]

CASE STUDY: BACULOVIRUS EXPRESSION VECTOR SYSTEM

Sierkstra notes that in most cases, affinity chromatography serves as a purification rather than a scavenging technology. Purification technologies isolate a single target molecule from a complicated mixture while discarding the rest. On the other hand, scavenging technologies remove a single undesirable molecule or class of molecules from a mixture.

In one case, however, researchers at the Institute of Experimental Biology and Technology in Portugal worked with Thermo Fisher Scientific to develop an affinity resin for scavenging unwanted virus particles from a manufacturing process. The baculovirus expression vector system uses a common class of insect pathogen to produce viral vectors and viruslike particles, which can be used for gene therapy or vaccine development. When the baculovirus gets used as an expression vector system in insect cells, it causes the host cell to produce baculovirus envelopes alongside the target molecule. These baculovirus envelopes are an unwelcome impurity, and separating baculovirus from viruslike particles is an understandably difficult task. But an affinity chromatography resin engineered for this purpose achieved baculovirus removal above 70% while recovering 60% of the desired viruslike particles.[9,10]



Transmission electron microscopy images of virus-like particles (VLPs) expressed in a Baculovirus expression system. The upper row shows images of clarified VLPs and the lower row of purified VLPs, demonstrating a purity increase after purification with the Baculovirus scavenging resin.

Source: Thermo Fisher Scientific

CONCLUSION

Antibody-based affinity chromatography is best viewed as the separation sciences' response to a rapidly diversifying landscape of biotherapeutics. By achieving a high degree of purification in a single process step, the method lets researchers trim lengthy purification processes that would erode product yield as well as cost margins. The powerful one-step purification that monoclonal antibody researchers have enjoyed through the use of protein A can be translated to other classes of

biological molecules by using antibody-based affinity chromatography. The unique structure of the camelid heavy-chain affinity ligand represents another advantage. More compact and sturdy than standard antibodies, these ligands can be produced with relative ease. That makes them useful for applications in GMP manufacturing, an important consideration when purifying compounds that may be destined for clinical studies as well as for final commercial manufacturing.

Although not a one-size-fits-all solution, antibody-based affinity chromatography is a versatile tool for biomolecule purifications of any stripe.

For more information, watch a webinar here.

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